उपक्रम एवं संकलन

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Extension and Training Division

आई सी एम आर – राष्ट्रीय पोषण संस्थान

ICMR - NATIONAL INSTITUTE OF NUTRITION

(भारतीय आयुर्विज्ञान अनुसंधान परिषद)

(Indian Council of Medical Research)

जामे उस्मानिया पोस्ट, हैदराबाद

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वार्षिक प्रतिवेदन
Annual Report

2016 - 17

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भारतीय आयुर्विज्ञान अनुसंधान परिषद

Indian Council of Medical Research

हैदराबाद, तेलंगाना, भारत

Hyderabad, Telangana, INDIA

Annual Report

2016-17

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29. Sreenu pagidoju
30. Sunu P.V
31. Srinivasu kurella
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33. P. Sailaja
34. B.R. Annapurna
35. Ch. Hanumantha reddy
36. V. Bhasker
37. P. Yadagiri reddy
38. M. Seshacharuyulu
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10. P.S.Rama Rao
11. Jagdish Buwade
12. R. Raghunath Babu
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16. Ravindranath Palika
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7. G.l. Stephen
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10. K. Balaji
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12. Srinivas Dheeravath
13. N. Peddi Reddy
14. K. Satyanarayana
15. Y. Agreepa Raju
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17. P. Bheem Shanker
18. J. Pochaiah
19. M. Balram
20. S. Chandraiah
21. B. Nagender Rao

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6. S. Devendran
7. Ramavath Ramsingh
8. Sriramulu Naidu
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11. Polishetty Naidu
12. P. Narendra Kumar
13. B. Bal Reddy

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2. Nigala Yadagiri
3. E. Krishna
4. Bommaka Srinu

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6. L. Dasu
7. D. Dasaratha
8. J. Nageswara Rao
9. C. Chandramouli
10. Abdul Sattar
11. K. Rama Rao
12. V. Rajkumar
13. Manupathi Bikshapathi
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25. K. Gopal
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3. D. Ravinder
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8. J. Yadagiri

9 Syed Mohd. Iqbal

10. Mabbu Ramulu
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15. J. Lakshmaiah
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22. G. Yadagiri
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RESEARCH HIGHLIGHTS

1. BASIC STUDIES

1.1 Amelioration of neuronal cell death in obese rat model by dietary restriction through modulation of ubiquitin proteasome system

Dietary restriction has been shown to increase longevity, delay onset of aging, reduce DNA damage, oxidative stress and prevent age-related decline of neuronal activity. We previously reported the role of altered ubiquitin proteasome system (UPS) in the neuronal cell death in a spontaneous obese rat model (WNIN/Ob rat). Now we demonstrate the effect of dietary restriction on obesity- induced neuronal cell death in a rat model by feeding the WNIN/Ob rats with either unlimited or restricted (equaling to lean rats) diet. Dietary restriction for 6.5 months improved metabolic abnormalities in obese rats. Alterations in UPS, increased ER stress, declined autophagy, increased expression of α-synuclein, p53 and BAX were observed in obese rats and dietary restriction alleviated these changes in obese rats. Further, diet restriction decreased apoptotic cells. Thus, dietary restriction in obese rats could not only restore the metabolic abnormalities but also preserved neuronal health in the cerebral cortex by preventing alterations in the UPS.

1.2 Ubiquitin-Proteasome system and ER stress in the retina of diabetic rats

Diabetic retinopathy (DR) is the most frequently occurring complication of diabetes. Alterations in ubiquitin–proteasome system (UPS) have been associated with several degenerative disorders. In this study, we assessed the role of UPS and ER stress in the retina of diabetic rats. Diabetes was induced in rats and ER stress, UPS, autophagy and apoptosis were investigated after 2- and 4-months of diabetes. Chronic diabetes increased acellular capillaries and pericyte loss in rat retina along with the decreased protein expression of UPS components. Further, increased ER stress in diabetic rats is associated with declined autophagy and apoptosis. Interestingly, treatment of diabetic rats with a chemical chaperone restored the UPS and ameliorated ER stress-induced retinal cell death in diabetic rats. These studies indicate that declined UPS components in the retina of diabetic rats could elicit ER stress, and the prolonged ER stress may trigger CHOP-mediated neuronal apoptosis.

1.3 Alzheimer's and Danish Dementia peptides induce cataract and perturb retinal architecture in rats

The familial Danish dementias (FDD) are autosomal dominant neurodegenerative disorders which are associated with visual defects. In some aspects, FDD is similar to Alzheimer's disease (AD). For example, amyloid deposits in FDD and AD are made of short peptides called dementia peptides: amyloid β (Aβ) in AD and ADan in FDD. Previously, we demonstrated an interaction between these dementia peptides and α-crystallin leading to lens opacification in organ culture due to impaired chaperone activity of α-crystallin. Herein, we provide the evidence for the *in vivo* effects of ADan and Aβ peptides on the eye. The ADan and Aβ peptides were injected intravitreally to the rats. The onset of cataract was seen after injection of the peptides, but the cataract matured by 2-5 weeks in the case of ADan peptides and after 6 weeks for of Aβ peptides. The severity of cataract is associated with insolubilization and alterations in crystallins and loss of chaperone activity of α-crystallin. Further, disruption of the architecture of retina was evident from a loss of rhodopsin, increased gliosis and the thinning of the retina. These results provided a basis for the visual impairment due to dementia.

1.4 Hsp90 regulation of fibroblast activation in pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is a severe fibrotic lung disease associated with fibroblast activation that includes excessive proliferation, tissue invasiveness, myofibroblast transformation, and extracellular matrix (ECM) production. To develop therapeutic agents, we queried IPF gene signatures against a library of small-molecule-induced gene expression profiles and identified Hsp90 inhibitors as potential therapeutic agents that can suppress fibroblast activation in IPF. In support of this, we found elevated Hsp90 staining in lung biopsies of patients with IPF. Notably, fibroblasts isolated from fibrotic lesions showed heightened Hsp90 ATPase activity compared with normal fibroblasts. 17-*N*-allylamino-17-demethoxygeldanamycin (17-AAG), a small molecule inhibitor of Hsp90 ATPase activity, attenuated fibroblast activation and also fibroblast to myofibroblast transformation. Further, knock-down of the Hsp90 resulted in reduced fibroblast proliferation, myofibroblast transformation, and ECM production. Finally, in vivo therapy with 17-AAG attenuated progression of established and ongoing fibrosis in a mouse model of pulmonary fibrosis, suggesting that targeting Hsp90 represents an effective strategy for the treatment of fibrotic lung disease.

1.5 Impact of dietary fatty acids on the progression of nonalcoholic fatty liver disease in fructose induced model of steatosis – Role of adipose tissue insulin sensitivity and secretory function

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease and includes spectrum of liver condition ranging from benign steatosis to non-alcoholic steatohepatitis (NASH), advanced fibrosis and ultimately liver failure. The recent phenomenal increase in prevalence of NAFLD may be associated with significant modification of dietary habits due to westernization of the diet. The changes in dietary habits have made fast food as an important component of today's diet. In addition to providing high calories, several components of the fast food based diets could play a significant role in the pathogenesis of NAFLD. Increased consumption of soft drinks which contain high levels of fructose along with high intake of saturated fat, trans fat, n-6 PUFA and low intake of n-3 PUFA may be the possible cause of increased prevalence of NAFLD. Although the exact cause of the progression from simple steatosis to NASH is not established, lipotoxicity has been suggested to play an important role. Studies were carried out to investigate the impact of specific fatty acids on the progression of NAFLD in the setting of hepatic steatosis. The results showed that fructose: saturated/trans fatty acid combination induced hepatic steatosis. However, compared to fructose: saturated fatty acid combination, fructose: trans fatty acid combination induced NASH as evidenced by increased inflammatory and fibrotic changes suggesting that fructose: trans fatty acids combination is detrimental to the liver. Further, substitution of n-6 PUFA with n-3 PUFA prevented high fructose, high cholesterol induced NASH. The protective effect of n-3 PUFA supplementation on high fructose, high cholesterol induced NASH could be attributed to the suppression of proinflammatory cytokines and oxidative stress by dietary n-3 PUFA. The results of the present study reinforce the current recommendations of restricting the intake of trans fats, moderate the intake of n-6 PUFA and increase the intake of n-3 PUFA for the prevention of diet related chronic diseases including NAFLD.

2. EXTENSION AND TRAINING

2.1 Health seeking behaviour, food beliefs and practices among Chenchu women during physiological changes – A gender based approach

This study was planned to bring awareness on health and nutrition among Chenchus. Data was collected from 16 Chenchu tribal villages in Telangana and Andhra Pradesh. From each state, 4 core villages (2 homogenous villages & 2 heterogeneous villages) and 4 buffer villages (2 homogenous villages & 2

heterogeneous villages) were selected for the research study. a total number of 322 women were interviewed to assess the information relating to food fads, myths and beliefs which are likely to affect the dietary practices during different physiological conditions of Chenchu women.

During lactation (first six months) most of the foods are avoided except rice and spicy chilli powder, leading to malnutrition of both mother and child. No additional food intake is given during pregnancy. Majority of women (79%) gott married before the age of 18 years. Millet acceptability is high but consumption is low due to easy availability of subsidized cereals. Seventy six percent of women used the services of government PHC for health care.

IEC material were developed based on information collected from chenchu tribe these include folders, charts which were distributed among the participants as a source material to educate the women. At the end of the project, a one-day dissemination workshop on "Health and nutrition awareness" was conducted for ICDS & Health functionaries working with Chenchus.

3. FOOD AND DRUG TOXICOLOGY RESEARCH CENTRE

3.1 Differential protein expression in liver tissues in fluoride intoxicated rats by proteomics approach

There was a significant decrease in body weight in the 50 and 100 ppm F groups compared to control group. There was a significantly higher excretion of fluoride in all the F groups than the control group. There is a significantly increased serum fluoride levels in the 50 and 100 ppm F groups compared to control groups. There was a significant increase in the teeth and bone fluoride levels in the F groups compared to control group. The differential protein expression in the liver of control and 100 ppm fluoride group was observed. 7 spots were identified as differentially expressed proteins by PDQuest software (BIORAD). The proteins highly expressed in control group as compared to fluoride group are glutathione–s-transferase and hemoglobin, alpha 2. The proteins highly expressed in the fluoride groups as compared to control group are dismutase, arginase-1, T-cell receptor beta chain, D-dopachrome decarboxylase.

I. COMMUNITY STUDIES

1. ASSESSMENT OF 24-HOUR URINARY SODIUM EXCRETION LEVELS IN URBAN MEN AND WOMEN IN ANDHRA PRADESH, TELANGANA AND ASSAM FOR VALIDATION OF CONSUMPTION LEVELS OF SODIUM ASSESSMENT THROUGH 24 HOUR DIETARY RE-CALL METHOD

The increase in the prevalence of hypertension in epidemic proportions has been significantly correlated with the daily consumption levels of sodium, which is a modifiable risk factor for hypertension. The International study of Salt and Blood Pressure (INTERSALT), showed a modest association between higher levels of sodium intake and higher blood pressure. However, INTERSALT was not large enough to determine whether, the association varied according to region, participant characteristics or levels of sodium or potassium intake.

In this context, the National Nutrition Monitoring Bureau (NNMB), had conducted a large and comprehensive urban Nutrition survey in 16 states of the country. About 13,000 Hhs were surveyed using 24hour dietary recalls and other investigations like anthropometry were done to determine NCDs among urban population. The consumption levels of salt through dietary recall were assessed for individual subjects in the study. However, the gold standard method for estimation of sodium consumption levels is through estimation of 24-hour urinary sodium excretion was not done in all the states. In this context, the National Institute of Nutrition, carried out a larger study to estimate 24-hour urinary sodium excretion levels in three cities of three different states, i.e., Telangana (Hyderabad) Andhra Pradesh (Vijayawada) and Assam (Guwahati), that would indirectly provide the salt intakes levels among the different population groups. The study population comprised a sub-sample of the large comprehensive study undertaken in 2015-16 by the Nutrition Monitoring Bureau (NNMB), in the urban population in 16 states of the country. The dietary salt intakes were estimated from the 24-hour dietary recall method of diet surveys carried on the study population.

The objective of the study was to determine sodium excretion levels from 24 hour urinary samples and spot urine samples and to assess dietary sodium consumption using 24 hour recall method subjects were adults of Vijayawada, Hyderabad and Guwahati cities. The study also aimed to validate the dietary sodium intake estimation and spot urinary sodium estimation levels with the 24 hour urinary sodium excretion.

OBJECTIVES

- To estimate 24-hour sodium excretion levels in those subjects whose dietary sodium consumption was already assessed by 24-hour recall method of diet survey, among adults in the urban population of the cities of Vijayawada and Hyderabad, and Guwahati,
- To estimate urinary creatinine and albumin
- To validate the 24-hour and spot urinary sodium excretion levels with the dietary sodium consumption levels.

RESULTS

A total of 437 samples of 24-hour urine samples and 542 spot urine samples were collected from urban adult population from three major cities viz. Hyderabad, Vijayawada and

Guwahati. The samples were stored < -80°C and analysed for sodium excretion levels and its salt (NaCl) equivalents were estimated. The information was collected on dietary consumption of salt through a one-day 24 hour recall method of diet survey from 267 adult subjects.

The mean salt (NaCl) intake through diet was 7.2 g in Vijayawada,8.4 g in Hyderabad and 12.8g in Guwahati. Likewise, the mean sodium intake was 0.49g in Vijayawada, 0.51g in Hyderabad and 0.77 in Guwahati. Similarly, the excretion levels of NaCl in the spot urine samples was 8.3g, which ranged from a low 5.7g in Guwahati to a high 8.9g each in the cities of Vijayawada and Hyderabad. While the mean sodium excretion from the spot urine samples was 3.3g, ranged from a low 2.3g in Guwahati to a high 3.5g each in Vijayawada and Hyderabad. Similarly, the mean NaCl excretion levels in the 24 hr urine samples was 7g,which ranged from a low 5.7g in Guwahati to a high 7.5g in Vijayawada. In case of mean sodium excretion from 24 hour urinary samples was 2.7 g, which ranged from a low 2.2 g in Guwahati to a high 3 g in Vijayawada. In general, the median salt equivalents for the Sodium and NaCl excretion levels in spot urine samples were 3g and 7.7g respectively, while the corresponding figures for 24-hour urine samples were 2.4g and 6g, respectively. The agreement of sodium excretion between the spot and 24hr urine samples was good as compared to dietary intakes of sodium.

In general, the prevalence of hypertension among urban adult men and women was 53.3% and 35.7% respectively and the prevalence was high among adult population in Vijayawada as compared to their counterparts in Hyderabad.

2. ASSESSMENT OF EFFECT OF 'ANNA AMRUTHA HASTHAM' ON NUTRITIONAL STATUS OF PREGNANT WOMEN, LACTATING MOTHERS AND THEIR <3 YEARS OLD CHILDREN IN THE STATE OF ANDHRA PRADESH

Studies in developing countries have shown that inadequate dietary intakes are associated with deterioration in maternal nutritional status and thereby the birth weight of their children. The dual stress of pregnancy and lactation further widens the already existing food and nutrient gap. In order to reduce the high maternal and infant deaths, higher prevalence of low birth weight among new born and high prevalence of iron deficiency anemia, as reported by the NFHS and National Nutrition Monitoring Bureau, the Govt.of Andhra Pradesh had launched a Full Meal Program (FMP), " Anna Amrutha Hastham". This modified supplementary program was designed to provide one hot cooked FMP to the pregnant and lactating mothers, as spot feeding at Anaganwadi centers for 6 days in a week for a duration of 150 days during pregnancy and another 150 days during lactation. On the request of the Government of Andhra Pradesh, a study was carried out by NIN, to carry out situational analysis of the FMP component of AAH in order to take necessary mid course corrections, if any, for strengthening the program.

RESULTS

The study covered a total of 578 currently pregnant women, 956 lactating mothers from 1918 households in the AAH villages and 538 currently pregnant women, 1034 lactating mothers from 2000 households in the Non AAH villages. A total of 516 households were covered for 24 hour recall diet survey and 128 AWCs were covered for institutional diet survey. In-depth interviews were conducted for Anganwadi workers (131), ICDS supervisors (109) CDPOs (58) to assess their knowledge and performance of AWCs with regard to implementation of FMP. The findings of the study in FMP areas were compared with the findings of the areas where Take Home Ration (THR) is implemented.

The results of the study indicated that study population was comparable in both the FMP and THR implemented areas, with respect to socio-economic and demographic characteristics, except for higher proportion of tribal population in FMP areas, by virtue of their natural selection. A majority (51-58%) of the male heads of the HHs were engaged as either agricultural or other labour, while most of the women were homemakers. The average percapita monthly income was ₹2103 and ₹2497 respectively in FMP and THR areas. The drinking water source was mainly piped water (66-69%). Only about half (49-58%) of the HHs had sanitary latrine and were using it presently. Almost all the HHs were electrified and about 59-67% of HHs were using LPG as cooking fuel. It is observed that <1% of the HHs were using adequately iodized salt (>15ppm), in both the areas.

The diet survey at household level indicated that, barring the intake of cereals the consumption of all the other foods was lower than the RDI, in both the areas, the intake of all the nutrients was also less than the RDA, in both the areas. The dietary assessment at individual intakes during household diet survey indicated that, the consumption of cereal intake alone among the pregnant and lactating mothers was higher than the RDI of NPNL sedentary women. With respect to intake of nutrients, the consumption of energy alone was more than the RDA of pregnant and lactating mothers. However, the total food intake by the beneficiaries which includes the household food intake as well as the FMP provided at AWC was less than the RDI and the nutrient intake was also less than the RDAs for the respective groups. The institutional diet survey conducted at the AWCs indicated that the percapita distribution of different foods and nutrients under FMP was marginally lower than the program norms.

Early marriages (<18years) were more frequent among pregnant women in FMP areas (20%) than in THR areas. All the currently pregnant were registered for ANC, of which early registration (<12 weeks of gestation) was 95%. Similarly, all the lactating mothers of <12 months children covered in the study had undergone antenatal check-up (ANC) during the previous pregnancy, and 91-93% among them registered to ANC before 12 weeks. The proportion of pregnant women who have undergone at least 3 ANCs during pregnancy was marginally lower (88%) in the FMP served areas as compared to the THR areas (94%). The pregnant women preferred private hospitals (60-66%) to the government health facilities for getting ANC done. Almost all the lactating mothers were covered for Tetanus Toxoid (TT) immunization of which 96% received two doses. In general, consumption of IFA tablets during pregnancy was about 90% in both areas for currently pregnant women and 98% for lactating mothers during pregnancy.

Almost all of currently pregnant women and lactating mothers (89%) during pregnancy were registered for FMP, of which, 50% of lactating mothers were registered before 12 week of pregnancy for FMP under AAH. The total number of feeding days for currently pregnant women and currently lactating mothers during lactation was 102 days and 130 days respectively, against the norms of 150 days, prescribed in the program. During lactation period, about 89% were registered for FMP among them 51% were registered within 7 days of delivery and the mean participation days in FMP were 94 days.

The consumption of IFA tablets along with cooked meals was very low among both pregnant women and lactating mothers. The regularity (≥ 21days/month) of participation in the FMP was also low. About 78% of pregnant women and 72% lactating mothers reportedly consumed meals at AWCs under the supervision of the AWWs. A majority of the beneficiaries stated that the quality of food in general was good or satisfactory. On the other side, the THR was received by 94% of the currently lactating mothers during pregnancy and 85% of them shared the food with other members of the family.

The prevalence of chronic energy deficiency among the pregnant women in FMP areas was 14%, while, in THR area it was 10%.The pregnant women who were nutritionally at risk constituted 9.4% owing to their low height of <145 cms and 5.9% owing to their low weight of

<39kgs. Similarly, in THR area, it was 5.8% and 3.9% respectively. The chronic energy deficiency among the lactating mothers in AAH area was 26% whereas in THR area it was 23%. About 73% of the lactating mothers were anaemic. The total prevalence of anaemia among currently pregnant women and currently lactating mothers was about 71%.

Weight monitoring was not regular, among both currently pregnant and during pregnancy of currently lactating mothers, in both FMP and THR areas, it was observed that only <4% of the pregnant women were weighed regularly at monthly intervals. Further, 16-24% of the beneficiaries were not weighed at all during entire pregnancy period. The proportion of pregnant women covered for weight monitoring was also low, ranging from 2-81% at different periods of gestation in FMP and THR areas.

With regard to the weight changes during pregnancy, it was observed that there was steady increase in mean weight of the pregnant women from 46.6kgs at <8weeks of gestation to 57.3 kgs at >33 weeks of gestation in FMP areas, while, in THR areas the mean weight at <8 weeks of gestation itself was on the higher side, which started from 49kgs, and gradually increased to 61.1kgs. The increase in mean weight was comparatively high in THR areas than FMP areas. The mean interval between successive weight recordings among currently pregnant women and during pregnancy of the currently lactating mothers, in FMP and THR areas was ranging from 4.1 – 5.6 weeks. The mean weight gain between two successive weight recordings was 1.9kgs and 1.8 kgs in FMP and THR areas, among currently pregnant women, while, it was 2.0 kgs and 2.1kgs, among currently lactating women during pregnancy in FMP and THR areas. About 64% of the currently pregnant women in FMP areas gained ≥ 1.5kgs of weight / month as compared to 66% in THR areas. Considering the mean weight of 46.6kgs at <8 weeks of gestation as base weight (NPNL women), it was observed that the pregnancy weight has improved with the increase in feeding days to 55.8kgs at 98 days of feeding till 33->36weeks of gestation. Similarly, the mean weights of the currently pregnant women in the THR areas also increased from 49.0 kgs at <8 weeks of gestation, to 62.2kgs at 33 ->36 of gestation. The total weight gain in FMP areas was 9.2kgs, which was less as compared to THR areas (13.2kgs). The results indicated that the weight gain is on par with the ideal weight gain during pregnancy (9-12kgs).

About 96-98% of the children's birth weight was recorded in FMP and THR. The prevalence of low birth weight (<2.5Kgs) was about 13% each in FMP and THR areas for infants and 10% in FMP and 11% in THR areas among the children of <3 years. The mean birth weight steadily increased from 2.81 kgs at <50 days of participation in FMP to 2.85 kgs at >150 days of participation, while, in THR areas it increased from 2.82 kgs to 2.88 kgs for the similar duration of feeding with THR. The prevalence of low birth weight also decreased from 13.7% to 11.9% with the increase of number of feeding days in FMP areas, while in THR areas, it decreased from 15.3% to 11.2%.

A majority (94% to 97%) of the deliveries were institutional in nature, mostly conducted in private health facilities. Early initiation of breast feeding (<1 hr) was observed among only 55-58% of lactating mothers. About 95% of mothers fed colustrum to their new born. It was observed that about 75-72% of the mothers practiced exclusive breast feeding upto six months of age in both areas.. About 10-13% of the mothers initiated complementary feed to their children before attaining 6 months. The practice of giving ORS to their children during the episodes of diarrhoea and proportion of children (<3 yrs) covered for de-worming was very low at 7-8%, in both the areas. About one third of Anganwadi centers were located in government owned buildings and 49% of the centers had separate kitchen. Drinking water facility was available in about 40% of the anganwadi centers within the premises and 39% AWC had functioning toilets. About 75% of the weighing scales for adults and children were in working condition. The knowledge about

objectives and services of AAH was found to be satisfactory. Only 34% AWWs underwent induction training for implementation of AAH programme. The knowledge about the objectives and services of ICDS programme was found to be good. In 56% of the villages, AAH convergence committees were formed by AWWs. The difficulties expressed by AWWs and the functionaries were two-fold low quality of rice (55%) and irregular supply of food grains (22%). Almost, all the supervisors and CDPOs were aware of the objectives and services of Full Meals Programme under AAH. In majority of the supervisors (80-93%) and CDPOs (78-93%) had periodically monitor the work of AAH during supervisory visits.

It can be concluded that the supplementary feeding in the present form had contributed in positive way in meeting the nutritional requirements of pregnant and lactating mothers in FMP areas. A higher benefit can be reaped if sharing of the supplement gets controlled.

Table 1: Coverage particulars and performance indicators in AAH and THR areas

Socio-economic	AAH	THR
Scheduled Tribes +Scheduled Castes	37	29
Average family Size	5.3	5.2
Male literacy	84	87
Female literacy	84	89
Per Capita income/ month (Rs.)	2103	2497
Type of House- *Kutcha*	7	9
Semi Pucca	35	26
Pucca	58	65
Households having and using sanitary latrine	49	58
Currently pregnant women		
Outcome of early pregnancy		
Abortions	11	8
Live births	54	52
Still births and Intra uterine deaths	1.2	0.9
Antenatal care		
Registration for Antenatal care	100	100
Early registration (< 12 weeks of gestation)	95	95
Supplementary feeding		
Registered for FMP	97	91
Mean feeding days	102	NA
Weight monitoring		
Monthly	1.7	2.8
Not weighed	21.5	16.0

Socio-economic variables	AAH	THR
Mean weight gain between two successive measurements	1.9	1.8
Proportion of pregnant women with weight gain of >1.5 kg/month	64	66
Nutritional status		
Women at risk with height <145cms	9	6
Women at risk with weight <39 kgs	6	4
Women at risk of low height and weight	2.3	0.9
Chronic energy deficiency	14	10
Lactating Mothers		
Outcome of earlier pregnancy		
Abortions	3	4
Live births	95	94
Still births and Intra uterine deaths	1	1
Antenatal Care		
Early registration (< 12 weeks of gestation)	92	93
Attended ANC =5 times	89	94
Received two doses of TT immunization	96	98
Institutional deliveries	94	97
Birth weight recording on the same day	95	97
Low birth weight (<2.5 kgs)	13	13

Supplementary Feeding during pregnancy		
Registered for FMP	89	94
Mean feeding days	130	NA
Proportion of women participated for > 150 days	28	NA
Proportion of women participated regularly (=21days/month)	2.4	NA
Consumption of IFA tablet along with FMP	18	NA
Weight monitoring – during pregnancy		
Weight recording for 5 times	27	16
Mean interval between two weight recordings	5.6 weeks	5.3 weeks
Gained between 6-18 kg during pregnancy	66	60
Nutritional status Chronic energy deficiency	26	23
IFA tablet distribution		
Received and/consumed =100 IFA tablets during pregnancy	48	47
Received and/consumed =100 IFA tablets during Lactation	0.5	1.2

Supplementary feeding during lactation (mothers of 6-11 months children)		
Registered for FMP under AAH	89	96
Mean feeding days	94	NA
Proportion of women who participated in feeding for = 150 days	6.8	NA
Proportion of women who participated regularly (=21 days/month)	14.2	NA
Consumption of IFA tablet along with FMP	9	NA
Registration for FMP < 7 days of delivery	54	NA
Child Feeding Practices – Infants (<12 months)		
Breastfeeding within one hour of birth	54.8	48.4
Initiation of complementary feeding by 6 months	9.6	12.8
Postnatal care		
Institutional deliveries	94	97
Birth weights		
Birth weight recording on the same day	94.9	97.2
Low birth weight (<2.5 kgs)	12.8	13.1
Prevalence of anaemia		
Pregnant women age 15-49yrs (Hb< 11gm/dl)	71.5	72.8
Lactating mothers (<12 months) (Hb< 12gm/dl)	73.5	72.8

3. DIET AND NUTRIENT ADEQUACY, NUTRITIONAL STATUS AND ITS DETERMINANTS AMONG ADOLESCENT AND ADULT WOMEN IN INDIA – SECONDARY ANALYSIS OF THE DATASETS OF RURAL AND TRIBAL POPULATIONS OF THE NATIONAL NUTRITION MONITORING BUREAU, INDIA

World Health Organization (WHO) has defined 'adolescence' as the period between 10 and 19 years of age. It is a period of rapid growth and maturation with high demand for adequate nutrition and thus vulnerable to effects of malnutrition such as reduced lean body mass, lack of muscular strength and decreased work capacity. About 88% of the world's adolescent population live in developing countries and adolescent girls constitute nearly one tenth of Indian population. Their current nutritional status will decide the well being of the present as well as the future generations. Undernourished adolescent girls are at greater risk of becoming stunted mothers who in turn deliver low birth weight babies. If not rehabilitated, they may become the next generation stunted mothers, thus, perpetuating the vicious cycle of malnutrition. After the 'first 1000 days of life', adolescence provides a second opportunity for girls to optimize nutritional status and health, attain 'catch up growth' and help break the intergenerational cycle. However,

data on dietary adequacy, nutritional status and its determinants during adolescence is sparse. As a part of the collaborative Initiative convened by the New York Academy of Sciences, USA calling for secondary analysis of available datasets on Adolescent Nutrition, the two large rural (2011-12) and tribal (2008-09) datasets of the National Nutrition Monitoring Bureau of India which included the anthropometric, socio-economic, demographic, dietary, hygiene, sanitation and health variables of adolescent and adult women living in rural and tribal areas of India were analysed for the purpose.

HYPOTHESIS & OBJECTIVES

Hypothesis: The diet and nutrient adequacy, nutritional status and their determinants among adolescent women differ from adult women

OBJECTIVES

- To describe the dietary intakes and identify nutrient gaps in relation to estimated average requirements (EAR) of adolescent women in comparison to adult women.
- To describe dietary quality in terms of dietary diversity scores (DDS) and probability of adequacy (PA) for each nutrient and mean probability of adequate micronutrient (MPA) intake by adolescent women in comparison to adult women.
- To describe nutritional status of adolescent women in comparison to adult women.
- To explore specific associations between adolescent girls and adult women as regards the dietary and non-dietary factors (socioeconomic, demographic, health care, hygiene and sanitation).
- To explore the dietary and non dietary factors that predict adequate nutritional status in adolescent women and how these factors differ from adult women.

All the objectives were tested using tribal and rural data sets separately.

METHODOLOGY

Study design and sample size: The two NNMB studies used a cross-sectional study design and a multistage stratified random sampling procedure. In the original survey, data has been collected on all available individuals belonging to different age, gender and physiological groups on the day of survey. In the present study, secondary data on 8,028 adolescent women and 28,035 adult women from rural households and 10,288 adolescent women and 35,232 adult women from tribal households were utilized. Dietary data was available on a subset i.e on 4,895 adolescent women and 14,837 adult women from rural households and 2,471 adolescent women and 9,608 adult women from tribal households.

Methodological Approach

The probability of adequacy (PA) for individual micronutirnts nutrients namely vitamins A, C, B1, B2, B3, B12, folate, iron, calcium and zinc was calculated as PA = CDFNORM [(observed individual intake - EAR)/SD]. The PA of individual nutrient was calculated based on age and gender specific EARs recommended by WHO and the MPA for each individual was calculated as an average of the sum of all PAs of ten nutrients. The percentage of calories from carbohydrates, proteins and fat was calculated and adequacy assessed by comparing with Acceptable Macronutrient Distribution Range (AMDR) suggested for India. Ten food groups were created to calculate the DDS as the sum of all food groups consumed by the individual in the previous 24Hr. Nutritional status was categorized according to BAZ scores in adolescents and BMI in adults as per WHO classification.

Statistical Evaluation

The data was analyzed using SPSS for Windows, version 19. Descriptive analyses were performed to provide general information on the socio economic and demographic

characteristics of the study population, their diet and nutrient intake and nutritional status. For continuous variables, mean and standard deviations for normally distributed data and median and inter quartile range for skewed data was computed. The differences between groups were compared by ANOVA and F test with post hoc of LSD. The non-parametric Kruskal Wallis test was performed for skewed distributions. Proportions were calculated for categorical variables and associations between nutritional status and various dietary and non dietary factors were studied using chi square test. All variables significant at P=0.10 in univariate analysis were considered for multivariate analysis. Stepwise logistic regression was performed to identify the dietary and non dietary determinants that predict adequate nutritional status in adolescent women and how they differ from adult women. The relationship between DDS and nutrient adequacy was assessed using Pearson's correlation coefficients. Multilevel model was used for adjusting nested nature of the data. A p value of 0.05 was considered as significant.

RESULTS

Dietary adequacy, nutritional status and its determinants among adolescent and adult women in the rural areas of India

- The mean SD age of adolescent girls was 14.2±2.79 y and of adult women was 42±14.88 y.
- The intake of 12 food groups (cereals & millets, pulses and legumes, GLV, other vegetables, roots and tubers, fruits, milk & milk products, eggs & flesh foods, nuts & oilseeds, fats & oils, sugar & jaggery, miscellaneous) was significantly lower in the younger adolescents and geriatric women compared to older adolescents and adult women respectively.
- In both adolescent girls and adult women, the percentage of calories contributed by carbohydrates (74.5 & 74.2 % respectively) was higher while that contributed from fats was lower (11.6 & 12.2 % respectively) compared to AMDR of 55-65% for carbohydrates and 20-30% for fats. Proteins contributed 10% of calories in both the groups and were comparable to the AMDR of 10-15%.
- The median micronutrient intakes in the younger adolescents and geriatric women was significantly lower compared to older adolescents and adult women respectively with respect to the intakes of all nutrients except thiamine and niacin which were below adequate levels (Table 1).
- PA of all nutrients & MPA in general was far from adequate in both the groups of women with the adolescent girls particularly younger girls being in more disadvantaged position (Table 2).
- The mean ± SD DDS score was significantly lower in adolescent girls (3.45 ± 1.20) compared to adult women (3.64 ± 1.21) when a minimum consumption of 15g for a food group to be counted was considered. A weak/moderate but significant correlation ($p<0.001$) was observed between the DDS and MPA in both adolescent and adult group, which further decreased after adjusting for dietary energy.
- The prevalence of undernutrition and overweight/obesity was higher in adult women (34 & 15% respectively) compared to adolescent girls (27 & 2% respectively).
- Factors; (OR(95%CI)) such as young age of adolescents; 1.88(1.56,2.26), a lower protein consumption 1.40(1.19,1.65), living in houses made of temporary material, 1.35(1.11,1.64) and absence of sanitary facility; 1.23(1.04,1.47) significantly predicted undernutrition (thinness) in adolescents.
- Factors such as advanced age 1.17(1.04,1.31), belonging to a scheduled 1.64(1,45,1.86) and backward community 1.16(1.06,1.28), living in houses made of temporary material 1.32(1.17,1.50), absence of electrification 1.32(1.20,1.5), a lower wealth status 1.63(1.41,1.87), engaged in labour work 1.24(1.15,1.34), a lower consumption of food energy 1.13(1.03,1.23), fat 1.11(1.02,1.20) and micronutrients 1.14(1.04,1.25) and absence of sanitary facility 1.72(1.57,1.88) significantly predicted undernutrition in adults.

Table 1. Median (IQR) intakes of micronutrients* – Age disaggregated – Rural

Micronutrients	Adolescents		Adults	
	10-14 y n = 2549	15-19 y n = 2346	20-59 y n = 12548	=60 y n = 2289
Calcium, mg	237.5 (150.3,380.7)	264.3 (171.4,424.8)	317.85 (200.4,510.3)	292 (173.9,479.9)
Vitamin A, µg	86.9 (46.5,181.3)	99.6 (54.1,189.1)	118.6 (64.0,224.8)	105.2 (53.8,214.0)
Thiamine, mg	0.9 (0.70,1.30)	1.1 (0.70,1.50)	1.2 (0.80,1.60)	1.0(0.70,1.40)
Riboflavin, mg	0.6 (0.40,0.80)	0.7 (0.50,0.90)	0.7 (0.50,1.0)	0.6 (0.40,0.90)
Niacin, mg	10.6 (7.70,14.30)	12.1 (8.70,16.80)	13.5 (10.0,17.9)	11.1 (8.10,15.10)
Vitamin C, mg	21.4 (10.70,40.70)	25 (12.80,48.12)	28.2 (14.50,54.50)	24.6 (11.90,48.45)
Iron, mg	9.5 (6.30,14.8)	10.9 (7.30,17.10)	11.9 (8.0,17.90)	10.5 (6.80,15.70)
Folate, µg	93.7 (60.2,138.6)	104.7 (68.9,153.4)	114.1 (73.6,166.2)	94.4 (59.9,139.8)
Zinc , mg	6.32 (4.73,8.51)	7.26 (5.44,9.55)	7.94 (6.09,10.16)	6.6 (4.81,8.72)
B_{12}, µg	0.0 (0.0, 0.10)	0.0(0.0,0.10)	0.1 (0.0,0.10)	0.1 (0.0,0.10)

**The intake of all nutrients are significantly different between age groups (p<0.001) by ANOVA*

Table 2: Probability (Mean ± SD) of nutrient adequacy – Age disaggregated – Rural

Micronutrients	Adolescents		Adults	
	10-14,y n = 2549	15-19, y n = 2346	20-59 y n = 12548	=60 y n = 2289
Calcium, mg	0.012 ± 0.09	0.02 ± 0.14	0.1 ± 0.27	0.07 ± 0.23
Vitamin A, µg	0.13 ± 0.31	0.15 ± 0.33	0.22 ± 0.38	0.19 ± 0.37
Thiamine, mg	0.52 ± 0.46	0.63 ± 0.44	0.70 ± 0.41	0.57 ± 0.45
Riboflavin, mg	0.25 ± 0.38	0.33 ± 0.42	0.41 ± 0.43	0.30 ± 0.42
Niacin, mg	0.40 ± 0.41	0.53 ± 0.43	0.67 ± 0.39	0.52 ± 0.42
Vitamin C, mg	0.32 ± 0.44	0.38 ± 0.46	0.38 ± 0.46	0.34 ± 0.45
Iron, mg	0.08 ± 0.23	0.02 ± 0.09	0.03 ± 0.11	0.02 ± 0.08
Folate, µg	0.008 ± 0.07	0.02 ± 0.10	0.02 ± 0.12	0.02 ± 0.09
Zinc , mg	0.22 ± 0.35	0.35 ± 0.41	0.62 ± 0.41	0.45 ± 0.43
B_{12}, µg	0.003 ± 0.06	0.01 ± 0.08	0.01 ± 0.08	0.01 ± 0.07
MPA	**0.19 ± 0.17**	**0.24 ± 0.18**	**0.32 ± 0.19**	**0.25 ± 0.19**

**The MPA & PA of all nutrients except B12 are significantly different between age group (p<0.001) by ANOVA*

Dietary adequacy, nutritional status and its determinants among adolescent and adult women in the tribal areas of India

- The mean ± SD age of the adolescent girls was 14.3 ± 2.92 y and that of adult women was 39.3 ± 14.41 y.
- The intake of cereals and millets, pulses and legumes, roots and tubers, fats & oils, sugar & jaggery was significantly lower in the younger adolescents and geriatric women compared to older adolescents and adult women respectively. The consumption of GLV, other vegetables fruits, milk & milk products, eggs & flesh foods, nuts & oilseeds was almost negligible in both the groups unlike the rural counter parts
- In both the adolescent girls and adult women, the percentage of calories contributed by carbohydrates (78% in each group) was higher while that contributed from fats was lower (8.7 & 8.9 % respectively) compared to AMDR of 55-65% for carbohydrates and 20-30% for fats. Proteins contributed 9.9 & and 9.6% of calories in adolescent and adult groups respectively and were comparable to the AMDR of 10-15%.
- The median micronutrient intakes in the younger adolescents and geriatric women was significantly lower compared to older adolescents and adult women respectively with the intakes of almost all nutrients being below the adequate level (Table 3).
- PA of all nutrients MPA in general was far from adequate in both the groups of women with the younger adolescent girls being the most disadvantaged (Table 4). The risk of micronutrient inadequacy was higher in tribal population compared to rural population.
- The mean ± SD DDS score was significantly lower in adolescent girls (3.53 ± 1.20) compared to adult women (3.58 ± 1.25) when a minimum consumption of 15g for a food group to be

Table 3: Median (IQR) intakes of micronutrients* – Age disaggregated – Tribal

Micronutrients*	Adolescents		Adults	
	10-14 y n = 1280	**15-19 y n = 1191**	**20-59 y n = 8803**	**=60 y n = 805**
Calcium, mg	178.4 (118.73,284.1)	210.8 (138.80,40.00)	217.7 (143.70,367.60)	191.6 (115.95,314.15)
Vitamin A, µg	65.60 (34.85,137.40)	75.0 (40.00,146.70)	76.2 (39.40,150.90)	60.6 (28.95,123.05)
Thiamine, mg	0.9 (0.6,1.20)	1.0 (0.6,1.40)	1.0 (0.70,1.40)	0.9 (0.60,1.30)
Riboflavin, mg	0.4 (0.30,0.60)	0.5 (0.40,0.60)	0.5 (0.40,0.60)	0.4 (0.30,0.60)
Niacin, mg	10.5 (8.10,13.90)	12.0 (9.40,16.4)	13.3 (10.0,18.10)	11.4 (8.50,15.60)
Vitamin C, mg	15.9 (6.73,37.80)	20.4 (8.10,43.10)	21.4 (9.10,44.90)	18.3 (6.60,37.25)
Iron, mg	8.3 (5.63,12.18)	9.2 (6.50,13.10)	9.7 (6.90,14.10)	8.3 (5.70,12.60)
Folate, µg	86.8 (57.02,125.50)	93.4 (63.50,136.20)	95.3 (63.40,138.10)	76.6 (52.70,116.05)
Zinc , mg	5.9 (4.60,7.60)	6.9 (5.40,8.50)	7.2 (5.60,9.00)	6.0 (4.40,8.00)
B_{12}, µg	0.0 (0.0,0.0)	0.0 (0.0,0.0)	0.0 (0.0,0.0)	0.0 (0.0,0.0)

**The intake of all nutrients are significantly different between age groups ($p<0.001$) by ANOVA*

Table 4: Probability (Mean ± SD) of nutrient adequacy – Age disaggregated - Tribal

Micronutrients	Adolescents		Adults	
	10-14,y n = 2549	15-19, y n = 2346	20-59 y n = 12548	=60 y n = 2289
Calcium, mg	**0.01 ± 0.09**	**0.03 ± 0.14**	**0.06 ± 0.23**	**0.05 ± 0.19**
Vitamin A, µg	0.15 ± 0.35	0.14 ± 0.34	0.16 ± 0.35	0.12 ± 0.31
Thiamine, mg	0.46 ± 0.45	0.56 ± 0.46	0.59 ± 0.45	0.48 ± 0.46
Riboflavin, mg	0.06 ± 0.20	0.12 ± 0.28	0.13 ± 0.29	0.09 ± 0.26
Niacin, mg	0.39 ± 0.40	0.54 ± 0.42	0.67 ± 0.39	0.55 ± 0.42
Vitamin C, mg	0.29 ± 0.44	0.33 ± 0.45	0.30 ± 0.44	0.25 ± 0.42
Iron, mg	0.07 ± 0.21	0.14 ± 0.08	0.02 ± 0.09	0.02 ± 0.11
Folate, µg	0.003 ± 0.03	0.009 ± 0.08	0.02 ± 0.11	0.01± 0.09
Zinc , mg	0.16 ± 0.31	0.27 ± 0.37	0.54 ± 0.41	0.37± 0.42
$B_{12,}$ µg	0.007 ± 0.08	0.003 ± 0.05	0.007 ± 0.09	0.003 ± 0.06
MPA	0.16 ± 0.14	0.20 ± 0.15	0.25 ± 0.16	0.19 ± 0.16

**The MPA & PA of all nutrients are significantly different between age group (p<0.001) by ANOVA*

counted was considered. A weak but significant correlation (p<0.001) was observed between the DDS and MPA in both adolescent and adult group, which further decreased after adjusting for dietary energy.

- The prevalence of undernutrition and overweight/obesity was higher in adult women (34 & 15% respectively) compared to adolescent girls (27 & 2% respectively).
- Factors; (OR(95%CI)) such as late adolescence; 0.27(0.22,0.33), a lower fat consumption 1.45(1.17,1.78) and unprotected source of potable water 2.03(1.32,3.11) significantly predicted undernutrition (thinness) in adolescents.
- Factors such as advanced age 1.44(1.21,1.71) and unprotected source of potable water 1.25(1.03,1.53) significantly predicted undernutrition in adults.

Study Limitations

- Use of one time 24hr. recall method to represent actual dietary intake which does not reflect usual intakes of the study population.
- The nutrients obtained only foods is reported and not that from supplements, this may not reflect total intake.
- Cross sectional study design does not allow for cause and effect relationship.
- FCT are based on raw foods and hence effects of processing/nutrition retention factors was not employed.

CONCLUSIONS

- There is a need to develop and promote food based recommendations to improve dietaries with reference to micronutrients among adolescents in particular in both the rural and tribal areas.
- Strategies such as targeted food supplementation through government safety net programs, fortification of staple foods with essential micronutrients through food industry networks, bio-

fortification through agriculture partners need to be formulated, tested by clinical trials and scaled up.

- Nutrition sensitive education programs needs to be taken up to generate awareness and uptake to help reduce nutrient gaps.
- More research is required to generate an evidence base on optimal diet diversity and nutrient adequacy among adolescents.
- There is a need to generate better quality data including multiple day recalls to account for intra individual variability in nutrient intakes to better guide nutrition interventions.
- The social, economic, well being determinants of nutritional status need further research.
- Targeted policies and programs central to the adolescence period are required to improve their growth and nutritional status in both the rural and tribal areas.

4. ASSESSMENT OF CURRENT NUTRITIONAL STATUS OF <5YR CHILDREN AND PERFORMANCE OF ABM PROJECT IN THE DISTRICTS OF MADHYA PRADESH

National Institute of Nutrition had carried out district specific survey in the year 2009-10, with the objective of assessing the nutritional status of under 5 year children and infant and child feeding practices of mothers of under 3 year children in rural areas of Madhya Pradesh. Based on the results, the Government of Madhya Pradesh had launched "Atal Bal Arogya Evam Poshan Mission" (ABM) in April 2010 to bring about a systematic reduction in child malnutrition and also child morbidity and mortality. The present study was undertaken to assess change in nutritional status over the period of 5 years and also performance of ABM in all districts of Madhya Pradesh.

OBJECTIVES

1. To assess the nutritional status of <5 year children in terms of anthropometry such as heights & weights, and prevalence of clinical signs of nutritional deficiency.
2. To assess the nutritional status of mothers by BMI and prevalence of anaemia by haemoglobin estimation, on all the pregnant and lactating mothers of the index children covered for the survey,
3. To assess the prevalence of morbidities among <5 year children during the preceding fortnight.
4. To assess the infant & young child feeding (IYCF) practices of mothers of under 3 years children,
5. To assess district wise performance of the functionaries of the ABM/ICDS projects,
6. To assess the changes, if any, in the prevalence of undernutrition among <5 year children over a period of time by comparing with the results of the earlier study (2009-10) carried out by the NIN and Annual Health Survey.

METHODOLOGY

Sampling Design: It was a community based cross sectional operational research study by adopting systematic random sampling procedure.

Salient findings of the study

- A total of 30585 HHs were covered from 51 districts and 37924 children were covered for anthropometric measurement, IYCF practices for the present study.
- Majority (93.2%) were Hindus, 28% belonged to scheduled tribe, 50% were living in nuclear families, about 80% fathers and 68% mothers were literate.
- About 55% families were residing in kutcha houses, 22% had access to tap water for drinking purpose and 33% HHs had sanitary latrine facility and were using it.
- There was improvement in the IYCF practices over the periods.
- Utilization of Antenatal care (ANC) during last pregnancy had improved from 79% during 2010 to 98% in current survey, utilization of at least 3 ANCs has increased from 36% to 57% in current study.
- About 76% of pregnant women registered for ANC before 12 weeks of gestation as against 40% in the previous survey.
- Consumption of ≥ 90 IFA tablets during pregnancy had improved from 20% to 59.4% in present survey, also the proportion of women who consumed any IFA tablets has improved from 70% to 95% in current survey.
- About 81% women had attended Mangal Diwas at AWC and 52% received hot cooked food on that day.
- Institutional delivery had also improved from 79% during previous survey to 85% in current survey.
- The prevalence of low birth weight (<2.5kg) is 14% in current survey as against 19% in the previous survey.
- Initiation of breastfeeding within 1 hour had improved from 26% during previous survey to 58% in current survey.
- About 93% of 0-5 month children were solely breast fed in current survey as compared to 71% in the previous survey, while 43% were exclusively breast fed up to 6 months during previous survey and is 64% in the current survey.
- About 71% children (6-11 months) and 95% children (12-35 months) received THR from Anganwadi center in current survey as against 72% in 2011-12.
- About 93% children (12-23 months) were fully immunized in present survey as against 84% during 2010-11.
- Hand washing practices with soap among mothers before feeding the child had improved from 24% during previous survey to 43% in the current survey.
- The prevalence of undernutrition (<Median -2SD) among <5 year children such as underweight, stunting and wasting had declined from 52%, 49% and 26% during 2010-11 to 41%, 43% and 22% respectively in the present survey.
- The prevalence of chronic energy deficiency among NPNL women was 36%.

5. DIET AND NUTRITION STATUS OF URBAN POPULATION AND PREVALENCE OF OBESITY, DIABETES, HYPERTENSION, DYSLIPIDEMIA AMONG URBAN ADULTS

NNMB has conducted its first survey in urban areas during 1984 among high income group (HIG), middle income group (MIG), low income group (LIG), slums and industrial laborers in the cities of Trivandrum and Cochin (Kerala), Chennai (Tamil Nadu), Bangalore and Mysore (Karnataka), Hyderabad (Andhra Pradesh), Nagpur and Pune (Maharashtra). Repeated surveys among slum population were carried out during 1993-94 in the cities of Ahmedabad, Bangalore, Bhopal, Bhubaneswar, Cuttack, Hyderabad, Lucknow, Chennai, Nagpur and Trivandrum, where the head quarters of the state units of NNMB are located.

Several studies were carried out in the developing countries, including India, have been reporting increased in an epidemic proportion in the prevalence of diet related chronic Non-Communicable Diseases (NCDs) like overweight and obesity, insulin resistance, diabetes mellitus, hypertension, other cardiovascular diseases (CVDs), cancers etc., especially among urban population. Major causes for the increasing incidences of NCDs may be attributed to nutrition transition, sedentary behavior and unhealthy lifestyles. In addition, demographic and health transition, epigenetic, gene-environmental interactions, maternal and childhood undernutrition are leading causes for increase in occurrence of NCDs in India.

The World Health Organization (WHO) reported that hypertension (HTN) is the third 'killer' disease, accounting for one in every eight deaths world wide. It has been estimated that the number of hypertensives may rise globally from 118 million in 2000 to 214 million in 2025. Prevalence of diabetes is also increasing in urban population of India and is important risk factors for hypertension and CVD. As per World Health Organization (WHO) reports, in India, about 32 million people had diabetes in the year 2005, and in fact, India is considered to be the diabetic capital of the world. The International Diabetes Federation (IDF) estimates the total number of diabetic subjects in India to be around 40.9 million, which is expected to rise to 69.9 million by the year 2025.

Therefore, keeping in view the magnitude of NCDs, a survey was carried out with the objective to assess 'diet and nutritional status of urban populations' as well as health and nutritional status and the prevalence of obesity, hypertension, diabetes and dyslipidemia among representative urban population in16 states of India, including 6 new NNMB States.

OBJECTIVES

Primary Objectives

To assess diet and nutritional status of urban population, prevalence and determinants of obesity, hypertension, diabetes mellitus and dyslipidemia among urban adults (≥18years) in all the 16 NNMB States viz., Andhra Pradesh, Gujarat, Karnataka, Kerala, Madhya Pradesh, Maharashtra, Odissa, Tamilnadu, Uttar Pradesh, West Bengal, Assam, Andaman and Nicobar Islands, Bihar, Rajasthan, Puducherry and New Delhi.

Secondary Objectives

1. To assess the current status of food and nutrient intake among different age, gender, physiological activity and groups of urban population in all the16 NNMB states in India.
2. To assess the current nutritional status of all the available individuals in the selected HHs in each state in terms of anthropometry and clinical examination.

3. To assess the history of morbidity during previous fortnight among all the individuals covered for anthropometry.
4. To assess the prevalence and determinants of obesity, hypertension, *diabetes mellitus* and dyslipidemia among the urban adult men and women (≥18years).
5. To assess body composition using measurements of fat fold thickness at various sites, bio-electrical impendence assessment (BIA) among adults covered for anthropometry.
6. To assess knowledge and practices on obesity, hypertension, diabetes and dyslipidemia, including lifestyles and risk behaviors of adults.
7. To assess the infant and young child feeding (IYCF) practices among the mothers of <3 year children.

METHODOLOGY

Study design

It is a community based cross sectional study with multi-stage random sampling procedure.

Study setting

In the first stage four cities with more than one lakh population from each state were selected randomly and one capital city of the respective NNMB states was selected purposely. In the second stage, about 15 Municipal wards were selected randomly from each of the 5 selected cities/towns in each state.

Sample size estimation

The sample size required for each state for various investigations among different target groups of individuals are given in the below Table.

Table: Sample size covered for different variables

Investigations	No. of HHs	Age/Gender/ Physiological Group (≥18yrs)	Assumed Prevalence	C.I	Relative precision	Sample size required	Design Effect	3SE groups	Total Sample required per State*
Anthropometry	3600	All the available individuals in the selected 3600 HHs							
Clinical Examination									
History of morbidity									
Diet survey	900	All the individuals part a king meals in the selected HHs							
Diabetes Mellitus	3600	Men	12%	95%	20%	704	1.5	3	3484
		Women				704	1.5	3	3484
Blood pressure	3600	Men	20%	95%	20%	384	1.5	3	1899
		Women				384	1.5	3	1899
Lipid Profile	3600	Men	20%	95%	20%	384	1.5	3	1899
		Women				384	1.5	3	1899
Knowledge & Practice on HTN &DM and lifestyles	3600	Men	-	-	-	-	-	-	3484
		Women	-	-	-	-	-	-	3484
BIA	3600	Men	-	-	-	-	-	-	3484
		Women	-	-	-	-	-	-	3484

**Inclusive of 10% non-response* SE-Socio-Economic group*

Procedure for selection of samples

Cities, wards and Census Enumeration Blocks (CEB)

In each NNMB state, capital city of the state was covered purposely for the present urban survey. For the selection of rest of the four cities/towns in each NNMB state, all the cities/towns with more than one lakh population were arranged in descending order and four cities/towns were selected from the list by systematic random sampling method. List of municipal wards for all the selected cities were obtained from the respective city administration. Fifteen municipal wards were randomly selected from the list from each city.

From each of the selected ward, 6 Census Enumeration Blocks (CEB)/ Geographical areas (ward divided into 6 areas based on streets/*mohallas*) were selected randomly. About 8HHs in each CEBs/ geographical area (GA), a total of 48 HHs were covered from each ward for all the required investigations.

Investigations

The following investigations were carried out in the selected HHs/ individuals:

- Collection of HH and Socio-demographic particulars,
- Measurement of anthropometric parameters such as height, weight, mid upper arm (MUAC), waist, and hip circumference and fat fold thickness at triceps, biceps, sub-scapular and suprailiac sites**,**
- Clinical examination for the presence of nutritional deficiency signs and symptoms and collection of history of morbidity in all the subjects covered for anthropometry.
- 24 hour recall method of diet survey in every 4th HH selected for anthropometry (2diets per CEB/GA).
- Food frequency questionnaire among all available adults of ≥18 years in the selected HHs.
- Measurement of blood pressure among all the available adults of ≥18 years in the selected HHs.
- Estimation of fasting blood glucose among all the available adults of ≥ 18 years in the selected HHs.
- Estimation of lipid profile in one adult men or women (alternatively) from each of the selected HHs in all nuclear families. In case of joint families at least one adult man and adult women covered for lipid profile.
- Collection of dry blood spot (DBS) samples for biodepository, for future analysis of epigenetic and genetic epidemiology.
- Gathering information on Infant & young child feeding (IYCF) practices of mothers of <3 year children.
- Information on knowledge and practices of adults about hypertension, diabetes, hyperlipidemia and health and nutrition and lifestyles, including risk behaviors.

Training and Standardization of investigators

All the medical officers, nutritionists, social workers and laboratory technicians of all the NNMB units were given orientation training and standardization in all the methodologies and the proposed investigations at the NIN for 3 weeks before initiation of the actual survey. During the training, emphasis was also given to achieve acceptable intra and inter-individual variations with respect to all the above measurements.

Quality control

To ensure the quality of data collection process, anthropometric measurements, clinical examination, blood pressure measurements etc., were repeated in a sub-sample of HHs in all the cities by the senior staff of CRL periodically, through surprise and random visits.

Salient findings of the study

- A total of 1,71,928 individuals were covered for socio-demographic particulars, anthropometry and clinical examination from 52,577 HHs in 1,097 wards from 16 NNMB states.
- The blood pressure measurements are available for 39,415 men and 54,436 women, while the lipid profile was estimated on 18,392 men and 22,989 women.
- A total of 5,642 mothers of <36 month children were interviewed to collect information on antenatal care, infant and young child feeding (IYCF) practices as well as coverage for immunization, iron & folic acid tablets and massive dose of vitamin A supplementation.
- The average intake of cereals and millets was 320.3 g/CU/day, which ranged from a low 218.2 g in Rajasthan to a high 503.1g in Assam. The consumption of millets was observed to be high in the states of Maharashtra (37.7g), Gujarat (17.4g) and Karnataka (13g) while its consumption was negligible in other States.
- The average consumption of pulses & legumes was about 41.8g/CU/day, which is higher than the suggested level of 40g, in majority of the States, except in Kerala (35.0g), Andhra Pradesh (31.7g), Maharashtra (34.4g), Gujarat (36.0g), West Bengal (27.9g), and Rajasthan (29.3g).
- The median intake of energy was 1931Kcal which is less than RDA of 2320Kcal in all the States except Assam (2999.4Kcal).
- The median intake of iron was 13.2mg and was less than RDA of 17mg. The consumption was higher than RDA in the States of Assam (19.9mg), Madhya Pradesh (18.2mg) and Orissa (17.4mg).
- The prevalence of underweight, stunting and wasting among urban preschool children was 25.1%, 28.7% and 15.6%, respectively.
- The prevalence of Bitot's spot, the objective sign of vitamin A deficiency was 0.1% and was found high in Uttar Pradesh (0.7%).
- The prevalence of hypertension among men and women was 38% and 29% respectively and it was high in the state of Assam (45.8%) in men and highest in the state of Kerala (38.2%) among women.
- The prevalence of diabetes among men was 28.1% and ranged from a low 17.6% in Madhya Pradesh to a high 41.2% in Puducherry, while among women, it was 23.3% ranged from a low 13.6% in Orissa to a high 36.6% in Puducherry.
- About 40% men and 28% women were observed to have high triglyceride (≥150mg/dL) levels in their blood.

The present urban diet and nutrition survey revealed that the intakes of majority of foods and nutrients were lower than the recommended levels of ICMR among urban population. The prevalence of wasting was very high even among urban preschool children. The prevalence of hypertension, diabetes, dyslipidemia was higher among urban population. Therefore, there is a need to educate the population on healthy diet and lifestyles to prevent and control non-communicable diseases among urban population.

II. MICROBIOLOGY AND IMMUNOLOGY

1. EFFECT OF PROBIOTICS SUPPLEMENTATION ON BACTERIAL VAGINOSIS (BV) IN PREGNANT WOMEN

***Primary Objective*:** To study the effect of oral probiotics supplementation on bacterial vaginosis (BV) cure and relapse in pregnant women.

Secondary objectives: Effect of Probiotics on infant stool flora and breast milk, birth weight and on maternal serum cytokine.

STUDY DESIGN

It was a double blind placebo controlled study conducted to determine the effect of probiotic supplements on BV in pregnant women. Pregnant women in third trimester (26 to 30 weeks of gestation), visiting the outpatient department of the government maternity hospital, was the study population. These subjects were screened for BV based on Nugent's score and Amsel's criteria and were randomized to receive either probiotic capsule or placebo daily till delivery. General and gynecological examinations were performed to evaluate general health. For randomization, computer generated grouping was performed using STATA. Institute Ethical approval (CRI/I/2014) and DCGI (CT-DRUGS/180/2012, Drugs Controller General of India) approval were obtained for the study. The study was registered in ICMR-CTRI (CTRI/ 2013/ 01/ 003337).

Sample size calculation

Sample size for cure and relapse of BV: Assuming 80 % cure from BV in the probiotic and antibiotic treated groups and 60% cure in the placebo and antibiotic treated group 40 women per arm was required to obtain significance at 5%, with 80% power. Expecting huge attrition in the post natal follow up, 140 women were randomized with 70 in probiotic and placebo (control) arms.

Sample size for vaginal and fecal (gut) microbiome: Due to funds constraint, vaginal and fecal microbiome analysis was performed only in 16 samples each from pregnant women with BV and pregnant women with normal vaginal flora. And after supplementation, 8 from placebo and 8 from probiotic were chosen.

Inclusion and exclusion criteria

The inclusion criterion was pregnant women in their third trimester, willing to participate in the study after a written and informed consent. Subjects with multiple gestation, cervical incompetence (circulage in current gestation), fetus with major congenital malformations in current gestation, insulin dependent diabetes mellitus, systemic arterial hypertension under medication, chronic asthma requiring intermittent therapy, HIV positive, continuous or recent corticotherapy (or any other medical or surgical complications in present gestation) were excluded. Also women aged less than 19 or more than 35, participating in other clinical trial and women who had intercourse in the last 24 hours were excluded.

INTERVENTION

The test preparation consisted of oral probiotic capsule containing 2-5X10^8 CFU each of L.rhamnosus-GR1 and L.fermentum (reuteri) RC14, and the placebo contained only dextrose

sugars. Both the capsules were similar in color and appearance and were packed in identical strips. Probiotics and placebo capsules were procured from CHR-HANSEN, Denmark.

Randomization and blinding

The random numbers were generated by computerized randomization process called STATA. One of the senior scientists at the Institute was designated to code the probiotic and placebo capsules. Investigators, statistician and the subjects of the study were blind to the treatment. The intervention was provided to the center. Enrolled 140 subjects were randomized into two groups, A and B, with 70 subjects per group, but all the enrolled 140 subjects were treated with local antibiotic (Clindamycin) for 7 days as per WHO guideline.

Follow-up (Flow chart - given in page 20)

Visit 1: Pregnant women in third trimester were enrolled based on inclusion criteria and were screened for BV after obtaining a written consent. For diagnosing BV by Nugent's score, vaginal samples were collected from the lateral vaginal wall by gentle rotation of Dacron swab. For diagnosing BV by Amsel's criteria, wooden spatula was used to collect samples using a non-lubricated speculum for pH determination by paper strips and amine odor evaluation.

Visit 2: Subjects found positive for BV were recruited and were given a serial number as per randomization. Clinical and anthropometric data were collected. For bacterial DNA isolation, vaginal discharge was collected from the lateral vaginal wall using sterile Dacron swabs under a vaginal speculum without any use of lubricants. Supplementation was initiated and compliance was ensured by collecting empty strips in the following visit. Subjects were also inquired and encouraged to take the supplements regularly over phone.

Visit 3: After 30 days supplementation, vaginal swab, blood sample and stool sample of the subjects were collected. Empty strips of the probiotic and placebo supplementation were collected and compliance recorded.

Visit 4: 5 days after delivery, neonatal anthropometry, neonatal fecal sample and mother's breast milk were collected.

CLINICAL AND EXPERIMENTAL METHODS

Collection of Biological Samples

i) Maternal blood was collected in EDTA tubes for Hb estimation and in BD vaccutainer blood collection tubes for serum separation and Cytokine estimation.

ii) *Maternal fecal sample:* Stool samples were collected in sterile DNAse and RNAse free containers. 250 mg was weighed and washed with PBS (phosphate buffer saline) and then processed for DNA isolation.

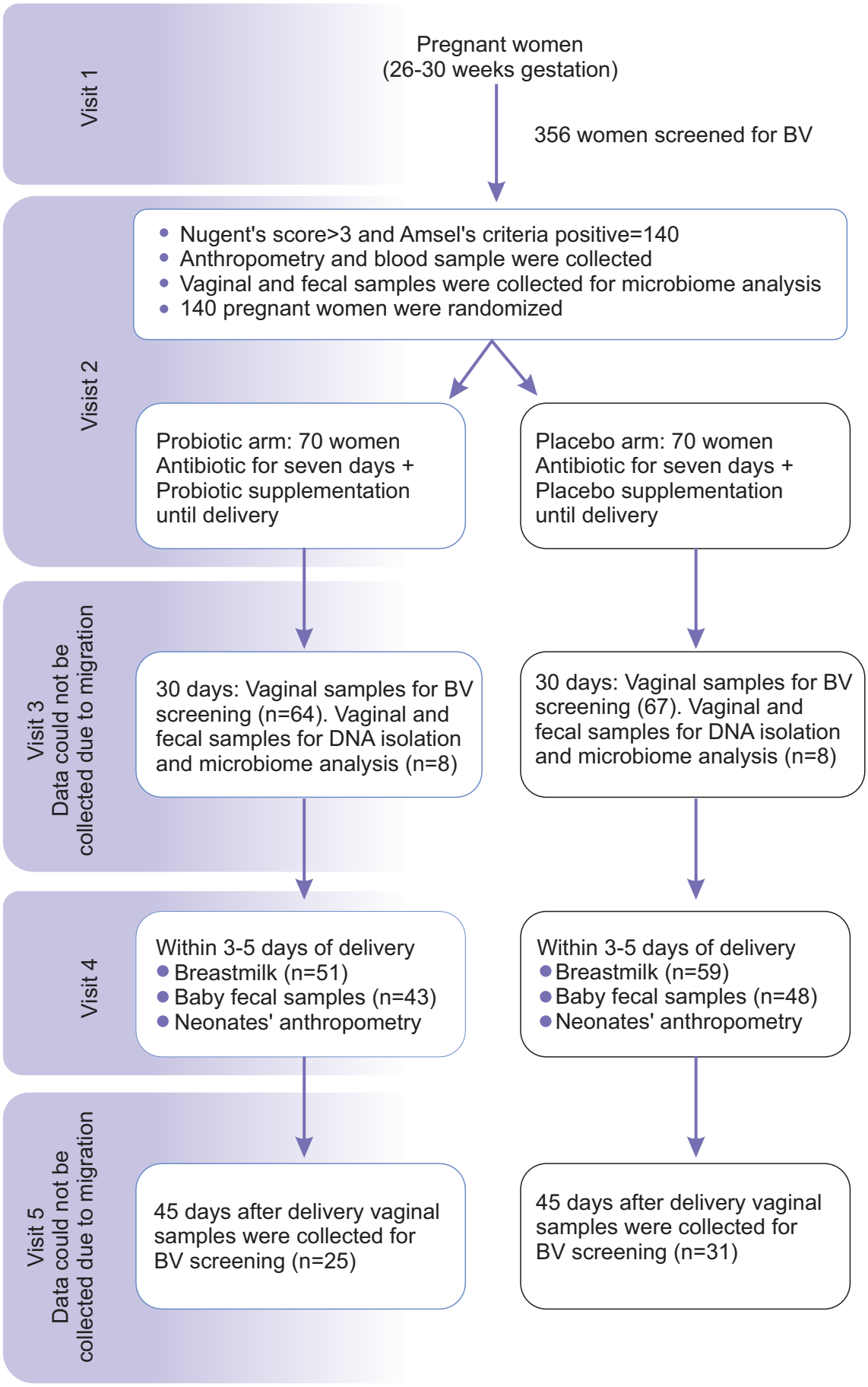
iii) Vaginal swab samples were collected in phosphate buffer saline (PBS) for Lactobacilli Species identification, quantification and typing.

iv) *Maternal Breast milk:* 3ml of breast milk sample (5 days post-natal) was collected in sterile DNAse and RNAse free non-pyrogenic tubes and processed for DNA isolation and for quantification of *lactobacillus* and bifidobacteria species.

Neonatal Fecal sample: Stool sample (5 days post natal) was collected in sterile DNAse and RNAse free containers. 250mg fecal sample was weighed and washed with PBS and then was processed for DNA isolation and for quantification and typing by RT-PCR.

Bacterial Vaginosis (BV) diagnosis

This syndrome is characterized by typical malodorous vaginal discharge, pH > 4.5, amine odour with Whiff test, presence of clue cells (vaginal epithelial cells with borders obscured by large numbers of bacteria called 'clue' cells) and altered vaginal flora.


Visit 1
Pregnant women
(26-30 weeks gestation)
356 women screened for BV
Visist 2
Nugent's score>3 and Amsel's criteria positive=140
Anthropometry and blood sample were collected
Vaginal and fecal samples were collected for microbiome analysis
140 pregnant women were randomized
Probiotic arm: 70 women
Antibiotic for seven days +
Probiotic supplementation
until delivery
Placebo arm: 70 women
Antibiotic for seven days +
Placebo supplementation
until delivery
Visit 3
Data could not be
collected due to migration
30 days: Vaginal samples for BV screening (n=64). Vaginal and fecal samples for DNA isolation and microbiome analysis (n=8)
30 days: Vaginal samples for BV screening (67). Vaginal and fecal samples for DNA isolation and microbiome analysis (n=8)
Visit 4
Within 3-5 days of delivery
Breastmilk (n=51)
Baby fecal samples (n=43)
Neonates' anthropometry
Within 3-5 days of delivery
Breastmilk (n=59)
Baby fecal samples (n=48)
Neonates' anthropometry
Visit 5
Data could not be
collected due to migration
45 days after delivery vaginal samples were collected for BV screening (n=25)
45 days after delivery vaginal samples were collected for BV screening (n=31)

Bacterial Vaginosis by Nugent's score

Vaginal samples were smeared on glass slides and were gram stained. Each Gram-stained smear was evaluated for the following morphotypes under oil immersion (x 1,000 magnification): large gram-positive rods *(lactobacillus* morphotypes), small gram-variable pleomorphic rods (G. vaginalis morphotypes), small gram-negative rods *(Bacteroides* spp. morphotypes), curved gram-variable rods *(Mobiluncus* spp. morphotypes), and gram-positive cocci. Each morphotype was quantitated from 1 to 4+ with regard to the number of morphotypes per oil immersion field (0, no morphotypes; 1+, less than 1 morphotype; 2+, 1 to 4 morphotypes; 3+, 5 to 30 morphotypes; 4+, 30 or more morphotypes) by a microbiologist who was unaware of the clinical or microbiological findings for these women. Ten fields were scanned under (x 1,000 magnification and average was taken to score bacterial morphotypes (Table 1).

BV by Amsel's criteria

Amsel's criteria were also used in diagnosing the infection. A homogenous white vaginal discharge, amine or fishy odor when KOH is added to vaginal discharge and a vpH of more than 4.5 were the typical symptoms of infection and the presence of any of these three symptoms indicates infection.

Table 1. Nugent's scoring system (0 to 10) for Gram-stained vaginal smears

Score	*Lactobacillus* morphotypes	*Gardnerella* and *Bacteroides* spp. morphotypes	Curved gram-variable rods
0	4+	0	0
1	3+	1+	1+ or 2+
2	2+	2+	3+ or 4+
3	1+	3+	
4	0	4+	

DNA extraction for vaginal and fecal microbiome analysis

Swabs were thawed on ice and re-suspended in transport buffer (Amies Liquid Medium) by vortexing. Cells were transferred to a sterile DNase/ RNase free 2 ml tube where an enzymatic lysis step was carried out for 1 h at 37^{0}C as with minor modification. Samples underwent additional mechanical disruption using a bead beater (FastPrep instrument, Qbiogene, Carlsbad, CA, USA) set at 6.0 m/second for 30 seconds. The resulting lysate was further processed and purified using QIAamp DNA Mini kit (Qiagen, Manchester, UK) and the DNA was eluted in 100 µl of TE (10 mM Tris–HCl, 1 mM EDTA) buffer, pH 8.0. Stool DNA was isolated by using QIAamp DNA stool kit (Qiagen).

Next Generation Sequencing (NGS) on illumina MiSeq platform

The V3 hypervariable regions of 16S rRNA genes were amplified for sequencing using a forward and reverse fusion primer. The forward primer was constructed with the Illumina i5 adapter (5'-3') and the reverse primer was constructed with (5'-3') the Illumina i7adapter with 6 bp barcode. Amplifications were performed in 25 µl reactions with Ex Taq DNA polymerase, 1 µl of each 5 pM primer, and 50 ng of template. Reactions were performed on T100 Thermal cycler (#Biorad) under the following thermal profile: 950C for 5 min, then 25 cycles of 95^{0}C for 1 min, 60^{0}C for 1 min, 72^{0}C for 1 min, followed by one cycle of 72^{0}C for 7 min and 4^{0}C hold. Amplification products were visualized with Agarose gels. Products were cleaned with Agencourt AMPure XP (Beckman Coulter, Indianapolis, Indiana) and quantified using Qubit fluorometer (#Thermofisher scientific).

The 96 multiplexed samples were pooled into a single library for sequencing on the MiSeq. The pooled library containing indexed amplicons was loaded onto the reagent cartridge and then onto the instrument along with the flow cell. Automated cluster generation and paired-end

sequencing with dual index reads of 2 × 250 bp were performed in a single 39-hour run. On the instrument, the global cluster density and the global passed filter per flow cell were generated. The MiSeq Reporter software (Illumina) determined the percentage indexed and the clusters passing the filter for each amplicon or library. The raw data were configured in fasta files for R1and R2 reads. All sequencing was performed at Sandor Life Science Pvt. Ltd. India.

Sequencing data analysis

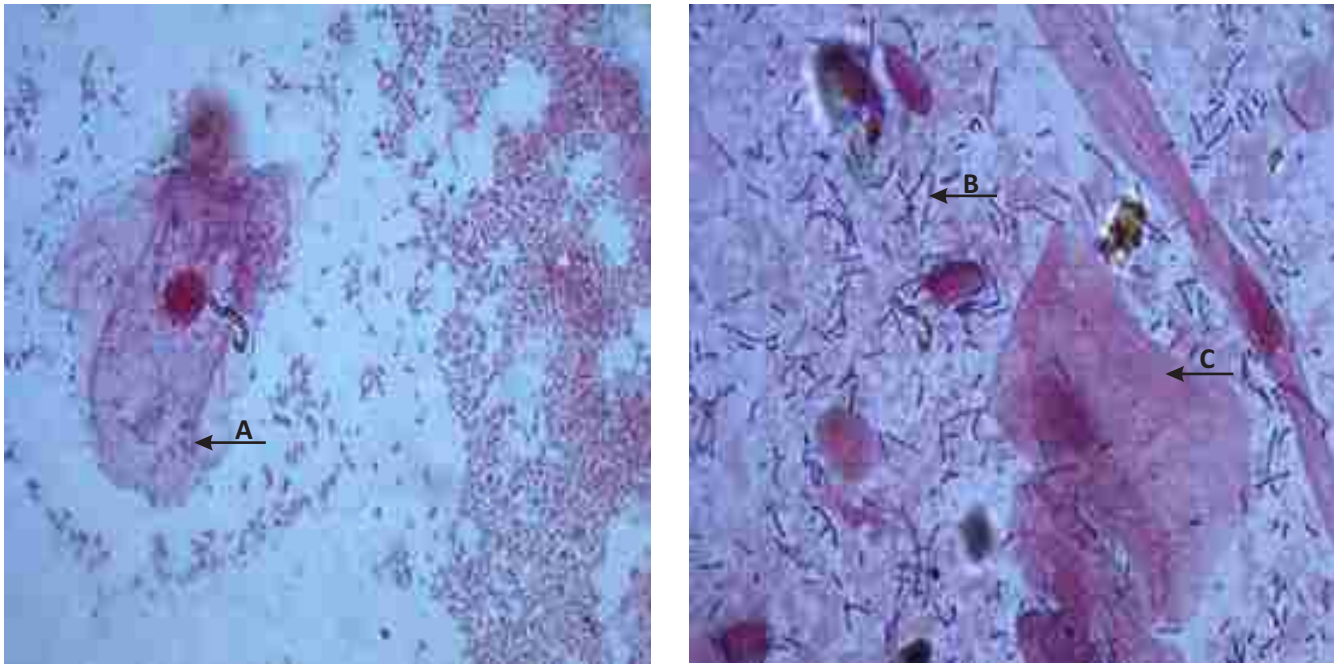
The short-read sequencing data sets were then analyzed using the operational taxonomic unit (OTU) approach. The trimmed sequences in FASTQ file was then uploaded to Metagenomic RAST server (MG-RAST). The analysis was performed in the MG-RAST server within Ribosomal Database Project (RDP) and taxonomic assignment was carried out with 97% homology match. Bacterial abundance data at phylum, class, order, family, genus and species levels were downloaded from the MG-RAST server.

STATISTICAL ANALYSIS

Principal Coordinate Analysis (PCoA) was performed to find clusters of similar groups of samples by QIIME. PCoA is an ordination method based on multivariate statistical analysis that maps the samples in different dimensions and reflects the similarity of the biological communities. A matrix using the UniFrac metric (unweighted) for each pair of environments was calculated. The first three principal dimensions were used to plot a three-dimensional graph. To test whether the results were robust to sample size we used a sequence-jackknifing technique in which a smaller number of sequences are chosen at random from each sample (1000 sequences). Unweighted distance metric accounts for presence/absence of taxons.

RESULTS

Fig 1. Vaginal smear images (x1,000 magnification) of infected (BV) and healthy flora

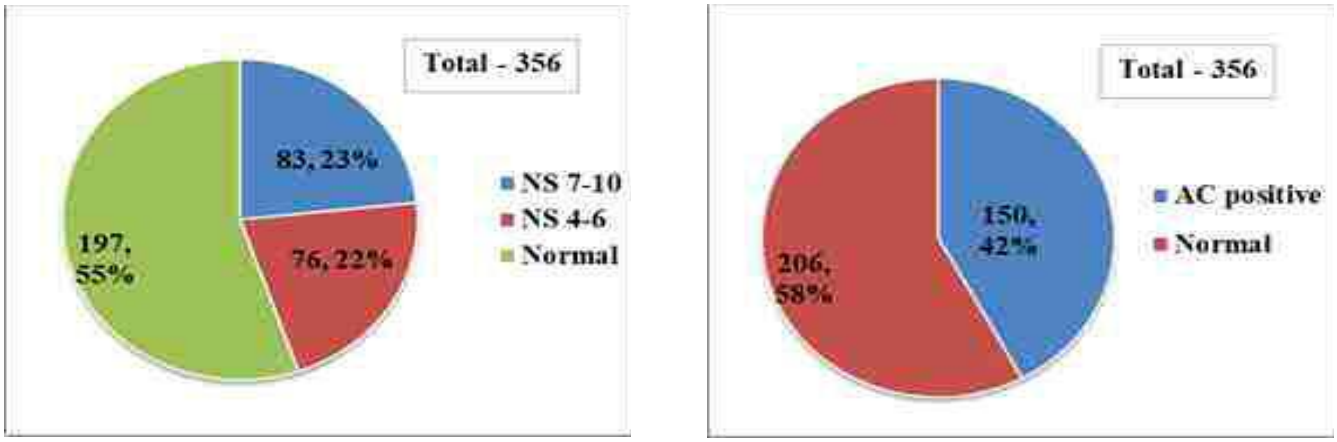


The first image depicts pleomorphic gram negative pathogenic bacteria in vaginal smear of BV positive women. Arrow A indicates typical clue cells (epithelial cell with obscured border and edged with pathogenic bacteria) commonly associated with BV. Second vaginal smear shows lactobacilli with healthy epithelial cells depicting vaginal flora of healthy women. B – Rod shaped gram positive lactobacilli, C – Healthy epithelial cell.

Clinical and Microbiome data before the Intervention:

Based on Nugent's score, of the 356, 83 (23.3 %) had BV, 76 (21.3%) had intermediate flora and 197 (55.3%) had normal vaginal flora. Based on Amsel's criteria, 150 (42.1%) had BV and the rest were normal 206 (57.8%) (Fig 2). Of these, 78 (22%) were positive for BV by both the methods, Nugent's score (7-10) and Amsel's criteria. While 188 (52.8%) were normal by both the methods.

Fig 2. Prevalence of BV by nugent's score and amsel's criteria subjects



NS- Nugent Score, AC- Amsel's Criteria

Vaginal microbiota diversity in pregnant women with normal flora and BV

For microbiome analysis, vaginal and fecal samples were collected from 16 pregnant women with normal vaginal flora and 16 pregnant women with BV. Using sequence-based method vaginal microbiota was characterized in pregnant women with BV. α diversity (Shannon matrix) (indicating bacterial diversity across BV subjects) ($p<0.0006$) and beta diversity (indicating diversity of bacterial species) in BV positive subjects were more in comparison with normal pregnant women ($p<0.0001$). At phylum level, firmicutes was the most abundant in normal pregnant women (98.2%) compared to BV subjects (68.7%). In BV subject, the next most abundant phylum was Actinobacteria (15.8%), whereas in pregnant women with normal vaginal flora Actinobacteria was negligible (0.31%).

Gut microbiota profile in pregnant women with normal flora and BV

Gut microbiome signatures between BV-negative vs. BV-positive pregnant women also revealed some differences in the intestinal carriage of several bacterial clades. The abundance of Firmicutes, was comparable between the two groups (60.9% control group; 61.2% BV group). Species-level analyses revealed less abundance of *P. copri* (8.2 vs. 16.7; P=0.11), *Dialister invisus* (0.7 vs. 4.1; P=0.001) and *Lactobacillus salivarius* (0.0 vs. 2.2; P=0.02) in women with BV. In contrast, Higher abundance of *Clostridium bifermentans* (5.2 vs. 0.0; P=0.0), *Clostridium disporicum* (5.0 vs. 1.1; P=0.04) and *Atopobium vaginae* (1.3 vs. 0.0; P=0.01) were observed in women with BV. Overall, the gut microbiota diversity in terms of Shannon-index appeared to be similar between the two groups; however, women with BV demonstrated a significantly higher observed species richness as compared with normal women (P=0.04).

Clinical and Vaginal and Fecal microbiome data in pregnant women with BV after oral intervention with probiotic (clindamycin for one week + probiotic for 30 days) versus placebo (clindamycin for one week + placebo for 30 days).

One hundred and forty (140) pregnant women, positive for BV by Amsel's criteria and with Nugent's score >3, were randomized and were supplemented probiotic or placebo till delivery.

Samples were collected from all supplemented women for Nugent's score and Amsel criteria at baseline, 30 days (after supplementation) and at 45 days after delivery. Delivery data were collected from all supplemented women soon after delivery. For microbiome analysis, at baseline and after 30 days of supplementation, vaginal and fecal samples were collected from 8 women from probiotic group and 8 women from placebo group (total 16 with BV). Breast milk and neonates' feacal samples were also collected for microbiome analysis from the same 16 women.

Maternal nutritional status, gestational age at recruitment and neonatal anthropometry are given in table 2. Age, height, weight and BMI of the pregnant women were similar between the groups. The mean hemoglobin of the total subjects was 10.2gm/dl. Low birth weight (LBW) and Pre-term births (PTB) were prevalent in 20.7% and 5.8% respectively. On comparing the probiotic and placebo arms, mean gestational age was non-significantly higher in the probiotic arm when compared to the placebo. Predictably, the proportion of LBW and PTB were non-significantly lower in the probiotic arm. The crown-heal length of the baby showed a higher trend in the probiotic arm (Table 2).

Table 2: Anthropometric data of the study subjects and their neonates

	Probiotic group	Placebo group	Total	p.value
Age (years)	22.50±2.34 (70)	22.57±2.65 (70)	22.54±2.49 (140)	0.866
Height (cms)	153±5.2 (70)	153±5.19 (70)	153±5.19 (140)	0.881
Weight (kgs)	54.21±9.33 (70)	54.53±7.97 (70)	54.3±8.6 (140)	0.853
BMI	23.14±3.73 (70)	23.31±3.23 (70)	23.2±3.47 (140)	0.918
Hemoglobin (gm/dl)	10.12±1.36 (70)	10.29±1.45 (70)	10.2±1.4 (140)	0.466
Gestational age at recruitment	28.6±1.27 (70)	27.8±1.82 (70)	28.57±1.36 (140)	0.872
Gestational age at delivery (weeks)	39.8±1.92 (68)	38.97±1.31 (68)	38.9±1.6 (136)	0.327
Birth weight	2.73±0.39 (67)	2.62±0.5 (69)	2.67±0.43 (136)	0.985
Crown heel length of neonates (cms)	48.79±1.7 (56)	47.25±2.3 (60)	47.9±1.8 (116)	0.083
Chest circumference (cms)	31.3±1.6 (56)	31.1±1.7 (60)	31.2±1.7 (116)	0.410
Head circumference (cms)	33±1.35 (56)	32.8±1.36 (60)	32.9±1.7 (116)	0.335
Mid upper arm circumference (cms)	9.58±0.84 (56)	9.55±0.74 (60)	9.56±0.79 (116)	0.828
Low birth weight	13%	15%	20.7%	0.30
Pre-term births	5.0%	6.0%	5.8%	0.63

Values mean ± SD. Number of subjects indicated in parenthesis

The mean Nugent's score, vaginal pH, proportion of women with Amsel's criteria and clue cells were similar between groups at baseline. After 30 days of probiotic and placebo supplementation, the mean Nugent's score, proportion of women with positive Amsel's criteria and clue cells percent decreased in both the groups. Post-natal follow-up showed significant reduction in mean Nugent's score and decreased proportion of women with positive Amsel's criteria and clue cells in both the probiotic and placebo arms.

When paired t-test was applied, a significant reduction in mean Nugent's score from baseline values was observed at all the three followups. However, though the mean Nugent's score was low compared to the baseline in the postnatal probiotic group, no statistical significance could be obtained due to a small sample size. Vaginal pH was not altered in both the groups. Proinflammatory cytokine, IL1 β reduced significantly ($p<0.05$) after 30 days intervention with probiotic.

Vaginal microbiota profile in BV positive pregnant women after oral intervention

At baseline, the alpha diversity was non-significant (p= 0.072) between intervention and placebo groups however, some primary pathogens such as *Gardnerella vaginalis, Acidaminococcus sp*. D21, *Atopobium vaginae* were significantly higher at baseline in the probiotic intervention arm. After 30 days probiotic treatment, α-diversity (p= 0.0312) and species richness (after, p= 0.0156) decreased in BV positive women and were comparable to normal vaginal flora. In the clindamycin + probiotic group there was significant (p = 0.053) decrease in the *Gardnerella vaginalis* (from 12.40%, 7/7 to 2.83%, 7/7) and increase in *Lactobacillus crispatus* (p 0.075), *L. iners* (p 0.017) and *Prevotella copri* (p 0.011), whereas there was decrease in *Lactobacillus helveticus*, *Megasphaera genomosp. type_1* (p 0.004) and Lactobacillus gasseri (p 0.011). In contrast, the clindamycin + placebo group had non-significant increase in *Gardnerella vaginalis* (from 2.609%, 6/7 to 3.81%, 7/7) *Lactobacillus helveticus*, *Lactobacillus gasseri, Lactobacillus crispatus* (p 0.075) and decrease in *Lactobacillus iners*. As for the *Acidaminococcus sp.* D21 and *Atopobium vaginae*, decreasing trend was observed in both the groups with reference to baseline.

Fecal microbiota profile in BV positive pregnant women after oral intervention

On comparing the bacterial communities at phylum level, there was significant difference in the abundance of Firmicutes. At baseline, Firmicutes proportion was 50.7%, which increased to 91.2% after treatment with probiotic group. In contrast, the abundance of Bacteriodetes and phylum Actinobacteria decreased from 0.59% to 0.096% and 25.07% to 4.28% respectively, with probiotic treatment. In clindamycin + placebo group, the phylum-level analysis showed no change in the relative abundance of Firmicutes (85.91%), as compared to baseline (88.10%). However, the abundance of Bacteriodetes decreased with placebo treatment (0.12%) as compared to baseline (0.78%). The other phylum present in baseline such as Actinobacteria (5.57%) and Proteobacteria (3.71%) increased to 6.032% and 6.07% respectively with placebo treatment.

Breast milk microbiota

In the breast milk, the predominant phylum was firmicutes (55%) followed by proteobacteria (33%) and actinobacteria (11%). The relative abundance of taxa, *Bifidobacteria* was 0.3% in the breast milk of women with probiotic intervention group, which was similar to women with normal vaginal flora who had 0.1% *Bifidobacteria* in breast milk samples. Surprisingly, *Bifidobacteria* was not detectable in the placebo group of women with BV.

Neonatal fecal sample microbiota profile

The relative abundance of taxa *Bifidobacteria* (32.7%), *streptococcus* (23%) and *Enterobacteriacea* (27%) were significantly different in the neonates born to the probiotic intervention group compared to the neonates of placebo group. In the neonates of placebo group,

Bifidobacteria (19%) was significantly lower, while, *Enterobacteriacea* (50%) was significantly higher. *Streptococcus* (23%) was similar in both the groups.

CONCLUSIONS

After 30 days of oral probiotic or placebo supplementation in pregnant women with BV (who were initially treated with local clindamycin for one week), the results are summarized as follows:

- Clinical evaluation and microbiological evaluation showed significant reduction in BV in both the groups (probiotic and placebo) during all the three follow-ups. These results suggest that local clindamycin for one week is effective in curing BV and also prevents relapse at least up till three months. However, the vaginal and fecal microbiome analysis showed very encouraging results with probiotic supplementation.
- The proportion of LBW and PTB were non-significantly lower in the probiotic arm. The crown-heal length of the baby showed a higher trend in the probiotic arm.
- A relatively higher abundance of *Gardnerella vaginalis* was observed in women with BV compared with non-BV counterparts However, its abundance was considerably decreased (p=0.053) following probiotic intervention.
- Based on high-throughput MiSeq sequencing approach on 16S rRNA gene amplicons of vaginal microbiota, the probiotic group demonstrated a significant decrease in the relative abundance of several clades such as *Megasphaera genomo sp.* type_*1* (p=0.004) and *Lactobacillus gasseri* (p=0.011) while showing a considerable increase in *Lactobacillus crispatus* (p=0.075) and *L. iners* (p=0.017).
- In contrast to this trend, women in the placebo group had no significant change from baseline except an increasing trend in *Lactobacillus crispatus* (p=0.075).
- While *P. copri* was present in the vagina of all pregnant women with normal vaginal flora, it remained undetected in women with BV.
- After probiotic intervention, *Prevotella copri* was detectable in vaginal flora, while it remained undetected after placebo supplementation.
- A considerable reduction in α-diversity (p= 0.031, vs. baseline) and species richness (p= 0.015, vs. baseline) was observed in the intervention (probiotic) arm. Whereas, no such change was observed in the placebo group.
- *Gut microbiota*: Analyses of gut microbiota revealed predominance of *Prevotella copri* in healthy women.
- *Gut microbiota*: *Prevotella copri* abundance increased significantly following probiotic intervention (16.495 %); whereas in the placebo group *P copri* reduced from 7.75% (baseline) to 4.84% (endpoint).

III. BASIC STUDIES

1. MANIPULATION OF DIETARY FAT TO ENHANCE CAROTENOID BIOAVAILABILITY AND BIOCONVERSION TO VITAMIN A: EFFECT OF SYNTHETIC AND NATURAL PPAR AGONISTS ON INTESTINAL CAROTENOID ABSORPTION

The vitamin A nutrition from a given plant food is governed by the extent of carotene micellarization during digestion, its uptake and its conversion to vitamin A in intestinal cells. Several studies reported that dietary fat is required for efficient micellarization and thus enhanced absorption of carotenoids from various plant foods. Nevertheless, the type and quantity of fat required for enhancing the micellarization of carotenoids remained unknown. Our preliminary investigation revealed that different oils (with varying fat composition) have varying influence on micellarization of carotenoids from drumstick leaves. Based on early rat studies, carotenoid uptake is believed to be mediated simply by a passive diffusion of carotenoid mixed micelles. Subsequent studies have demonstrated specific and saturable uptake of carotenoids through scavenger receptor B1 (SRB1). Once inside the cell, the provitamin A carotenoids are converted to vitamin A by the actions of β-carotene mono-oxygenase (BCMO1). Isolated observations suggest that activation of PPAR-γ, induces the expression of both SRB1 and BCMO1. It's well known that conjugated linoleic acid and omega 3 fatty acids, a constituent of ruminant milk and fish oils, are potent agonists of PPAR-γ. Therefore, together these observations raise a theoretical possibility, where in rational selection of dietary fat and its inclusion with PPAR agonists, might promote micellarization, intestinal uptake and bioconversion to vitamin A from carotenoid rich plant foods, which could be tested and developed in to functional food.

In the current report, the data related to the effect of dietary fat composition and food matrix on micellarization of carotenoids from vegetables and fruits, as assessed by coupled *in vitro* digestion/ Caco-2 cell model have provided.

AIMS AND OBJECTIVES

Hypothesis

The dietary fatty acid quantity, composition and food matrix influence the carotenoid micellarization from vegetables and fruits.

OBJECTIVES

1. To assess the effect of dietary fat on micellarization and intestinal uptake of carotenoids from vegetables and fruits.
2. To study the effect of food matrix and polarity on carotenoid micellarization.

METHODOLOGY

Vegetables and fruit processing: Carrots, spinach, drumstick leaves and papaya are the most commonly consumed carotenoid rich vegetables and fruit, respectively in India and therefore, selected for the study. Orange carrots (*Daucus carota*), tender leaves of spinach (*Spinacia oleracea*) and drumstick leaves (*Moringa oleifera*) and fully ripened papaya (*Carica papaya, local variety Surya*) were purchased fresh from the local market. The vegetables and fruits were cleaned with milli-Q water twice and pat-dried on a blotting paper. The vegetables were cut into

small pieces, microwaved for 3 min at maximum power to simulate cooking, cooled and pureed in a kitchen blender. The papaya fruit pulp was prepared and used directly for the *in vitro* digestion without cooking as it was generally consumed raw. The vegetable puree or fruit pulp were used immediately for *in vitro* digestion.

Vegetable oils and fatty acid composition*:* Vegetable oils were procured locally. The fatty acid composition of the oils was analyzed as fatty acid methyl esters by gas chromatography using an SP-2330 capillary column (30m X 0·25 mm; Supelco, Bellefonte, PA, USA) as described earlier.

Simulated in vitro digestion: The simulated gastric and small intestinal phases of digestion were carried out as described previously. The digestion reactions (in 50 mL screw cap tubes) contained either 2.0 g of pureed vegetables or fruit pulp in the absence and presence of dietary fat (0-10%, w/w), which was added prior to the initiation of gastric phase of digestion. Briefly, 2g of food homogenate was mixed with 35 mL of saline, and pH of the contents was adjusted to 2 with 2M HCl followed by addition of 2 mL porcine pepsin stock solution (40 mg/mL in 100mM HCl). The final volumes were made to 40 mL with saline, blanketed with nitrogen gas and incubated submersed in a 37 °C shaking water bath for a period of 1h. At the end of gastric digestion, the pH was increased to 6 with 1M NaHCO3, followed by addition of 3 mL bile extract (60 mg porcine bile extract/mL in 100 mM $NaHCO_3$) and 2 mL of pancreatin (10mg/mL)-lipase (10 mg/mL) stock solution. The pH of all the samples was adjusted to 6.5 with 1M NaOH, and the final volumes were made to 50 mL. The samples were then blanketed with nitrogen and incubated as above for 2h. At the end of digestion, an aliquot of the product (referred as "digesta") was centrifuged (5810R, Eppendorf, Germany) at 20,000 g at 4°C for 60 min to separate the aqueous fraction containing mixed micelles. The aqueous fraction was filtered through 0.2µ surfactant free cellulose acetate membrane (Corning, NY 1831, USA) to remove microcrystalline carotenoid aggregates, if any and microbial contamination. The filtrate is referred as the "micellar fraction". Aliquots of digesta and micellar fractions were stored at -20°C under a blanket of nitrogen and analyzed within 2-3 days.

Caco-2 cell culture: Caco-2 cells were obtained from the National Centre for Cell Sciences (NCCS, Pune, India). Briefly, cells, between passages 28-35, were seeded at a density of 50,000 cells/cm^2 in 6-well plates and cultured in complete DMEM with 10% fetal bovine serum (FBS, supplemented with 1% NEAA, 0.4 mM glutamine and 1% antibiotic-antimycotic solution) and maintained at 37°C in an incubator with a 5% CO_2/95% air atmosphere at constant humidity.

Carotenoid uptake: Briefly, aliquots of the micellar fraction obtained after intestinal digestion were immediately diluted in DMEM (1:4) and fed to the differentiated Caco-2 cells for 3h. At the end of incubation, the spent medium was removed; monolayers were washed once with ice-cold phosphate-buffered saline (PBS, pH 7.2) containing 0.5% bovine serum albumin to remove residual carotenoids adhering to the cell surface and twice with PBS only. At the end, cells were scraped into 1mL PBS, and stored under nitrogen at -20°C for a maximum of 2-3 days before analysis.

Extraction of carotenoids: Briefly, the vegetable puree or fruit pulp (2g) was mixed with methanol and saponified. The carotenoids were then extracted with petroleum ether:acetone (2:1 vol/vol), dried under nitrogen and analyzed by HPLC as described below. The carotenoids from frozen samples of digesta (2mL), micellar fraction (3mL), cell pellets (sonicated in 1ml PBS for 30 sec.) were thawed and extracted thrice with an equal volume of petroleum ether:acetone (2:1 vol/vol). The extracts were combined and dried under nitrogen at 37°C. The residue was resolubilized in methanol/dichloromethane (80:20 v/v) and analyzed immediately. Using apo-8 carotenal as a recovery standard, extraction efficiency was routinely 95–105%.

HPLC analysis of Carotenoids: Briefly, carotenoids were analyzed using Agilent 1100 HPLC system (Agilent, Model 1100, Puala Alto, CA, USA) equipped with an auto-sampler, and UV-Vis

detection system controlled by Chemstation software. Either 50 µL or 100 µL aliquots of reconstituted carotenoid extracts from digesta or filtered aqueous fraction, respectively were injected and fractionated on a reverse-phase column (ODS, C-18, 250X4.6mm, 5µm, Thermo) with methanol: dichloromethane (80:20, v:v) at a flow rate of 1 mL/min. The flow was monitored at 450 nm for a period of 30 min. Carotenoids were identified and quantified by comparing retention times and peak areas with authentic standards.

Statistics: The percent (%) micellarization of carotenoids in the filtered aqueous fraction was computed considering the total carotenoid content in digesta as 100%. Data were analyzed using SPSS version 7.0 (SPSS, Chicago, IL). Descriptive statistics including mean and SD were calculated for the efficiency of micellarization (%) of carotenoids from digested foods. Means were compared using one-way analysis of variance (ANOVA) followed by least significant differences test. Linear regression analysis and Pearson's coefficients were calculated to compare the relationship between β-carotene content in the micellar fraction to that of its intestinal cell uptake. All experiments were conducted in triplicate and each experiment was repeated twice to provide six independent observations. Differences were considered significant at $P<0.05$.

RESULTS

Carotenoids content of vegetables and fruits and fatty acid composition of dietary fat: The carotenoid content of test foods is given in table. 1. The β-carotene content of carrot was highest followed by drumstick leaves, spinach and papaya. Lutein content of drumstick leaves was 1.5 times higher compared to spinach. α-carotene was detectable only in carrot while lycopene was detected only in papaya. The unsaturated fatty acid content was higher in sunflower oil, olive oil and soybean compared to peanut>palm>coconut oil. The medium chain fatty acid content (<c14) was higher in coconut oil compared to all other oils. However, soybean alone contained linolenic acid (18:3, 6.6%) and peanut oil contained docosanoic acid (C22, 4%), while the oleic acid (C18:1, 78%) content was higher in olive oil.

Table 1. Carotenoid content (mean±SD) of vegetables and fruit

Name of sample	β-carotene	α- carotene	Lutein	Lycopene
	µg/100g fresh weight			
Carrot	4423 ± 42	2123± 28	-	-
Spinach	2605 ± 51	-	3890±65	-
Drumstick	3200 ± 65	-	5360 ±79	-
Papaya	1050 ±42	-	-	76.7 ± 4.6

Effect of dietary fat content on micellarization of carotenoids*:* Addition of 0.5-5% olive oil increased the micellarization of lutein, β-carotene, α-carotene and lycopene from carrot, drumstick leaves, spinach, and papaya (Table 2). However, lutein micellarization from spinach and drumstick leaves was higher with 1% olive oil, and remained similar thereafter. The extent of increase in carotenoid micellarization remained similar after 5% dietary fat, indicating saturation. Therefore, all the subsequent experiments were performed with 2.5% dietary fat.

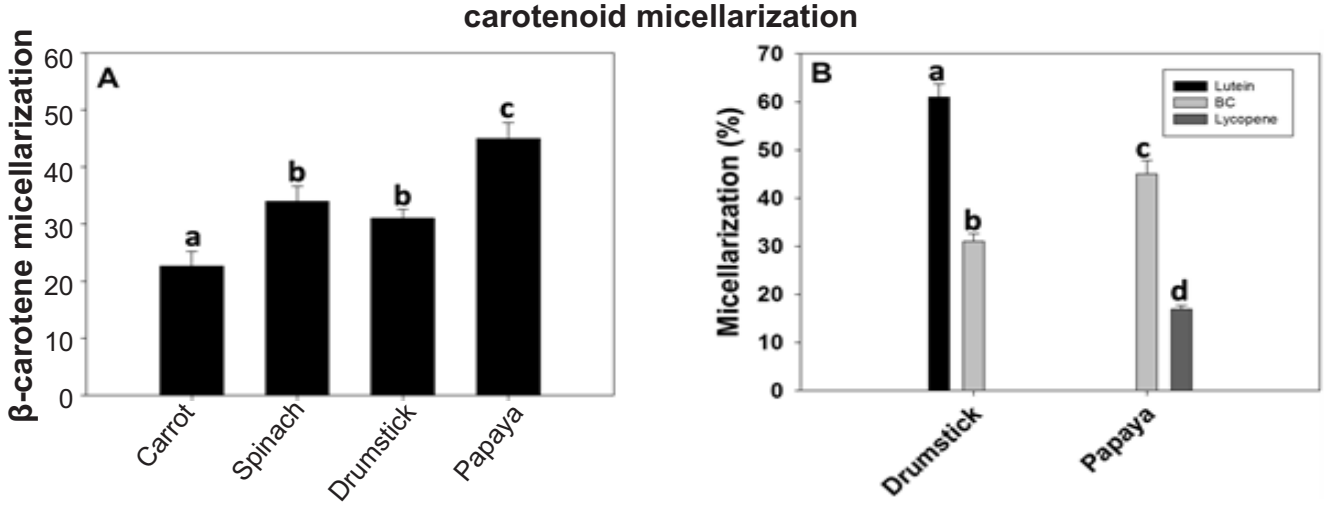
Effect of food matrix and carotenoid polarity on micellarization: Extent of β-carotene micellarization was dependent on the food matrix in the order of papaya>spinach=drumstick leaves>carrot (Fig. 1A). Among the carotenoids, the micellarization of lutein was higher from drumstick leaves compared to β-carotene while it was higher from papaya compared to lycopene (Fig. 1B).

Table 2. Effect of olive content on efficiency of carotenoid micellarization from vegetables and fruit*

Vegetable/Fruit	% olive oil	% micellarization			
		Lutein	β-carotene	α-carotene	Lycopene
Carrot	0	-	4.8±0.9[a]	4.6±0.8[a]	-
	1	-	13.2±1.2[b]	12.6±1.8[b]	-
	2.5	-	22.6±2.6[c]	24±2.2[c]	-
	5	-	23.2±2.9[c]	26±2.7[c]	-
	10	-	26.2±3.9[c]	28±2.2[c]	-
Spinach	0	22±2.7[a]	7.2±0.2[a]	-	-
	1	59±2.2[b]	13±1.4 [b]	-	-
	2.5	62±2.6[b]	34±2.6[c]	-	-
	5	67±2.9[b]	36±3.1 [c]	-	-
	10	62±2.2[b]	32±3.1 [c]	-	-
Drumstick	0	27±1.7[a]	5.2±0.8[a]	-	-
	1	61±2.6[b]	16±1.5 [b]	-	-
	2.5	61±2.7[b]	31±1.6[c]	-	-
	5	62±1.9[b]	32±1.9 [c]	-	-
	10	59±3.8[b]	29±2.5 [c]	-	-
Papaya	0	-	8.2±0.6[a]	-	3.4±0.2 [a]
	1	-	21.5±1.7 [b]	-	6.7±0.8 [b]
	2.5	-	45±2.8[c]	-	16.9±0.8 [c]
	5	-	47±1.9 [c]	-	22.1±1.4 [d]
	10	-	48±2.7 [c]	-	19.2±2.1 [d]

** The percent efficiency of micellarization was computed considering the carotenoid content in test food as 100%. Data are means ± SD for 6 observations generated in two independent experiments. Means without a common letter in a column and within a test food differ significantly ($p < 0.05$). - refers to not determined.*

Fig 1. Effect of carotene distribution and polarity on efficiency of carotenoid micellarization



A. β-carotene micellarization was compared among test foods to understand the effect of food matrix on micellarization (B). The lutein, BC and lycopene micellarization was compared amongst drumstick leaves and papaya (both contain carotenoids in fat globules) to understand the effect of polarity of carotenes with that of efficiency of micellarization. The bars represent means ± SD for 6 observations generated in two independent experiments. Means without a common letter in a column differ significantly (p < 0.05).

***Effect of type of vegetable oil on micellarization carotenoids**:* The micellarization of carotenoids increased due to addition of all types of vegetables oils (2.5 w/w) from carrot (BC and AC), spinach (Lutein, BC), drumstick leaves (Lutein, BC) and papaya (BC and lycopene) (Table 3). However, the extent of increase in micellarization of carotenes was higher with oils rich in unsaturated fatty acids (olive=soyabean=sunflower) compared to oils rich in saturated fat (peanut=palm>coconut), with the exemption of lutein which remained independent of the type of dietary fat. Interestingly, micellarization of all the carotenoids was lowest with coconut oil, rich in saturated, but medium chain fatty acids.

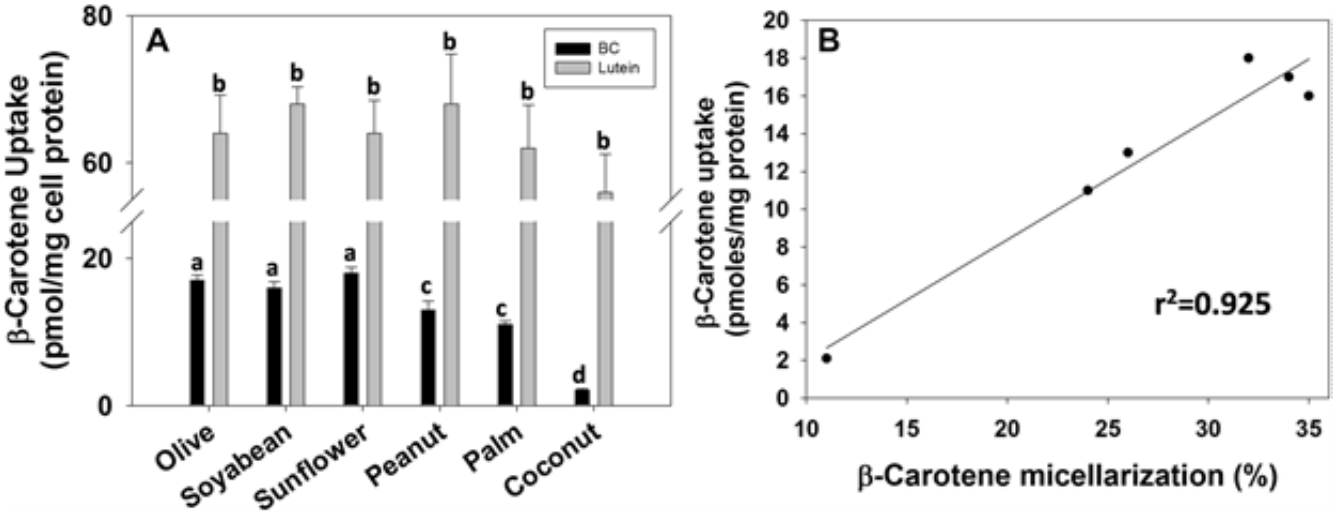
Table 3. Effect of type of vegetable oil (2.5%) on micellarization of carotenoids*

Vegetable/ Fruit	Carotene	olive	Soybean	Sunflower	Peanut	Palm	Coconut
Carrot	BC	22.6±2.2^{a}	24±2.8 ^{a}	22.6±2.2^{a}	16±2.8^{b}	15±2.1^{b}	7.8±0.8^{c}
	AC	23.2±2.9^{a}	26±2.7 ^{a}	24.6±2.6^{a}	14±1.8^{b}	13±2.5^{b}	7.2±0.8^{c}
Spinach	LuT	66±4.6^{a}	67±5.6 ^{a}	68±5.9^{a}	66±5.8^{a}	68±6.2^{a}	42±4.7^{b}
	BC	34±2.6^{a}	35±2.9 ^{a}	32±1.2^{a}	26±0.8^{b}	24±0.6^{b}	11±0.2^{c}
Drumstick	Lut	61±2.7^{a}	61±3.6 ^{a}	64±4.9^{a}	62±2.8^{a}	62±7.2^{a}	48±3.7^{b}
	BC	31±0.6^{a}	30±0.8 ^{a}	29±0.5^{a}	22±0.6^{b}	21±1.1^{b}	8.1±0.8^{c}
Papaya	BC	45±1.8^{a}	49±2.1^{a}	43±1.5^{a}	31±2.1^{b}	29±1.4^{b}	12±0.6^{c}
	Lyco	16.9±0.8 ^{a}	17.2±0.7 ^{a}	15.8±0.6 ^{a}	10.6±0.5 ^{b}	10.6±0.8 ^{b}	8.4±0.2 ^{c}

** The percent efficiency of micellarization was computed considering the carotenoid content in test food as 100%. Data are means ± SD for 6 observations generated in two independent experiments. Means without a common letter in a row differ significantly (p < 0.05).*

Effect of dietary fat on Intestinal cell uptake carotenoids: Cellular accumulation of lutein from the micellar fraction of spinach was higher compared to β-carotene either in the absence or presence of dietary fat (Fig 2A). The uptake of lutein was increased with the addition of dietary fat, but it remained independent of the type of dietary fat used. In contrast, β-carotene uptake was dependent on the type of dietary fat used, akin to its micellarization. Further, a positive relationship was observed between β-carotene content in the micellar fraction with that of its cellular accumulation as evidenced by Pearson correlation (R=0.975 and p<0.001) and regression analysis (r^2=0.925, Fig. 2B).

Fig 2. Effect of type of dietary fat on β-carotene uptake in intestinal cells



The aqueous micellar fraction obtained after digestion of spinach puree with 2.5% dietary fat from indicated vegetable oils was fed to the differentiated Caco-2 cells for a period 3h. The cellular β-carotene content was estimated as described in methods (A). The micellarization of BC was plotted against its cellular uptake, and the line represents the linear regression (r^2=0.92) (B). The bars represent means ± SD for 6 observations generated in two independent experiments. Means without a common letter in a column differ significantly (p < 0.05).

CONCLUSIONS

Micellarization is a prerequisite to intestinal absorption of dietary carotenoids, which in turn is influenced by food matrix, physicochemical properties of carotenoids and dietary fat. Our results demonstrate that addition of dietary fat (0 to10%) increases the micellarization of lutein from spinach (22 to 62%) and drumstick leaves (27 to 59%), β- carotene from carrot (4.8 to 26.2%), spinach (7.2 to 32%), drumstick leaves (5.2 -29%) and papaya (8.2 to 48%), α-carotene (4.6 to 28%) from carrot and lycopene from papaya (3.4 to 19.2%). Further, the micellarization of carotenes is increased dose dependently from 0 to 2.5% dietary fat followed by saturation, while the saturation of lutein micellarization required only 1% dietary fat. The β-carotene micellarization varied among different test foods in the order of papaya>spinach=drumstick>carrot. The efficiency of micellarization decreased in the order of lutein>β-carotene=α-carotene>lycopene independent of the amount of dietary fat. Although lutein micellarization increased with dietary fat, it remained independent of the type of dietary fat. Interestingly, micellarization of other carotenoids was dependent on the type of dietary fat in the order of olive=soyabean=sunflower >peanut=palmcoconut oil. These results suggest that food matrix, carotenoid polarity and dietary fat determine the extent of carotenoid micellarization and therefore, bioavailability from plant foods.

PART B

In the current report, we have provided the data related to the effect of synthetic and natural agonists of PPARs on intestinal cell carotenoid absorption & lipid transporter expression.

Hypothesis

Synthetic and Natural agonists of PPARs modulate intestinal carotenoid absorption via modulation of lipid transporter expression.

OBJECTIVES

1. To assess the effect of synthetic and natural agonists of PPARs on intestinal carotenoid absorption.
2. To characterize the mechanism of PPARs induced modulation of intestinal carotenoid absorption.

METHODOLOGY

Caco-2 cell culture

Caco-2, clone TC-7 cells, was a gift from Dr Emmanuelle Reboul. (INRA, UMR 1260, France). Briefly, cells were cultured in Dulbecco's modified eagles medium (DMEM) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino acid, and 1% antibiotics (complete medium) as previously described.

Delivery of fatty acids, PPAR ligands and β-carotene

The stock solutions of fatty acids (10 mmol/L) and taurocholic acid (38 mmol/L) in alcohol were introduced into a sterilized glass tube, solvents evaporated, and the dried residue was then solubilised in serum-free DMEM, vigorously mixed and filtered to achieve final concentration of 200 µmol/L fatty acid and 250 µmol/L taurocholic acid. Wherever required this stock was diluted with DMEM containing 250 µmol/L taurocholic acid. The stock solutions of PPAR agonists and antagonist were prepared in DMSO (50 mmol/L) and added to the cell culture medium at indicated concentrations such that the DMSO content of the cell culture medium was always <0.01%. Wherever present, the PPAR-α (25 µmol/L) and PPAR- (1 µmol/L) antagonist were added 2 h prior to the addition of fatty acids. β-carotene was delivered to the cells as tween-40 micelles as described previously. Briefly, β-carotene in hexane was mixed with 40 µL of 20% tween-40 in acetone in a glass tube, solvents evaporated, solubilised in 20 mL of basal DMEM (90 nmoles β-carotene in 1 mL media) by vigorous mixing and sterile filtered before addition to the cell culture media.

Carotene uptake in Caco-2 cells

For uptake experiments, cells were grown in 6 well plates. The medium was changed every alternate day for 3 weeks to ensure differentiation of cells. At this stage the spent medium from the differentiated Caco-2 cells was replaced with 1 mL of serum-free DMEM (containing 250 µmol/L taurocholic acid), 2 h prior to the initiation of treatments. The cells were treated with 1 mL DMEM containing 250 µmol/L taurocholic acid without (control) or with CLA (0-100 µmol/L), DHA (0-100 µmol/L), EPA (0-100 µmol/L), WY14643 (25 µmol/L), rosiglitazone (25 µmol/L), GW0742 (1 µmol/L) for a period of 72 h. At the end of treatment, 1 mL media was withdrawn and replaced with equal volume DMEM supplemented with 90 nmoles of β-carotene, and incubated for further 3 h. At the end, the monolayers were washed twice with 10 mmol/L phosphate buffer saline (PBS) containing 1% bovine serum albumin (BSA) followed by once with PBS without BSA. The cell associated β-carotene was extracted and analyzed as described below.

Carotene transport in Caco-2 cells

For transport experiments, cells were seeded and grown on transwells with 24 mm polycarbonate membrane (6-well plate, 0.4 µm pore size, Corning, USA). The medium was

changed every alternate day for 21 days to ensure differentiation of cells. On the day of the experiments, the spent media in the apical chambers was replaced with fresh serum-free DMEM while the basal medium was replaced with DMEM supplemented with 2.5% delipidated serum. The cells were then treated with fatty acids or PPAR agonists exactly as above, except that the β-carotene uptake and transport to the basolateral media were measured after 16 h of incubation. To ensure the integrity of monolayers, 10 µL of FITC-inulin (10 mmol/L) was added to the apical chambers 4 h prior to end of experiment, and the same was measured in basolateral media at the end of experimental period at 480 nm excitation and 540 nm emission wavelength using Cary Eclipse spectrofluorometer (Varian, USA).

Extraction of carotenoids from cells

β-carotene from cells and media was extracted as described previously. Briefly, frozen cell pellets (1-2 mL) were thawed and extracted thrice with 2 mL of 2-propanol: dichloromethane (2:1, v/v) at 20°C for 60 min. The extracts were combined and evaporated under a stream of nitrogen. The carotenoids from media were extracted with petroleum ether: acetone as described previously. The dried lipid extracts were reconstituted in 250 µL of dichloromethane/ methanol (10:90, v/v) and analyzed by HPLC as described below. Routinely, 20 ng of *trans*-8-apo-carotenal (dissolved in methanol) was added to the cell pellets or media before extraction, to monitor the extraction efficiency, which was varied between 95-103 %.

HPLC analysis of carotenoids

β-Carotene was analyzed using HPLC method. Briefly, carotenoids were fractionated on a reverse-phase column (ODS, C-18, 4.6 × 250 mm, Thermo) with methanol: dichloromethane (90:10, v:v) containing ammonium acetate (0.07%) at a flow rate of 1 mL/min. The flow was monitored at 450 nm for a period of 15 min. β-carotene (retention time 11.4 min) was quantified from the peak area of standard β-carotene.

Immunoblotting

At the end of treatments, the monolayers were washed thrice with 10 mM phosphate buffer saline pH 7.2, and lysed in RIPA buffer containing protease inhibitor cocktail. The protein content was estimated using micro-BCA kit method. Equal amount of protein (20-60 µg) was fractionated on either 12 % (for SRB1) or 4-12 % (for NPC1L1) gradient SDS-gels under reducing conditions and transblotted onto the nitrocellulose membranes. The blots were blocked with 5 % non-fat dry milk, and probed with primary antibodies for SRB1, NPC1L1 and GAPDH followed by respective secondary antibodies. The blots were developed using ECL detection kit (Bio-Rad, USA) and images were acquired on G-box imaging system (Syngene, USA). The images were quantified densitometrically using Image J software (NIH, USA). The densities of target proteins were normalized with respective housekeeping protein (GAPDH) levels.

Real-time qPCR

Differentiated Caco-2 cells were treated with either EPA (100 µmol/L) or WY14643 (25 µmol/L) for 72 h. Briefly, at the end of incubation, total RNA samples were prepared from Caco-2 cells, using TRI Reagent (Sigma-Aldrich, Bangalore, India) as per manufacturer's instructions. RNA concentration and purity was assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, DE, USA). RNA integrity was assessed using the RNA 6000 Nano Lab Chip Series II Assay with the 2100 Bioanalyzer System (Agilent Technologies, Palo Alto, CA, USA). Samples with RNA integrity number (RIN) value of >7.5 were reverse transcribed using cDNA synthesis kit using Oligo dT_{18} primers (Thermo Scientific, Waltham, MA USA). The cDNA reactions were diluted to 50 µL in nuclease-free water (Invitrogen Life Technologies), aliquoted and stored at -20°C till further use. Real-timeqPCR reactions were carried out in duplicate for each sample using SYBR Premix Ex Taq (TliRNase H Plus, DSS Takara Bio India Pvt. Ltd, India)

in a 20 µL reaction volume containing each primer at 500 nM concentration. Reactions were temperature cycled, and SYBR green levels were measured using a CFX-96 system (Bio-Rad Laboratories, Berkeley, California).The cycling parameters were as follows: 95 °C for 15 s, 61 °C and 59 °C for 30 s for CPT1A and TBP, respectively for a total of 40 cycles. The initial target concentration for each gene was calculated by relative standard curve method using a pool of sample cDNA as calibrator. Primers were synthesized from Eurofins Genomics India Pvt Ltd (Bangalore, India) with the following sequences:humanCPT1A,Forward5'-CAAACTGGACCG GGAGGAAA-3and Reverse5'-TGTGCTGGATGGTGTCTGTC-3'; human TBP, Forward5'-GCCAAGAGTGAAGAACAG-3'andTBPReverse5'-GAAGTCCAAGAACTTAGCTG-3'.

Statistics

The β-carotene uptake and transport experiments were conducted in triplicate and repeated twice to generate 6 independent observations. All protein expression experiments were performed thrice to generate three independent observations. Data are presented as mean±S.D. Means between groups or between time points within a group were compared by one-way analysis of variance and post hoc t-test (SPSS software, version 11.0). $P<0.05$ was considered significant.

RESULTS

Effect of DHA, EPA and CLA on β-carotene uptake and lipid transporter expression in Caco-2 cells

The representative HPLC chromatograms of β-carotene analysis in Caco-2 cells treated with and without DHA and EPA were depicted in fig 1A. The chromatogram demonstrates clear separation of recovery standard (*apo*-8-carotenal) and β-carotene and their relative quantities during different treatment regimens. Treatment with EPA (25-100 µmol/L), but not with DHA (25-100 µmol/L) for 72h led to dose-dependent inhibition of β-carotene uptake in differentiated Caco-2 cells (Fig 1B). At 100 µmol/L EPA, the β-carotene uptake was inhibited by 55±4.6 % compared to control. Treatment with 100 µmol/L EPA but not DHA for 72 h also downregulated the SRB1 abundance compared to control cells (Fig 1C). However, NPC1L1 abundance was decreased by both DHA and EPA, respectively (Fig 1D). Similar to that of DHA, CLA treatment had no effect on either β-carotene uptake (Fig 2A) or SRB1 (Fig 2B), NPC1L1 expression (data not shown) but increased the PPAR-γ expression (Fig 2C) in Caco-2 cells.

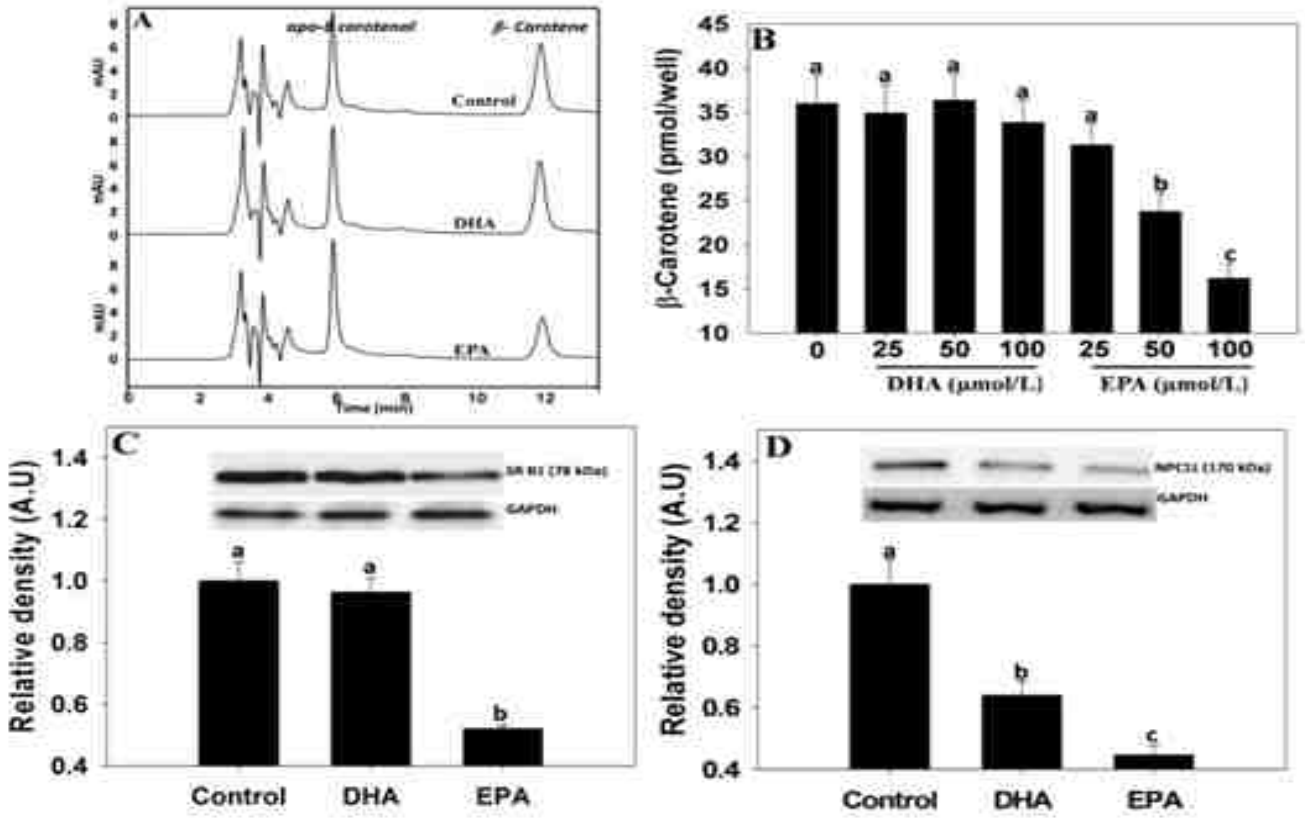
And without DHA and EPA were depicted in fig 1A. The chromatogram demonstrates clear separation of recovery standard (*apo*-8-carotenal) and β-carotene and their relative quantities during different treatment regimens. Treatment with EPA (25-100 µmol/L), but not with DHA (25-100 µmol/L) for 72h led to dose-dependent inhibition of β-carotene uptake in differentiated Caco-2 cells (Fig 1B). At 100 µmol/L EPA, the β-carotene uptake was inhibited by 55±4.6 % compared to control. Treatment with 100 µmol/L EPA but not DHA for 72 h also downregulated the SRB1 abundance compared to control cells (Fig 1C). However, NPC1L1 abundance was decreased by both DHA and EPA, respectively (Fig 1D). Similar to that of DHA, CLA treatment had no effect on either β-carotene uptake (Fig 2A) or SRB1 (Fig 2B), NPC1L1 expression (data not shown) but increased the PPAR-γ expression (Fig 2C) in Caco-2 cells.

Effect of PPAR agonists on β-carotene uptake and transporter expression in Caco-2 cells

Treatment of differentiated Caco-2 cells with WY14643 (PPAR-α agonist, 25 µmol/L), but not with rosiglitazone (PPAR-γ agonist, 25 µmol/L), or GW0742 (PPAR-δ agonist, 1 µmol/L) for 72h, significantly inhibited the β-carotene uptake and abundance of SRB1 levels compared to control cells (Fig 3A & 3B). However, NPC1L1 abundance was reduced by both PPAR-α and PPAR-δ agonists, but not by PPAR-γ agonist compared to control (Fig 3C).

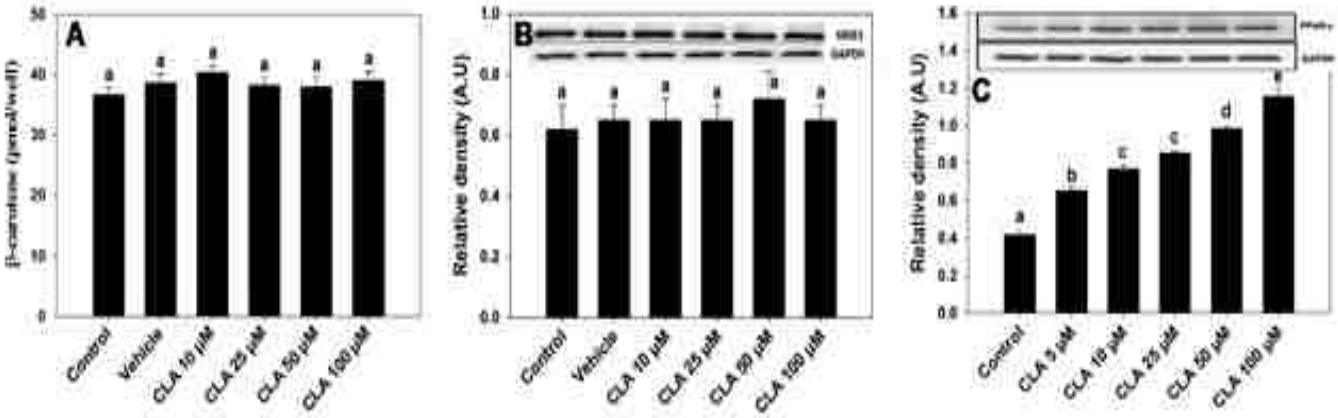
The mRNA expression of CPT1A, classical target gene for PPAR-α was also upregulated by both EPA and WY 146643 compared to control (Fig 3D).

Fig 1. Effect of DHA and EPA on β-carotene uptake and lipid transporter expression in Caco-2 cells



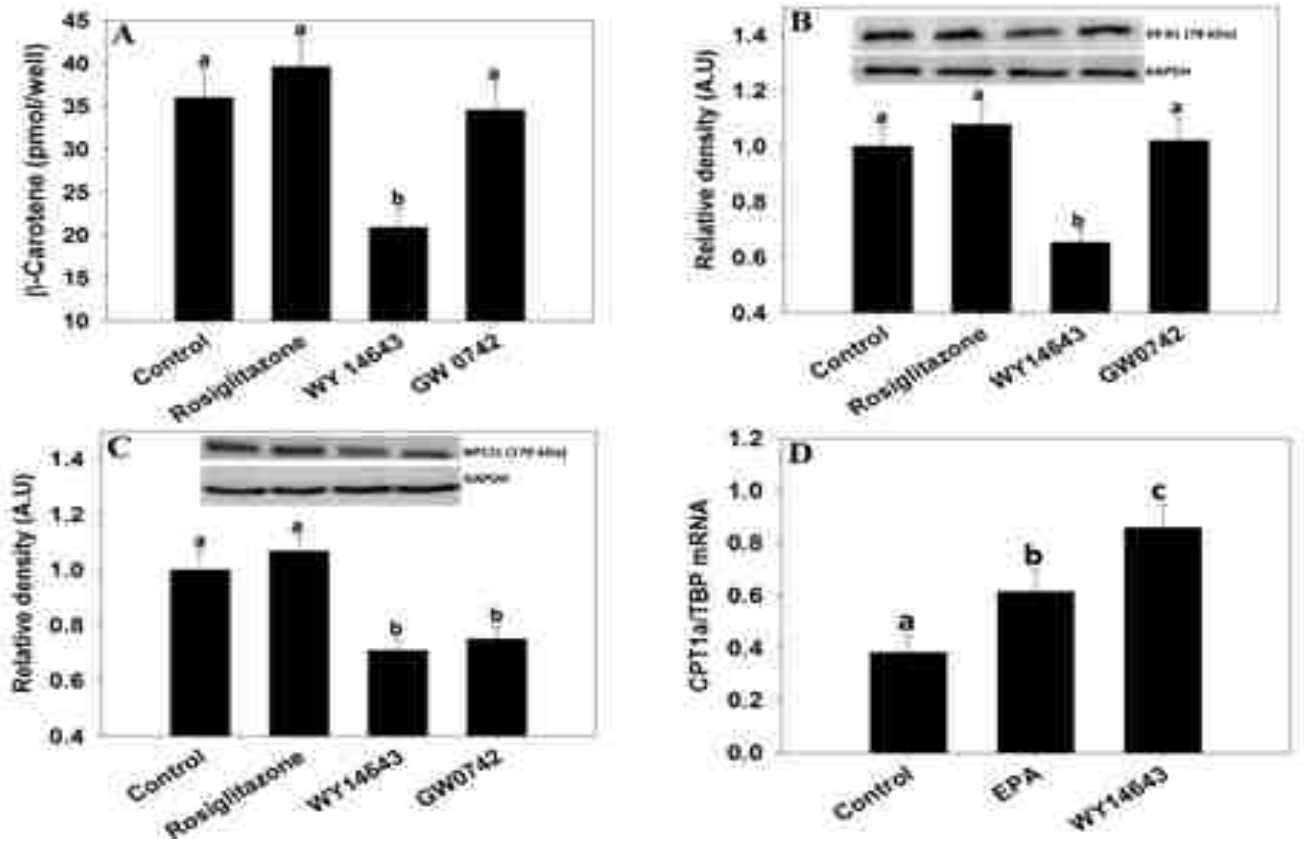
Differentiated Caco-2 cells grown in 6-well plates were treated with 250 µmol/L taurocholic acid (control) either in the presence or absence of DHA or EPA for a period of 72 h. (A). Representative HPLC chromatograms of β-carotene analysis in Caco-2 cells treated with or without DHA and EPA. (B) Effect of DHA or EPA treatment for 72h on β-carotene uptake in Caco-2 cells. (C). Immunoblotting of SR B1 and (D). NPC1L1 in Caco-2 cell lysates treated with 100 µmol/L DHA or EPA for a period of 72h. The bars represent mean β-carotene uptake±SD (n=6) or mean relative density±SD (n=3) of target protein with GAPDH, and the bars without common superscript differ significantly (P<0.05).

Fig 2. Effect of CLA on β-carotene uptake, SR B1 and PPAR-γ expression in Caco-2 cells



Differentiated Caco-2 cells grown in 6-well plates were treated with 250 µmol/L taurocholic acid (control) either in the presence or absence of 0-100 µmol/L CLA for a period of 72 h. (A). Effect of CLA on β-carotene uptake. (B). Effect of CLA on SRB1 expression (C). Effect of CLA on PPAR-γ expression with increasing concentration. The bars represent mean β-carotene uptake±SD (n=6) or mean relative density±SD (n=3) of target protein with GAPDH, and the bars without common superscript differ significantly (P<0.05).

Fig 3. Effect of PPAR agonists on β-carotene uptake and lipid transporter expression in Caco-2 cells



Differentiated Caco-2 cells grown in 6-well plates were treated with 5 µL of ethanol (0.05%) either in the absence (control) or presence of rosiglitazone (25 µmol/L, PPAR-γ agonist), WY14643 (25 µmol/L, PPAR-α agonist) or GW0742 (1 µmol/L, PPAR-δ agonist) for a period of 72h. (A). Assessment of β-carotene uptake in cells was done as described in methods. (B). Immunoblotting of SR B1 and (C). NPC1L1 in Caco-2 cell lysates. (D). qPCR analysis of CPT1a expression in Caco-2 cells. The bars represent mean β-carotene uptake ± SD (n=6) or mean relative density ± SD (n=3) of target protein with GAPDH, and the bars without common superscript differ significantly (P<0.05).

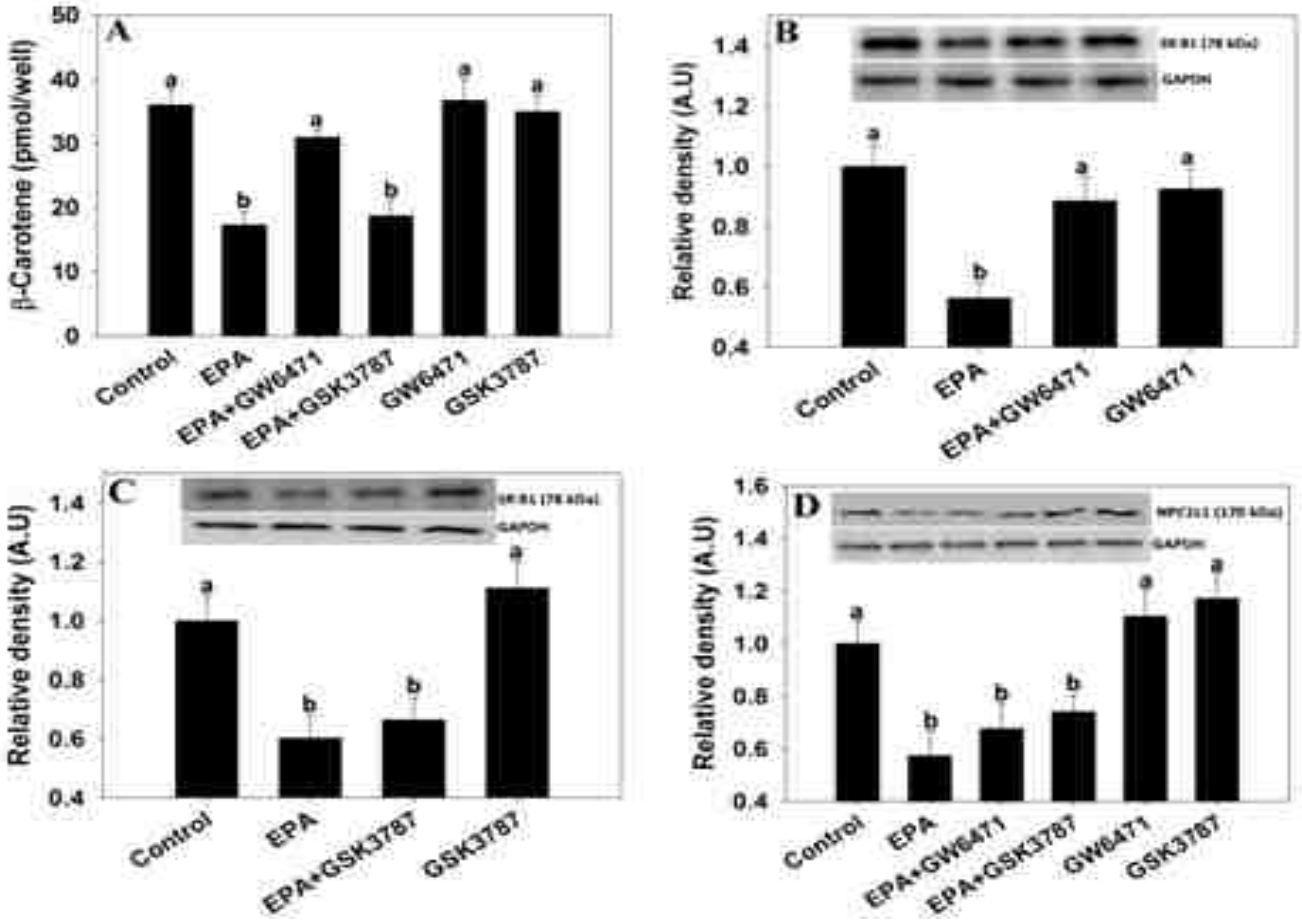
Effect of PPAR-α and PPAR-δ antagonists on EPA induced inhibition of β-carotene uptake in caco-2 cells

EPA induced inhibition of β-carotene uptake was abrogated in Caco-2 cells pre-treated with 1 µmol/L GW6471 (antagonist of PPAR-α), but not with 1 µmol/L GSK3787 (antagonist of PPAR-δ) (Fig 4A). PPAR-α but not PPAR-δ antagonist abrogated the EPA induced downregulation of SRB1 expression (Fig 4B & 4C). However, neither PPAR-α nor PPAR-δ antagonists abrogated the EPA induced down-regulation of NPC1L1 expression (Fig 4D).

Effect of EPA and PPAR-α agonist on basolateral secretion of β-carotene in Caco-2 cells

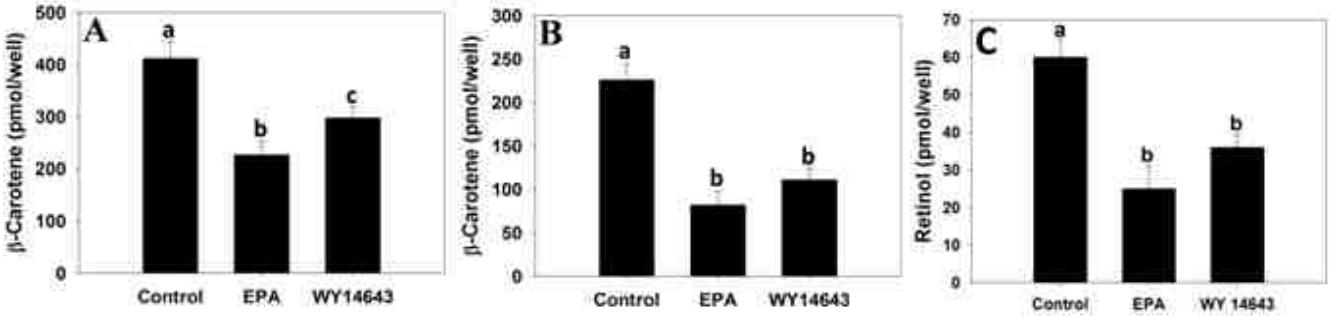
The barrier function of Caco-2 cells grown on transwell inserts is not influenced by treatment with either EPA or PPAR-α agonist, as measured by FITC-inulin transport (<0.03%). Both EPA (227±26 and 82±16 pg/well) and PPAR-α agonist (298±22 and 111±13 pg/well) significantly inhibited the carotene uptake (Fig 5A) and transport to the basolateral media (Fig 5B) compared to control (412±32 and 226±18 pg/well), respectively. Further, EPA and PPAR-α agonists also significantly inhibited the retinol content in basolateral compartment compared to control (Fig 5C), consistent with decreased β-carotene uptake and transport. However, the ratio of uptake/transport was lower in controls (1.82) compared to EPA (2.76) or PPAR-α agonist (2.68).

Fig 4. Effect of PPAR-α and PPAR- antagonists on EPA induced inhibition of β-carotene uptake and lipid transporter expression in Caco-2 cells



Differentiated Caco-2 cells grown in 6-well plates were treated with 250 µmol/l taurocholic acid (control) and GW6471 (PPAR-α antagonist) or GSK3787 (PPAR-δ antagonist) in the absence or presence of EPA (100 µmol/L) for 72h (A). Assessment of β-carotene uptake in cells was done as described in methods (B&C). Immunoblotting of SR B1 and (D).NPC1L1 in Caco-2 cell lysates. The bars represent mean β-carotene uptake ± SD (n=6) or mean relative density ± SD (n=3) of target protein with GAPDH, and the bars without common superscript differ significantly (P<0.05).

Fig 5. Effect of EPA and PPAR-α agonist on transcellular transport and conversion of β-carotene to vitamin A in Caco-2 cells



Caco-2 cells grown in transwell plates for 3 weeks were treated with 250 µmol/L taurocholic acid (control) either in the presence or absence of EPA (100 mmol/L) or WY 14643 (25 µmol/L, PPAR-α agonist) for a period of 72 h. (A). β-carotene uptake in cells and (B). β-carotene secretion (C). Retinol secretion and transport to the basolateral media was analysed as described in method section. The bars indicate mean±SD of 6 observations and the bars without common superscript differ significantly (p<0.05).

CONCLUSIONS

Recent studies have indicated that the intestinal absorption of carotenoids is a facilitated process, involving specific lipid transporters. The PPAR family of receptors are known regulate the expression of lipid transporters. Further, ω-3 fatty acids and CLA are known agonists of PPARs. However, the effect of either synthetic or natural agonists of PPARs on intestinal carotenoid absorption remained unknown. In this study we demonstrate that EPA but not CLA or DHA inhibits the β-carotene absorption via downregulation of SRB1 and NPC1L1 expression. DHA, though down regulated the expression of NPC1L1, did not influence the β-carotene uptake in intestinal cells. Further, PPAR-α, but not PPAR-γ or PPAR-δ agonists emulated the effect of EPA on β-carotene absorption and SRB1 expression. We also demonstrated that specific PPAR-α antagonist abrogates the inhibitory of effect of EPA on β-carotene uptake. Moreover, both EPA and PPAR-α agonist also inhibited the basolateral transport of β-carotene in Caco-2 cells grown on permeable supports. These results together suggest that EPA, but not DHA inhibits the intestinal β-carotene absorption by downregulating the SRB1 expression in a PPAR-α dependent mechanism.

2. STUDIES ON THE EFFECTS OF OBESITY AND OBESITY INDUCED TYPE-2 DIABETES ON ADVANCED AGING PHENOMENON

WNIN/Ob lean, WNIN/Ob and WNIN/Gr-Ob male rats of 9-10 weeks age were procured from NCLAS, NIN, Hyderabad. They were grouped as: Group1 – WNIN/Ob lean controls, Group 2 – WNIN /Ob controls, Group 3 – WNIN/Gr-Ob controls and Group 4 – WNIN/Gr- Ob with diabetes. Rats in groups 1-3 received control diet ad lib and those of group 4 received a high sucrose diet (HSD) to induce type 2 diabetes in them. The rats were fed with sterile diet and water for a period of 6months. The body weight gain and FER of HSD fed WNIN/Gr-Ob rats were significantly higher after 6 months of feeding. Fasting insulin levels and insulin AUC were significantly higher in HSD fed rats after both 3 and 6 months of feeding indicating aggravated insulin resistance condition, a pre-diabetic condition. Histopathological examinations indicated higher percentage of liver steatosis and a greater degeneration of kidney tubules in WNIN/Gr-Ob rats fed HSD compared to SBD fed controls. Interestingly, IHC studies also indicated the highest intensity and percentage of pancreatic tissue stained for insulin in HSD fed WNIN/Gr-Ob rats compared those fed SBD, at both time points of feeding. Similarly, the β-cell function and HOMA β were significantly higher in WNIN-Gr/Ob rats fed HSD, *albeit* after 3 months of feeding but not later. These findings appear to indicate that despite the low count of β-cells in WNIN/Gr-Ob rats fed HSD – your observation of β cell function and HOMA β do not seem to support this conclusion but the other way round; there is the maximum efficiency and functioning of β –cells in HSD than SBD fed rats. Biochemical and histological data suggest that feeding high sucrose diet to WNIN/Gr-Ob rats aggravated their insulin resistance, a pre-diabetic condition which supports the preliminary data. DNA damage, which is considered as a prime indicator of accelerated ageing, is measured in terms of single (SSB) and double strand breaks (DSB) through the "comet" assay. But the total DNA damage indicate that the rats fed with high sucrose showed significantly higher DNA damage in brain cells when compared with the control animals. The survival analysis data also indicates that the rats fed high sucrose diet have decreased lifespan when compared to their counterparts.

The ^{13}C labeling of brain amino acids from [1-^{13}C] glucose was measured *ex-vivo* in tissue extracts using ^{1}H-[^{13}C] -NMR spectroscopy. The concentrations of ^{13}C labeled amino acids and the levels of neurometabolites were measured in the three brain regions (Cortex, Striatum and Thalamus). The total concentrations of glutamate, glutamine and aspartate were significantly increased in the cortex, striatum and thalamus regions respectively of WNIN/Gr-Ob rats fed HSD compared to WNIN/Gr-Ob rats fed SBD. The ^{13}C labeling of plasma glucose was also found significantly decreased in HSD fed rats.HSD feeding significantly reduced glucose oxidation by glutamatergic neurons in cortex and thalamus regions of the brain while a similar trend was observed in the striatum region. Also, glucose oxidation was reduced significantly in GABAergic neurons but only in the cerebral cortex. Additionally, the total glucose oxidation was reduced significantly in the cortex and striatum but not in the thalamus region of the brain. This indicates an overall reduction in the neuronal glucose oxidation / utilisation in the brains of WNIN/Gr-Ob rats fed High Sucrose Diet.

Reactive oxygen species (ROS) is a major contributing factor underlying the aging process, although ageing is multifactorial. In fact, we found significant differences in the stress levels in liver tissue, as evident from the increased protein oxidation levels and decreased catalase, reduced glutathione levels in WNIN/Gr-Ob rats fed HSD when compared to their counterparts fed SBD after 6 months on their respective diets; but only a trend was seen in the increased lipid peroxidation and decreased SOD activity of these rats. These discrepancies in the stress mechanism are in line with our finding that rats fed HSD had only aggravated insulin resistance but were not actually diabetic. These observations indicate that HSD fed obese rats exhibited moderately higher hepatic oxidative stress which was probably due to moderate changes seen in enzymatic and non enzymatic anti oxidants.

Further, the effects of high sucrose feeding were observed in the regulation of ROS mechanisms in the brain. We found the increased neuronal oxidative stress (lipid peroxidation) and decreased activity of antioxidant status (reduced glutathione and SOD activity) in HSD than Starch fed rats; which together could result in greater macromolecular damage suggesting that HSD feeding decreased longevity of WNIN/Gr-Ob obese rats probably by aggravating IR, a condition that precedes T2D and increasing oxidative stress.

Work progress in continuation and the objective is

- To determine the telomere length, a key marker of ageing studies.

Experimental design

Animal experimentation

The animal experimental procedure was approved by the “Institute's ethical committee on animal experiments” at National Institute of Nutrition, Hyderabad, India. WNIN/Ob, WNIN/Ob-Lean and WNIN/Gr-Ob male rats of 9-10 weeks age were procured from NCLAS, NIN, Hyderabad. The rats were housed individually in standard polycarbonate cages at 22 ± 2°C, with 14-16 air changes per hour at a relative humidity 50-60 per cent with a 12 hour light/dark cycle. These rats were given sterile powdered diet along with water, *ad libitum* for 6 months. They were grouped as: Group1-WNIN/Ob-Lean controls, Group 2-WNIN /Ob controls, Group 3-WNIN/Gr-Ob controls and Group 4-WNIN/Gr- Ob with diabetes. Diets were prepared according to AIN-93G formulation. The control diet composed of 54.5% of starch and in High Sucrose (HS) diet the starch is replaced with sucrose. Rats in groups 1-3 received control diet *ad lib*, those of group 4 received a high sucrose (HS) diet *ad lib* to aggravate insulin resistance and / or induce Type 2 Diabetes in them. The rats were sacrificed after 3months and 6months of feeding.

Telomere length determination

Telomere length determination was done by Quantitative/Real Time PCR method using

Step One System (Applied Biosystems, Foster City, CA). Nucleic acid amplification and detection techniques are among the most valuable tools in biological research today. Absolute quantification is based on a standard curves. Telomere standard curves were generated using StepOne software v2.1 (Applied Biosystems). Real Time PCR was carried out using SYBR Green PCR master mix using 20ng template DNA. The sequences for the primers were carefully examined and checked for their specificity. SYBR Green dye binds to each new copy of double-stranded DNA, resulting in a net increase in the fluorescence intensity proportional to the quantity of double-stranded PCR product produced and detected by the instrument.

1. Plate Set up

Components	Vol. in ul
sybr mix	10
Telo F (10pm)	0.6
Telo R (10pm)	0.6
ROX	0.4
NFW	6.4
Template	2
total reaction vol.	20ul

2. Turn on PCR machine.
3. Input sample identifiers on qPCR running software, including NTC, positive control and quantities of standards.
4. Cycling conditions, followed by a dissociation (or melt) curve are as below

Reaction Conditions	Time In Sec.	
95°C	30	
95°C	5	40 Cycles
60°C	34	
melt curve analysis		

Once PCR sets completed, analysiis of remove plate and discard

Processing and analyzing the data

Export values (kb/reaction for telomere and genome copies/reaction for SCG) to csv format. The kb/reaction value is then used to calculate total telomere length in kb per human diploid genome.

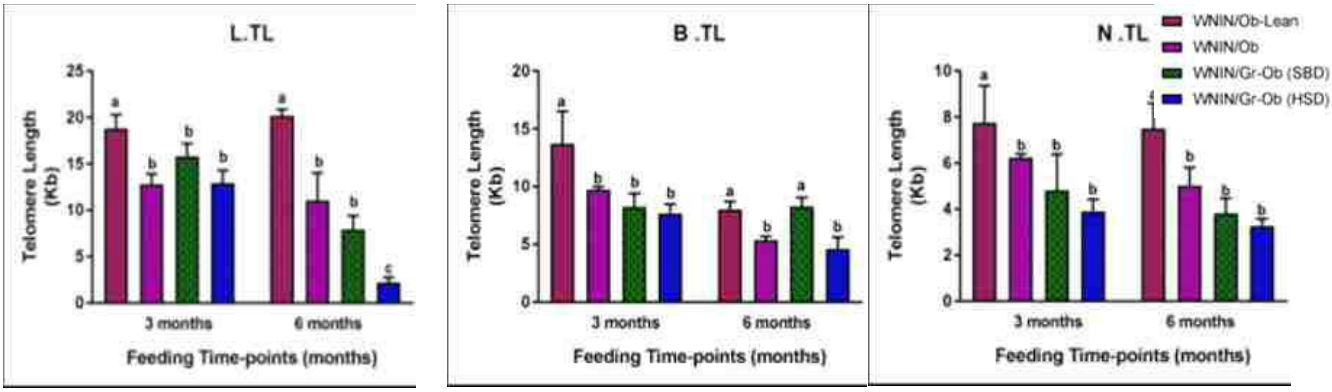
The telomere kb per reaction value is divided by diploid genome copy number to give a total telomeric length in kb per human diploid genome.

RESULTS

Telomere length was determined in brain tissue and also other tissues like liver and neuronal cells. A significant reduction was observed in the telomere length of brain and liver tissues in HSD fed rats after 3 months on their respective diets in comparison to their controls fed SBD.

Salient findings of the study so far

- Tissue specific variation was observed in the length of the telomeres.
- Telomere length was significantly decreased in the liver and the brain tissues after feeding for 6 months on their respective diets.



L.TL = Telomere length determined in the liver tissue. B.TL= Telomere lengths in the brain tissue. N.TL= Telomere length determined in the isolated neuronal cells.

- Telomere length was comparable among the obese rats (ie group2-4) but significantly lower than the WNIN/Ob lean controls.

CONCLUSION

The results appear to indicate that, high sucrose feeding accelerates ageing of WNIN/Gr-Ob rats as evident from the decreased telomere length.

3. AMELIORATION OF NEURONAL CELL DEATH IN OBESE RAT MODEL BY DIETARY RESTRICTION THROUGH MODULATION OF UBIQUITIN-PROTEASOME SYSTEM

According to the World Health Organization, 13% of the world's adult population was obese, and 39% were overweight in 2014. The worldwide prevalence of obesity was more than doubled between 1980 and 2014. Strong evidence exists indicating that obesity accelerates the onset and exaggerates the severity of a myriad of age-related disorders including type 2 diabetes, hypertension, myocardial infarction and stroke. Thus, it is possible that obesity might symbiotically interact with the aging process to significantly expedite the development of the age-related diseases. Emerging research has shown that obesity is also a potent risk factor for cognitive decline and various types of neurodegenerative dementias. The Central nervous system may be one of the most crucial sites at the intersection of age and obesity, and the relationship is highlighted by observations of gross abnormalities in overall brain structure and architecture associated with obesity. Alterations of brain morphology in obese young adults particularly in the frontal lobe were reported earlier.

Impaired function of the ubiquitin-proteasome system (UPS) has long been implicated as a contributing factor in various neurodegenerative diseases. However, the role of UPS in age-related and obesity-induced neurodegeneration is still to be elucidated. In this context, previously we described the neuronal damage in a spontaneously developed obese rat model, WNIN/Ob rat and provided the molecular basis in the form of altered UPS. Because, these animals were shown to be more prone to the age-related abnormalities such as vision impairment (retinal degeneration and cataract) (4-6), impaired immunity and development of tumors.

Neuronal cell death in WNIN/Ob rat is due to the unbalanced ubiquitin-proteasome system: wherein the up-regulated UCHL-1 stabilized p53 that drive apoptosis through BAX and caspase-3 were demonstrated. Further, the decreased proteasomal activity fails to clear the misfolded and unfolded proteins targeted to ER for degradation and thereby eliciting ER stress in the brain of WNIN/Ob rats. This was the first report of its kind that provides a role of UPS in neuronal damage or cell death in obesity. However, further studies are warranted to establish the involvement of altered UPS in neuronal damage under obese conditions conclusively.

Variations of the basic dietary regime, now known as dietary restriction (DR), is the most efficient way of extending the lifespan and health span of mammals. DR has been shown to have many benefits against obesity, oxidative stress, aging and age-related complications in various models. Studies show that neurons in the brains of rodents maintained on DR regimen exhibit increased resistance to metabolic, oxidative and excitotoxic insults. However, no attempts were made to explore the role of UPS and ER stress in obesity-induced neuronal health under DR. Therefore; we investigated the effect of DR on obesity-induced neuronal cell death in an obese rat model and its influence on UPS and ER stress.

METHODOLOGY

Animals: The WNIN/Ob rats of 40 days old along with their respective lean littermates were maintained in a temperature- and light-controlled animal facility. A group of Ob rats was fed ad libitum, and another group was pair-fed (PF) with lean (same quantity of diet is given to Ob rats as that of the food intake of lean rats). A group of respective lean littermates received stock diet ad libitum served as control. These three groups of animals were maintained for 6.5 months on their respective diet regime. Food intake and body weights were monitored periodically. After completion of the experimental period, rats were sacrificed by cervical dislocation. The animals were dissected, and cerebral cortex was collected. Animal care and protocols were in accordance with and approved by Institutional Animal Ethics Committee.

TUNEL assay: To determine apoptosis in the cerebral cortex, TUNEL assay was done using the *In Situ* Cell Death Detection Kit as described previously.

Immunoblotting: Tissues were homogenized in a buffer using a glass homogenizer, and the homogenate was centrifuged at 12,000xg for 20 min. The supernatant was collected and used for immunoblot analysis. An equal amount of protein from cerebral cortex was subjected to 12% SDS-PAGE and proteins were transferred onto PVDF membrane and incubated overnight at 4°C with respective primary antibodies diluted in PBS. After washing with PBST, membranes were then incubated with anti-rabbit IgG or anti-mouse IgG secondary antibodies conjugated to HRP. The immunoblots were developed with enhanced chemiluminescence detection reagents, and digital images were recorded.

Quantitative real-time polymerase chain reaction (qRT-PCR): Total RNA was extracted from the cerebral cortex using Tri-reagent. Isolated RNA was purified by RNeasy Mini Kit (Qiagen) and reverse transcribed to cDNA. qRT PCR was performed in triplicates with 20 ng cDNA templates using SYBR green master mix with gene-specific primers. Data were compared between lean, obese and pair-fed samples according to a comparative threshold cycle ($2^{-\Delta\Delta ct}$) method and expressed as fold change over lean.

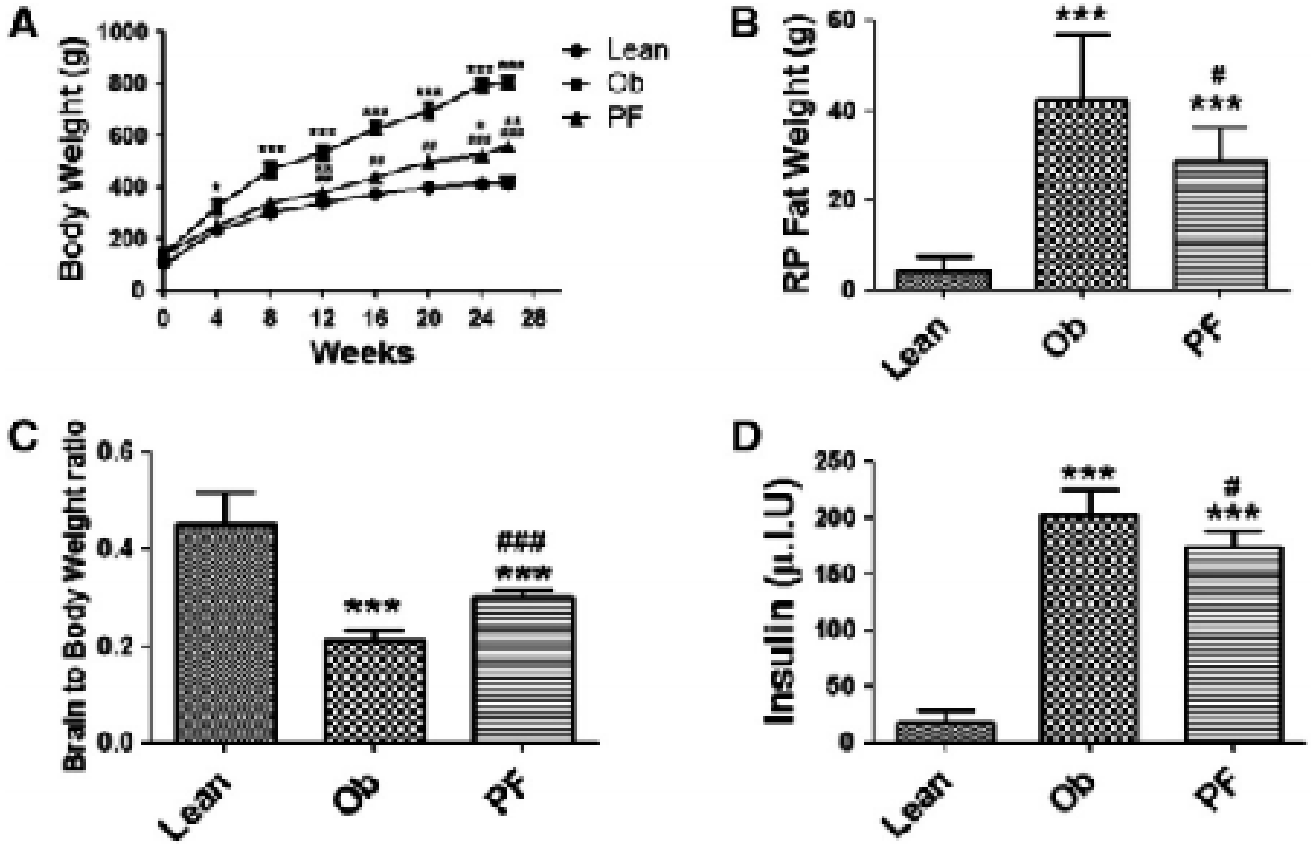
Proteasome activity assay: The enzymatic activity of the proteasome in the cerebral cortex was assayed using a kit. The kit takes advantage of the chymotrypsin-like activity, utilizing an AMC-tagged peptide substrate which releases highly fluorescent free AMC in the presence of proteolytic activity. The kit also includes a specific proteasome inhibitor MG-132, which suppresses all proteolytic activity due to proteasomes. This permits differentiation of proteasome activity from other protease activity that may be present in samples.

Immunohistochemistry: The paraformaldehyde-fixed cerebral cortex of rats was embedded in paraffin, and transverse sections (4µm) were mounted on silane-coated slides. Immunohistochemical study was performed for UCHL1, UCHL5, p53 and BCL2 using Vectastain Elite ABC kit (Vector Laboratories) that exploits the Avidin–Biotin Complex method as described previously. Sections were visualized using a Leica fluorescence microscope at magnification ×630.

RESULTS

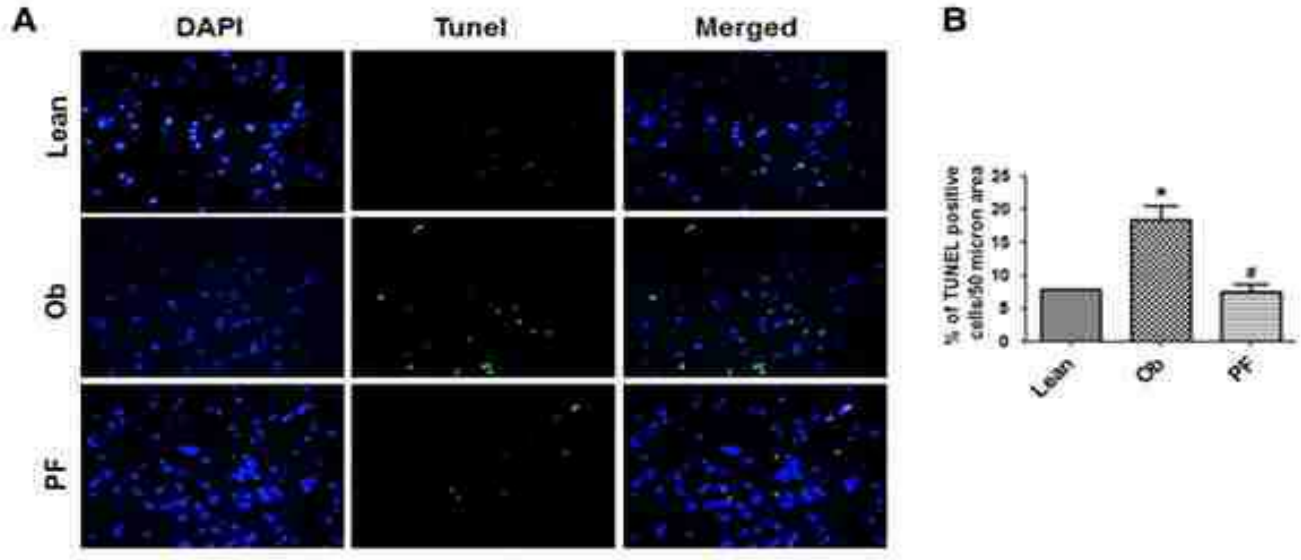
- Body weight gain of PF obese rats was significantly lower than ad libitum fed obese rats throughout the experimental period (Fig. 1A). Obese rats showed significantly more retroperitoneal (RP) fat when compared with lean rats while PF showed significantly less weight than Ob group (Fig. 1B).
- When the ratio of brain weight to body weight was calculated, the Ob rats were found to have lowered ratio compared to their lean counterparts (53.4% reduction), and PF rats showed a significant increase in organ-to-body weight ratio than Ob rats (Fig. 1C). Plasma insulin levels of PF rats were significantly lower than those of Ob rats (Fig. 1D).
- TUNEL assay indicated very few TUNEL-positive cells in lean rats (Fig. 2). In contrast, there was an apparent increase in the number of apoptotic neuronal cells in the cerebral cortex of Ob rats. Interestingly, PF rats showed significantly less TUNEL-positive cells (Fig. 2).

Fig 1. Metabolic and physiological parameters of rats



*Panel A: Body weight during the study. Panel B: RP fat weight at the end of the study. Panel C: Brain-to-body weight ratio. Panel D: Plasma insulin levels at the end of the experimental period. Data represent mean±SEM (n=8) (*p<0.05, **p<0.01, ***p<0.001 when compared to lean, #p<0.05, ##p<0.01, ###p<0.001 when compared to Ob).*

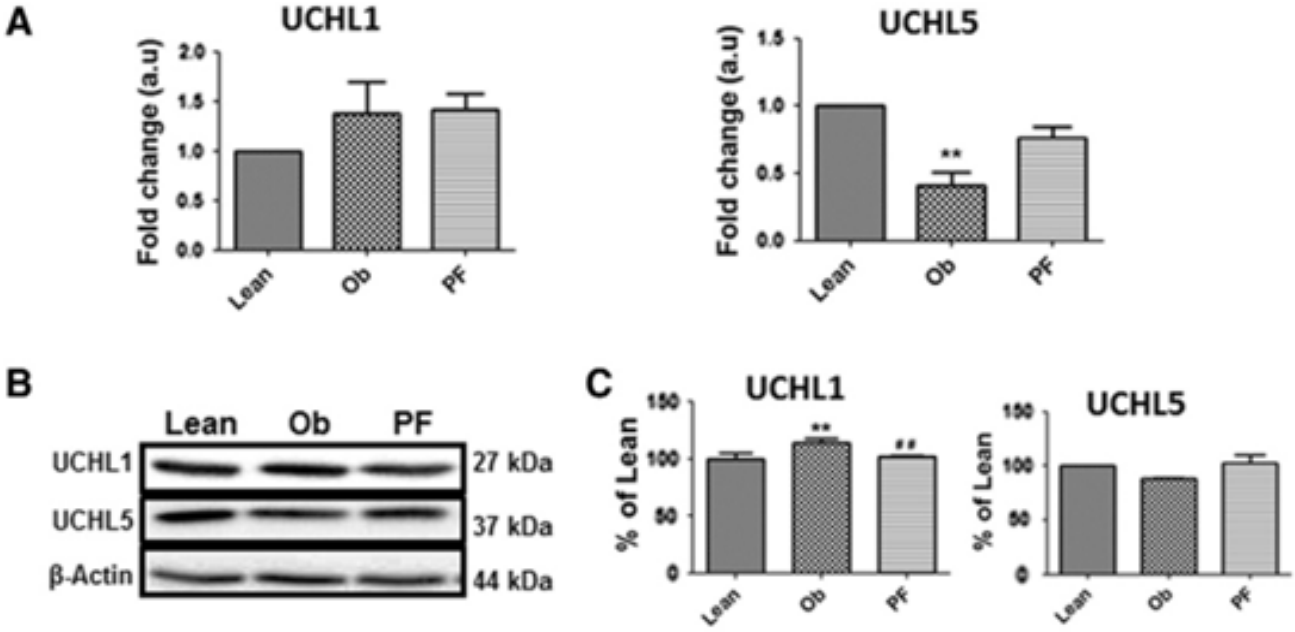
Fig 2. TUNEL-positive neurons in the cerebral cortex of rats



*Panel A: Representative fluorescence microscopic images of the cerebral cortex of lean, Ob and PF rats. Panel B: Bar graph shows % TUNEL-positive cells. Data represent mean±SEM from five independent experiments (*p<0.05 when compared to lean, #p<0.05 when compared to Ob).*

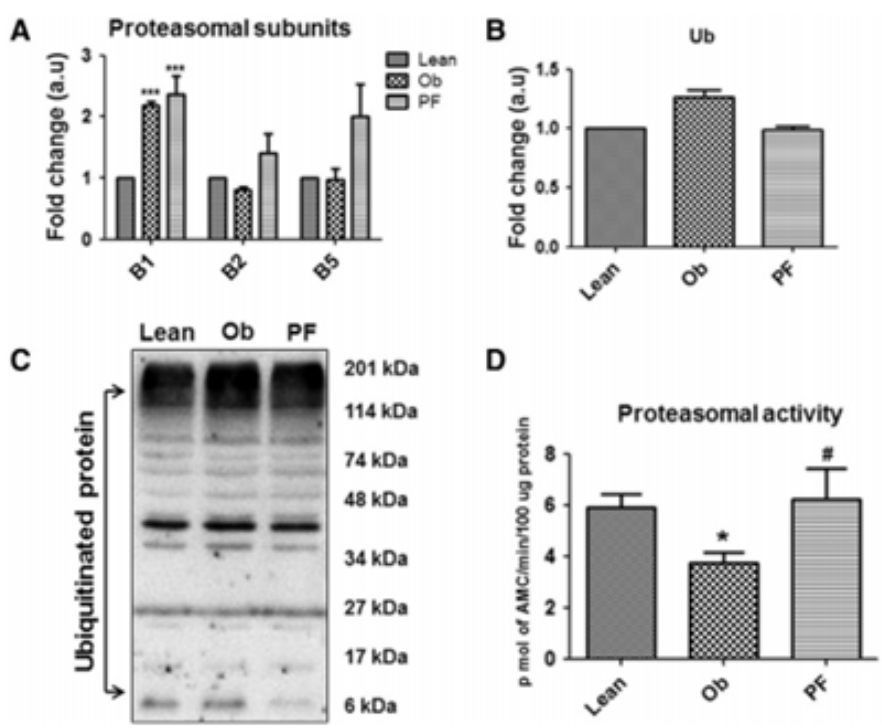
- Ob rats showed increased UCHL1 expression and decreased UCHL5 expression when compared to lean rats, and expression pattern of these two deubiquitinating enzymes in PF rats was similar to that of lean rats (Fig. 3).
- Declined proteasomal activity was observed in the Ob rats when compared to lean, and interestingly, PF rats showed activity similar to that of lean rats (Fig. 4D).
- The declined proteasomal activity could be a result of decreased expression of proteasome subunits or other extrinsic factors. qRT-PCR data showed upregulation of B1 and unchanged B2 and B5 levels in Ob rats (Fig. 4A). As a result of decreased proteasomal activity, there is an increased ubiquitinated protein in Ob rats that is prevented by DR (Fig. 4C). There was an increased trend in ubiquitin at the gene level in Ob rat when compared to lean and PF rats (Fig. 4B).
- Gene expression by qRT-PCR of various ER stress markers such as ATF6, XBP1, IRE1, PERK and CHOP and found up-regulation of these markers, except PERK, in the Ob rat (Fig. 5A) was studied. Further, immunoblot for CHOP, total ATF6∝, and total as well as active XBP1 (spliced) clearly showed increased protein levels of ATF6∝, XBP1 and CHOP in the cerebral cortex of Ob rats but not in PF rats (Fig. 5B & C). The results of immunoblot are in accord with qRT-PCR.
- Since persistent ER stress triggers apoptosis, p53 levels by qRT-PCR, immunoblot, and immuno-histochemistry were examined. The results showed a significant increase in protein level but not in mRNA level in Ob rats (Fig. 6A, B, & D).
- Co-immunoprecipitation assay indicated ubiquitination of p53 in the cerebral cortex of rats and the ubiquitin pulled from p53 is relatively lesser in Ob rats compared with lean and PF rats (Fig. 6C). Further, protein levels of BAX and BCL2 by immunoblot and immunohistochemistry, respectively, two important mediators that regulate apoptosis was analyzed. There was an increased protein expression of BAX but decreased expression of BCL2 in the cerebral cortex of Ob rats (Fig. 6B & E).

Fig 3. Expression of UCHL1 and UCHL5 in the cerebral cortex of rats



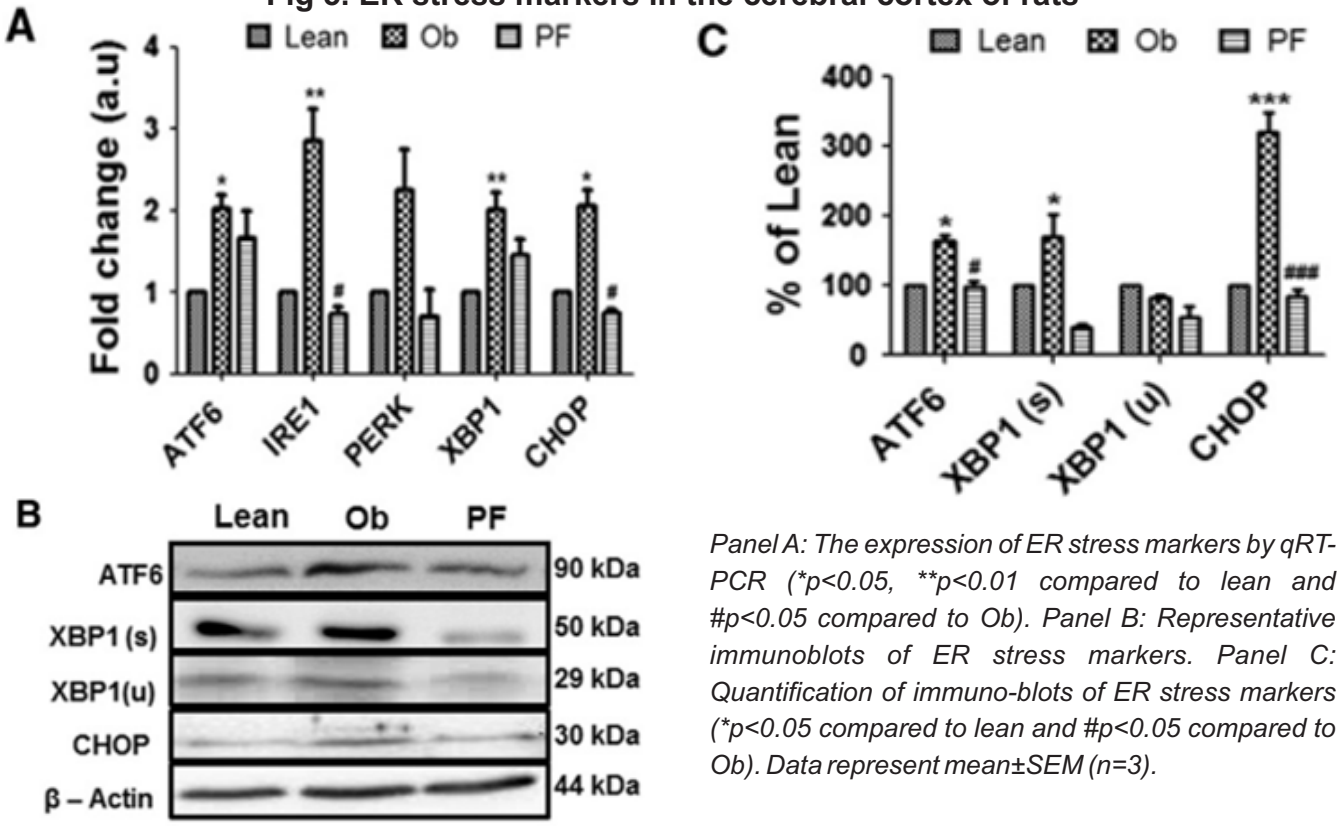
*Panel A: Expression pattern of UCHs by qRT-PCR.. Panel B: Representative immunoblots of UCHs. Panel C: Quantification of immunoblots of UCHs (**p<0.01 when compared to lean, ##p<0.01 when compared to Ob). Data in panels A and C represent mean±SEM (n=3).*

Fig 4. Ubiquitination and proteasomal activity in the cerebral cortex of rats



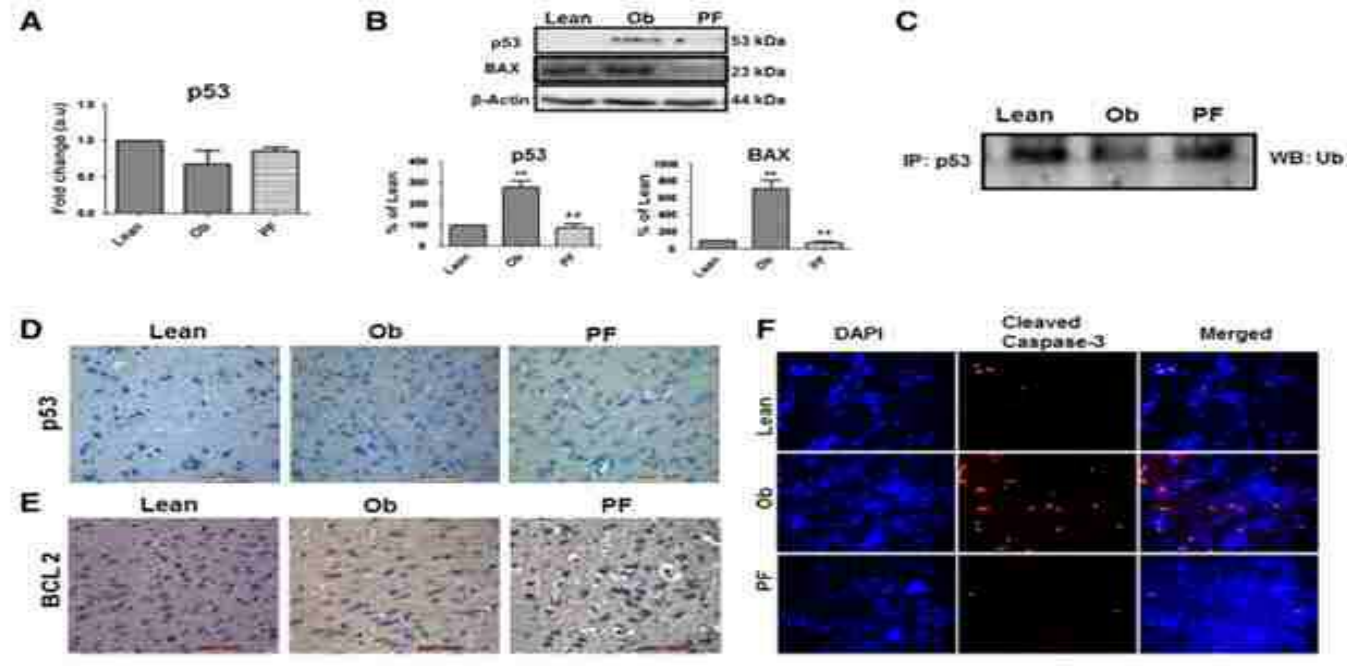
*Panel A: Expression of catalytic subunits B1, B2 and B5 of the proteasome by qRT-PCR (***p<0.001 when compared to lean). Panel B: The expression of ubiquitin by qRT-PCR. Panel C: Representative immunoblot of ubiquitinated proteins. Panel D: The chymotrypsin-like proteasomal activity (*p<0.05 when compared to lean, #p<0.05 when compared to Ob). Data in panels A, B and D represent mean±SEM (n=3).*

Fig 5. ER stress markers in the cerebral cortex of rats



*Panel A: The expression of ER stress markers by qRT-PCR (*p<0.05, **p<0.01 compared to lean and #p<0.05 compared to Ob). Panel B: Representative immunoblots of ER stress markers. Panel C: Quantification of immuno-blots of ER stress markers (*p<0.05 compared to lean and #p<0.05 compared to Ob). Data represent mean±SEM (n=3).*

Fig 6. Apoptotic regulators in the cerebral cortex of rats



*Panel A: The expression of p53 by qRT-PCR. Panel B: Representative immunoblots and quantification of p53 and BAX (*p<0.05, **p<0.01 compared to lean, ##p<0.01 compared to Ob). Panel C: Coimmunoprecipitation of p53 and Ub. Panel D: Expression of p53 by immunohistochemical staining. Panel E: Expression of BCL2 by immunohistochemical staining. Sections were counterstained with hematoxylin (blue). Panel F: Immunofluorescence images of cleaved caspase-3. Sections were counterstained with DAPI (blue).*

CONCLUSION

Obesity is a major risk factor associated with a variety of human disorders and its involvement in age-related diseases such as diabetes, coronary heart disease, and cancer has been well characterized. As the diet/calorie restriction is the most effective intervention for the age-related disorders, we examined whether DR could ameliorate neuronal cell death due to obesity using a spontaneous obese rat model. The findings indicate that DR prevented significant gain in body weight, fat deposit accumulation, elevation of insulin levels in the obese rat and thereby improving overall metabolism that could abolish alterations in the UPS. Prevention of alterations in the UPS resulted in alleviated ER stress and p53 levels thereby ameliorating neuronal apoptosis and generating a healthy environment in the neurons.

4. UBIQUITIN-PROTEASOME SYSTEM AND ER STRESS IN THE RETINA OF DIABETIC RATS

Over the last two decades, diabetes mellitus has become one of the most challenging public health problems worldwide. It is estimated that 415 million people had diabetes in 2015 and by 2040 this will rise to 642 million worldwide. Chronically high blood sugar from diabetes is associated with damage to the tiny blood vessels in the retina, leading to diabetic retinopathy (DR). DR is one of the leading causes of vision impairment with high social impact affecting one-third of the diabetic people worldwide. DR is a multi-factorial progressive disease of the retina with an extremely complex pathogenesis that involves a variety of different cells, molecules, and factors. Metabolic changes in the diabetic retina result in the altered expression pattern of many mediators resulting in vascular lesions and cell death.

The ubiquitin–proteasome system (UPS) is the primary intracellular pathway for modulated protein turnover, playing an important role in the maintenance of cellular homeostasis. Impairments of UPS function underlie an increasing number of genetic, lifestyle and idiopathic diseases including diabetes and its complications. UPS plays an essential role in pancreatic β-cell health and insulin sensitivity, alterations in which leads to β- cell apoptosis and insulin resistance. Studies have shown that hyperglycemia and oxidative stress can modulate UPS activity that plays a detrimental role in the development of diabetic complications. One way by which altered UPS can affect cell survival is by the induction of endoplasmic reticulum (ER) stress that can trigger apoptosis. In recent years, studies have demonstrated that dysfunction of the ER, or ER stress, is involved in the pathogenesis of diabetes and its complications. However, the status of ER-associated degradation (ERAD): the interface between unfolded protein response (UPR) and UPS is not reported in the diabetic rat retina. A better understanding of the major players of protein quality control machinery under normal and diabetic conditions is essential for the development of novel strategies targeting its activity. Hence, we investigated the status of UPS, ER stress, and autophagy in the retina of type 1 diabetic rat model.

METHODOLOGY

Animals: Two-month-old male Sprague-Dawley rats with an average body weight of 200±15 g were maintained at a temperature of 22±2°C, 50% humidity, and 12 h light/dark cycle. Institutional and national guidelines for the care and use of animals were followed, and all experimental procedures involving animals were approved by the Institutional Animal Ethical

Committee. A group of rats received a single intraperitoneal injection of streptozotocin (STZ) (35 mg/kg) in citrate buffer for inducing diabetes while another group of rats received 0.1 M citrate buffer [pH 4.5] as a vehicle and served as control. After 72 h, fasting blood glucose levels were monitored. Animals having fasting blood glucose levels ≥ 150 mg/dL were kept under diabetic group. Both control and diabetic animals were fed with AIN-93 diet *ad libitum*. Body weight and blood glucose concentration of each animal were measured weekly. Two and four months after the diabetes induction, animals were sacrificed, and the retina was isolated from the eyes of diabetic and control rats.

Immunoblotting: Retina was homogenized in buffer with protease inhibitors. Homogenate was centrifuged at 12,000g at 4°C for 30 minutes, and the cytosolic fraction was collected. The protein concentration was measured by Folin's Lowry method. An equal amount of protein from control and diabetic rat retina was subjected to 12% SDS-PAGE and proteins were transferred onto NC membrane. NC membrane was incubated overnight at 4°C with appropriately diluted primary antibodies. After washing with PBS, membranes were then incubated with secondary antibodies conjugated to HRP. The immunoblots were developed with enhanced chemiluminescence detection reagents, and digital images were recorded by an Image Analyzer. Images were analyzed and quantitated using ImageJ software.

Proteasome activity assay: The proteasomal activity in the retina was assayed using Biovision Proteasome Activity Assay Kit. The kit takes advantage of the chymotrypsin-like activity, utilizing an AMC-tagged peptide substrate which releases highly fluorescent AMC in the presence of proteolytic activity. The kit also includes a specific proteasome inhibitor MG-132, which suppresses all proteolytic activity due to proteasomes. This permits differentiation of proteasome activity from the activity of other proteases present in samples.

Immunohistochemistry: Immunofluorescence staining was performed in paraffin sections. The procedure was similar to that of immunohistochemistry with slight modifications. After blocking, the primary antibodies were added to the sections and allowed to incubate overnight at 4°C. The binding of primary antibodies was visualized by Alexa Fluor-conjugated secondary antibodies and mounted with Vectashield mounting medium containing DAPI as a nuclear counterstain. Fluorescently labeled sections were visualized using a Leica fluorescence microscope at x400. Images were analyzed and quantitated using ImageJ software.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay: To determine apoptosis in the retina, TUNEL assay was performed using the *In Situ* Cell Death Detection Kit. The apoptotic cells were analyzed using the fluorescent microscope at x400. Images were analyzed and quantitated using ImageJ software.

Trypsin digestion: Trypsin digestion was carried out according to the protocol as described by Chou et al.,(). Briefly, rat eye was fixed with 10% neutral buffered formalin for 24 h. Retina was dissected and was placed in a 24-well dish and covered with water. Water was changed every 30 min-1 h, for 4-5 times. Retina was left overnight in water with gentle shaking at room temperature. Next day water was removed, and retina was incubated in a solution of 3% trypsin in 0.1 M Tris buffer (pH 7.8) at 37 °C with gentle shaking. When the tissue showed signs of disintegration, digested vessels were transferred onto the slide and allowed to dry completely. Slides were stained with PAS/hematoxylin stain and mounted with DPX. Images were taken with Leica microscope.

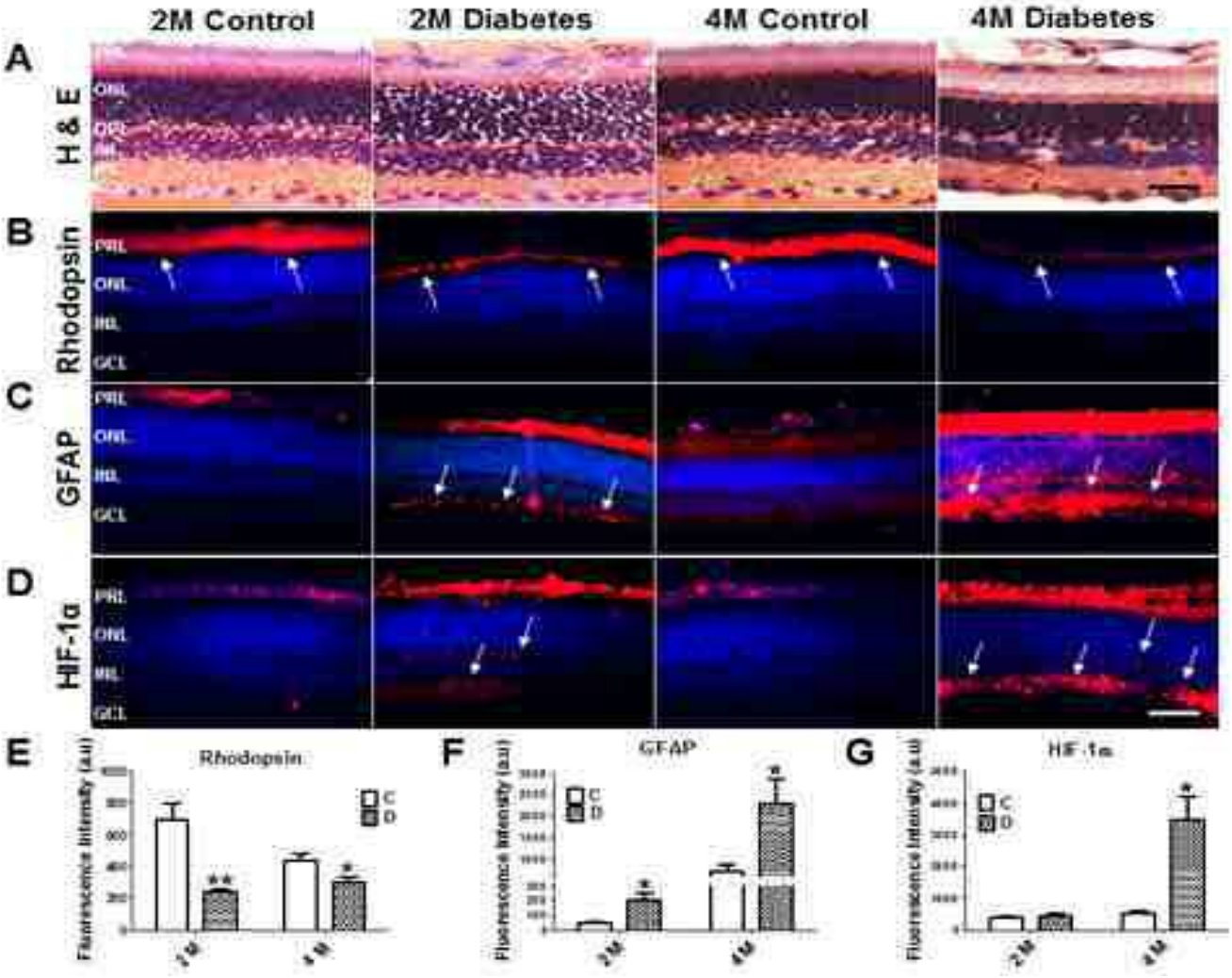
Flat mounts: The eyes were enucleated and immediately fixed in 4% paraformaldehyde in PBS for one hour. The anterior segments were removed, and the retinas were detached and separated from the optic nerve head. After washing with PBS, the retina was blocked with 5% normal donkey serum, 5% BSA and 0.3% Triton-X-100 in PBS for 2 h. The eyecups were

incubated for 24 h with a 1:100 dilution of a 1 mg/mL solution of isolectin GS IB4 conjugated with Alexa Fluor 594 (in a cold chamber at 4°C, followed by washing with PBS. Retina was mounted with Vectashield mounting medium containing DAPI as a nuclear counterstain. Fluorescently labeled retina was visualized using the fluorescent microscope.

RESULTS

- Hematoxylin and eosin (H & E) staining was done to identify the morphological changes in the various retinal layers. Loss of nuclei was observed in outer nuclear layer (ONL) and a decrease in retinal thickness was observed in diabetic rats of 4 months when compared to its respective control (Fig. 1A). Decreased rhodopsin staining was observed in the diabetic rats of 2 and 4 months when compared with the control rats (Fig. 1B & 1E).
- Significant increase in the fluorescence intensity of GFAP and HIF-1α were observed in diabetic rats of 2 and 4 months when compared with the control rats (Fig. 1C-1G).

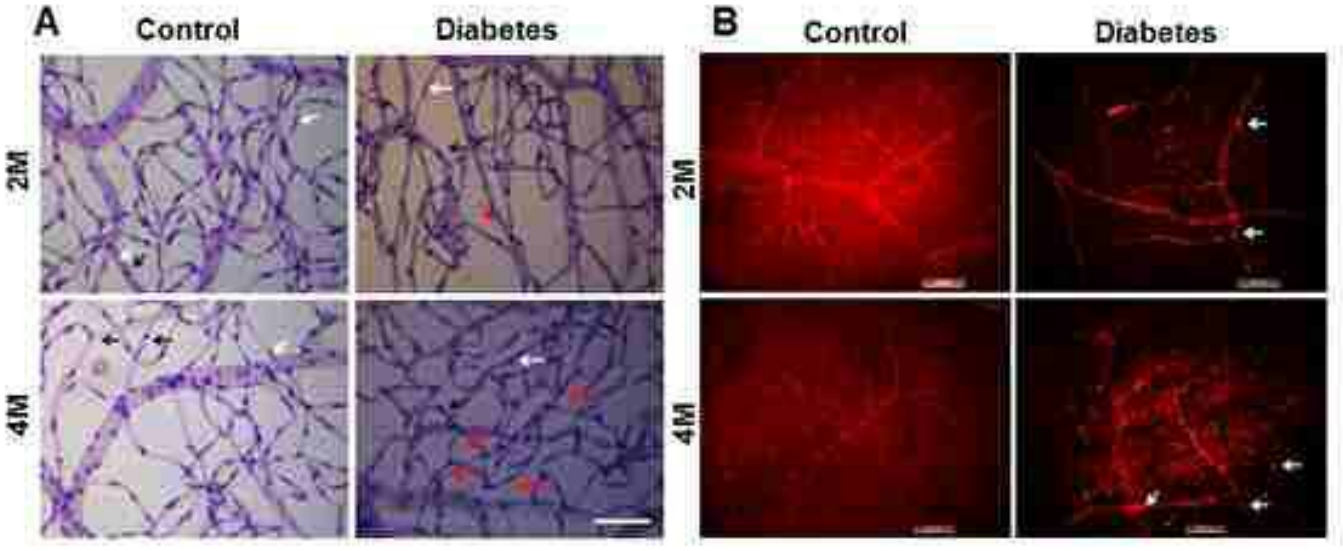
Fig 1. Morphology of retina and expression of rhodopsin, GFAP, and HIF-1α



*Representative histology (A), immunostaining of rhodopsin (B), GFAP (C) and HIF-1α (D) of 2 and 4-month control and diabetic rat retina. The relative fluorescent intensity of rhodopsin (E), GFAP (F) and HIF-1α (G) in 2 and 4-month control and diabetic rat retina. PRL-photoreceptor layer; ONL-outer nuclear layer; INL-inner nuclear layer; OPL-outer plexiform layer; IPL-inner plexiform layer; GCL-ganglion cell layer. *p <0.05 and **p <0.01. (C-control; D-diabetes; 2M- 2 months; 4M- 4 months)*

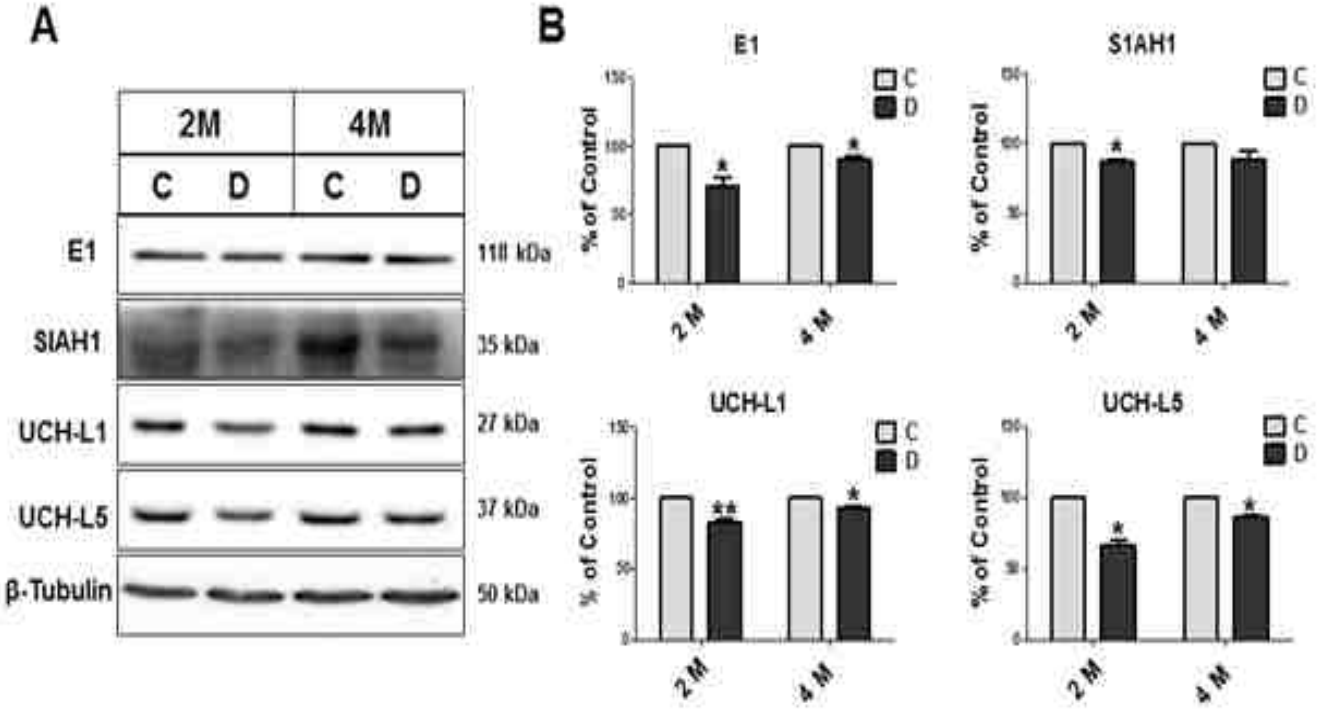
- Control rats showed normal retinal vascular architecture. Diabetic rats by 4 months begin to develop vascular pathology such as capillary degeneration (red arrow) (Fig. 2A).
- Retinal flat mounts suggested that 4 months of hyperglycemia induces more vessel leakages in the peripheral areas of the retina when compared with 2 months of diabetes (arrows in Fig. 2B) while control rats showed normal retinal vasculature. Together, the histological, immunostaining, vasculature pattern indicated diabetes-induced lesions in retina starting from 2-month after diabetes and progressed with 4-months diabetes.
- We detected the levels of UPS components: E1 (ubiquitin activating enzyme), S1AH1 (eye-specific ubiquitin ligase), and UCHs (deubiquitinating enzymes) by immunoblot. The immunoblot indicates that the level of E1, UCHL1, and UCHL5 were decreased significantly in the 2 and 4-month diabetic rats respectively when compared with the controls. (Fig. 3A & 3B).
- The difference in proteasome activity was observed between control and diabetes retina, particularly at 4-months (Fig. 4C). There was a significant decrease in ubiquitinated proteins and monoubiquitin in both 2 and 4-month diabetic rats when compared with their respective controls (Fig. 4A & 4B).
- The status of UPR and ERAD- important protein quality control systems located in the ER was assessed. We detected ATF6, XBP1, and CHOP protein levels by immunofluorescence in the 2 and 4-month rats (Figure 5A-C). Significant increase in the fluorescence intensity of ATF6, XBP1, and CHOP were observed in diabetic rats of 2 and 4 months when compared with the control rats (Fig. 5D-F).
- We detected ERAD components: HRD1 and VCP protein levels in the retina of diabetic rats by immunoblot. The results of immunoblot suggested that the HRD1 and VCP expression were decreased significantly in the 2 and 4-month diabetic rats compared with their respective control groups (Fig. 6A- 6B).

Fig 2. Trypsin digestion and flat mounts



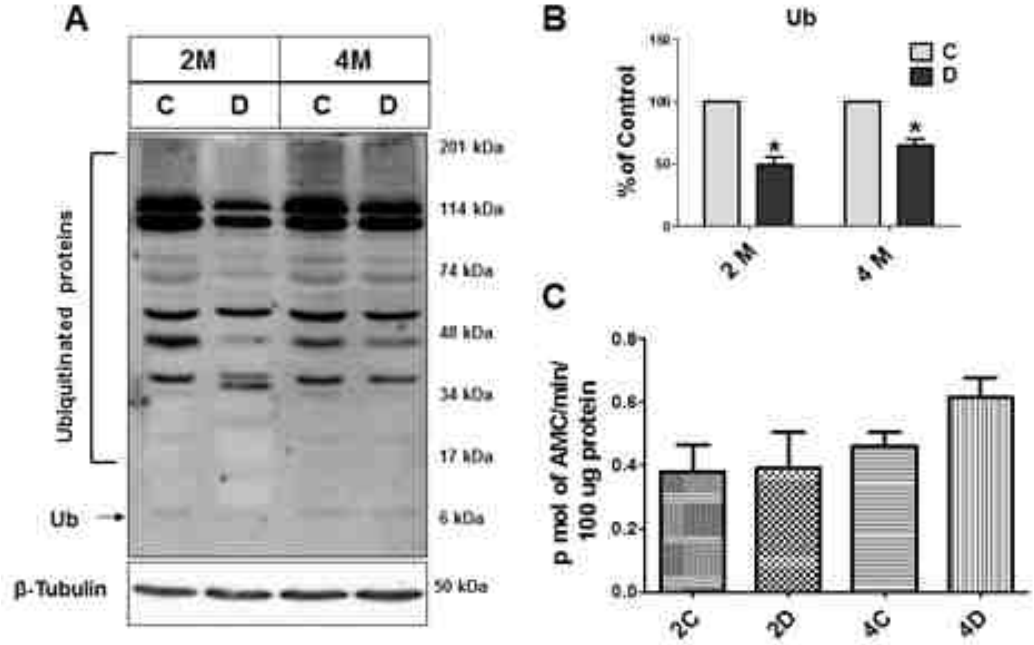
Panel A-Retinal digest of control rats shows normal vascular architecture. Diabetic rats begin to develop vascular pathology such as capillary degeneration (red arrow) by 4 months. Endothelial cells (white arrow) and pericytes (black arrow) are highlighted. Panel B-The flat-mounted retina of diabetic rat show vessel leakage in the peripheral region indicated by white arrows, while the control rats show normal retinal vasculature. (2M-2mon.; 4M-4 mon.).

Fig 3. Expression of components of UPS



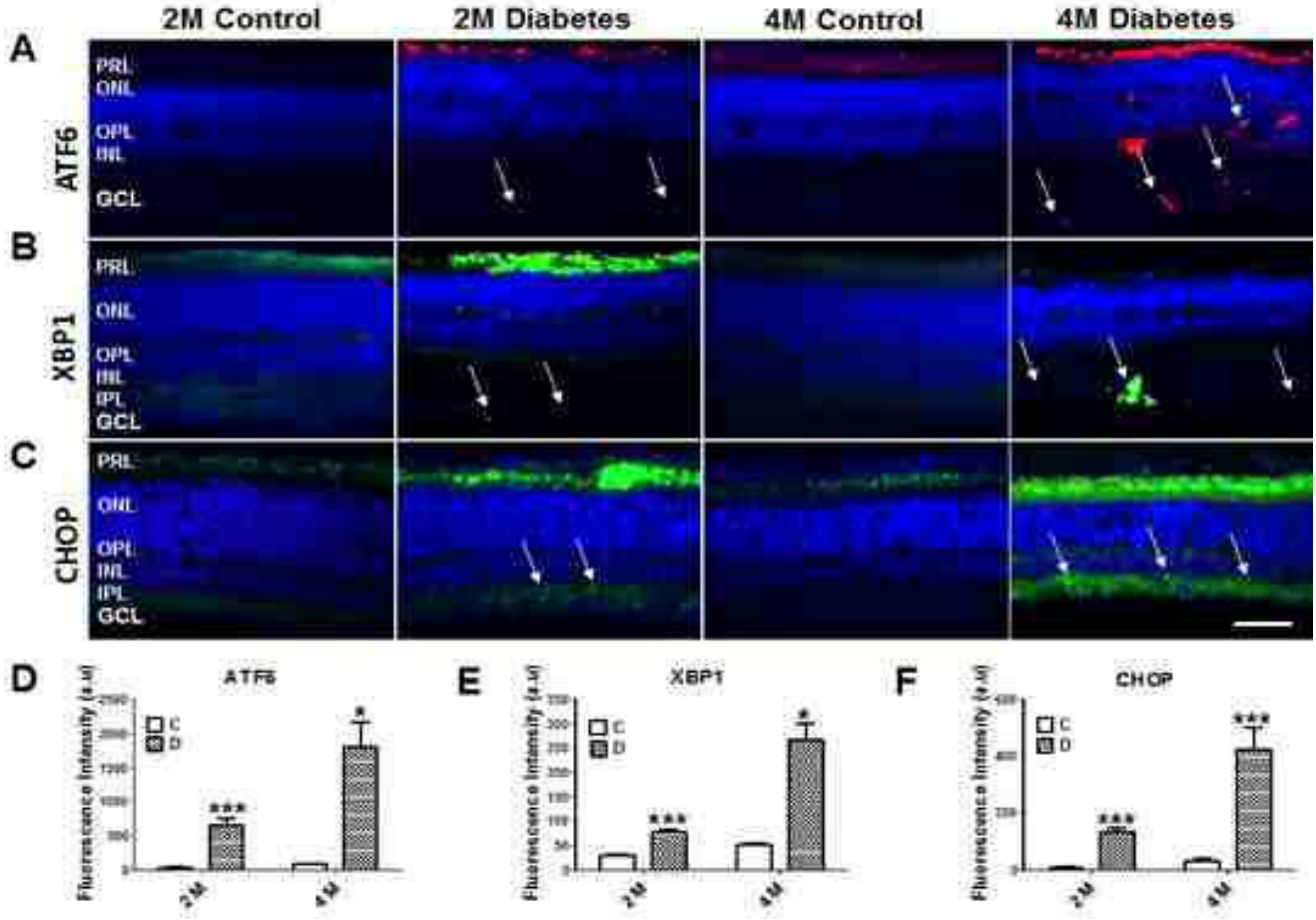
*Panel A- Representative immunoblots of E1, SIAH1 and UCHs in 2 and 4-month diabetic rat retina. Panel B- Quantification of immunoblots: expression was normalized for β-tubulin and are represented as the percentage of control. Data represent mean ± SEM of three independent experiments (*p <0.05 and **p < 0.01). (C-control; D-diabetes; 2M- 2 months; 4M- 4 months).*

Fig 4. Ubiquitination and proteasomal activity in rat retina



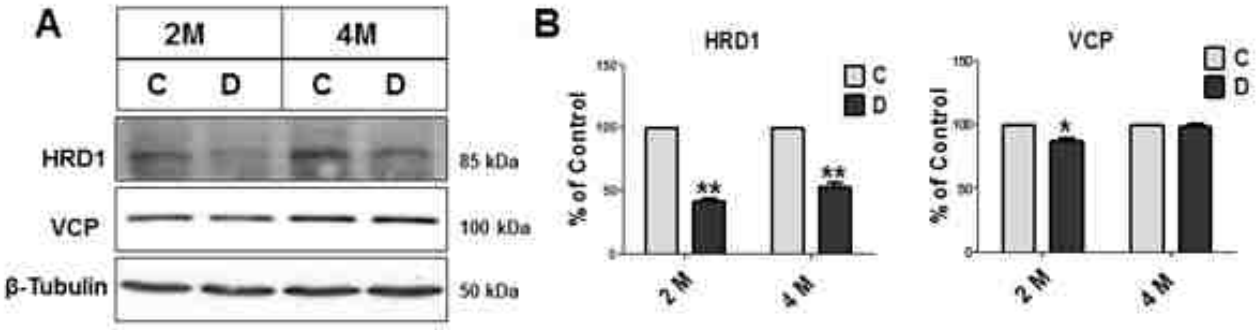
*Panel A- Representative immunoblot of ubiquitin and ubiquitinated proteins. Panel B- Quantification of immunoblot: expression was normalized for β-tubulin and is represented as the percentage of control. Panel C- Chymotrypsin-like activity in the rat retina. Data represent mean ± SEM of three independent experiments. (*p < 0.05). (C-control; D-diabetes; 2M- 2 months; 4M- 4 months)*

Fig 5. Expression of ER stress markers in rat retina



Representative immunofluorescence of ATF6 (A), XBP1 (B) and CHOP (C) in the retinas of 2 and 4- month diabetic rat. The relative fluorescent intensity of ATF6 (D), XBP1 (E) and CHOP (F) in 2 and 4-month control and diabetic rat. PRL-photoreceptor layer; ONL-outer nuclear layer; INL-inner nuclear layer; OPL-outer plexiform layer; IPL-inner plexiform layer; GCL-ganglion cell layer. (C-control; D-diabetes; 2M- 2 months; 4M- 4 months)

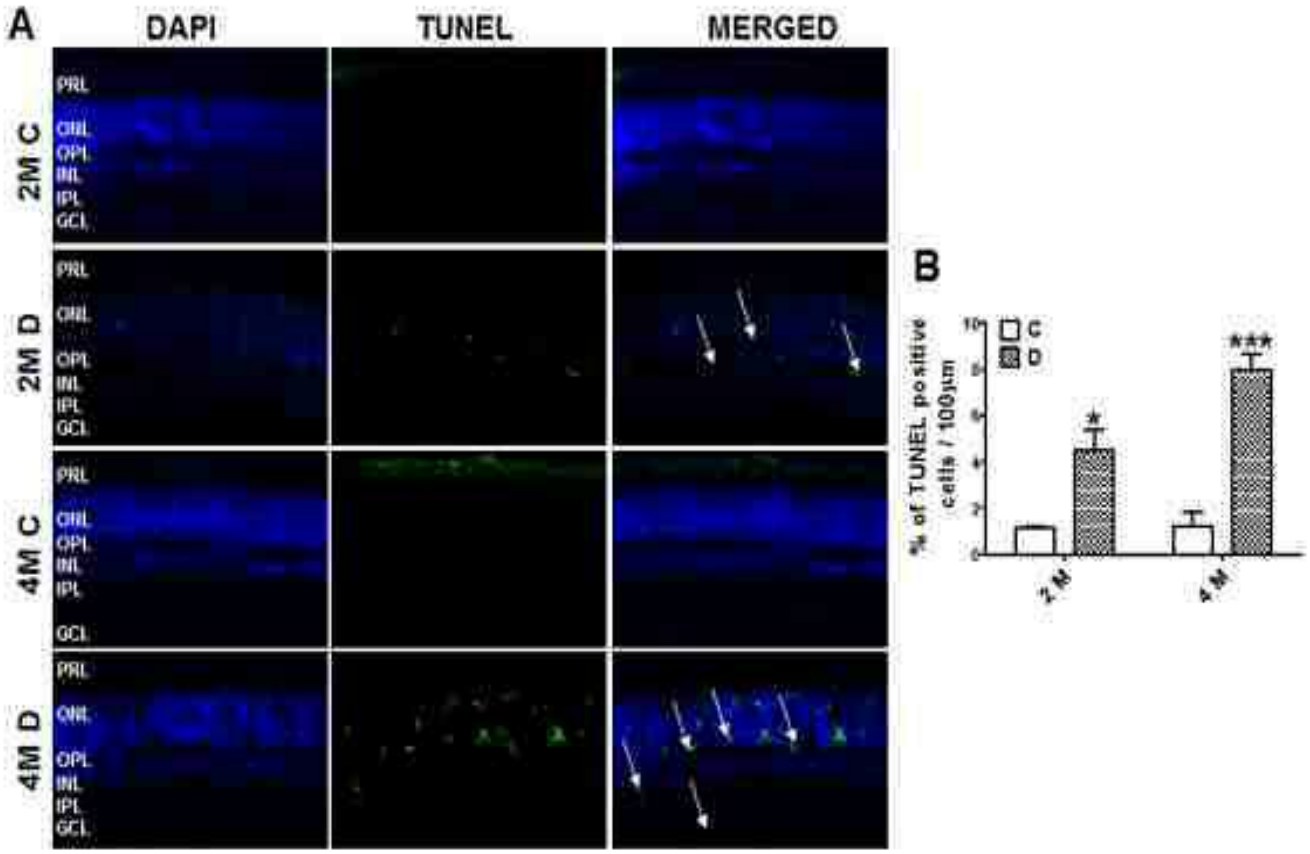
Fig 6. Expression of ERAD components in rat retina



*Panel A-Representative immunoblots of HRD1 and VCP in 2 months and 4 months' rat retina. Panel B-Quantification of immunoblots of HRD1 and VCP: expression was normalized for β-tubulin and is represented as the percentage of control. Panel C-Representative immunoblot of Autophagy marker, LC3B in 2 and 4-month rat retina. Panel D-Quantification of the immunoblot of LC3B; expression was normalized for β-tubulin and is represented as a ratio of LC3B-II to LC3B-I. Data represent mean ± SEM of three independent experiments (*p <0.05, **p <0.01 and ***p <0.001). (C-control; D-diabetes; 2M- 2 months; 4M- 4 months)*

- TUNEL assay indicated a few TUNEL-positive cells in the ONL of 2 months diabetic rats when compared with their controls. However, at 4 months of diabetes, there was an apparent increase in the number of apoptotic cells in the ONL, INL, and GCL of the retina when compared with their controls (Fig. 7A & 7B).

Fig 7. TUNEL assay in rat retina



Panel A-Representative fluorescence microscopic images of TUNEL Assay in 2 and 4-month diabetic rat retina. White arrows show the apoptotic cells. Panel B-Quantification of TUNEL Positive cells. PRL-photoreceptor layer; ONL-outer nuclear layer; INL-inner nuclear layer; OPL-outer plexiform layer; IPL-inner plexiform layer; GCL-ganglion cell layer. All the pictures were taken at 400X magnification (n=3). Scale bars, 100 µm. (C-control; D-diabetes; 2M- 2 months; 4M- 4 months).

CONCLUSION

The results of the study showed increased HIF-1α and decreased rhodopsin in the diabetes rats. The morphology of the retina as observed by H&E staining showed declined thickness in the different layers of diabetic rat retina. TUNEL assay showed more apoptotic cells in diabetic rat retina when compared to control. Vascular abnormalities in the retina are also observed in diabetic rats by flat mounts and trypsin digestion procedures. All these observations clearly confirmed the prevalence of DR-related lesions in diabetic rats. The down-regulated E1 protein in diabetic rat retina could be one reason for inducing UPR and cell death by apoptosis. The declined UCH-L1 levels in the diabetic rats as observed in this study might have an impact on the functioning of retinal neurons. The declining levels of UPS components: E1 and HRD1 as observed in the retina of diabetic rats could elicit ER stress. The prolonged ER stress triggers CHOP-mediated apoptosis in addition to autophagy induced apoptosis.

5. ALZHEIMER'S AND DANISH DEMENTIA PEPTIDES INDUCE CATARACT AND PERTURB RETINAL ARCHITECTURE IN RATS

Improved technology and health and nutrition have led to increased lifespan which in turn has contributed to the growth of aging population. With the rise in the average age of the population, there is an increased risk of chronic diseases such as diabetes, cancer, cardiovascular disorders, and neurodegenerative diseases, leading to disability and related mortality. Neurodegenerative diseases are becoming increasingly common resulting in a greater burden on the health care system of most countries. Many of these bewildering disorders are known to arise from the conformational instability leading to protein misfolding and aggregation (2). Conformational diseases arise when a constituent protein undergoes a change in size or fluctuation in shape, with resultant self-association and tissue deposition. There are at least a dozen diseases known to arise due to protein misfolding or aggregation, which have potentially devastating medical and social consequences.

Cataract, characterized by cloudiness or opacification of the eye lens, is the leading cause of blindness all over the world. Crystallins are the major structural proteins in the lens whose structure, stability and short-range interactions are important for lens transparency. There are three distinct crystallins: α-, β- and γ-crystallins. α-Crystallin, a member of the small heat shock protein family, constitutes a major portion of the eye lens cytoplasm and exists as a hetero-oligomer with two subunits, αA and αB. Increased levels of αB-crystallin have been observed in many neurodegenerative disorders, tumors and diabetic conditions. Both these proteins are known for their chaperone activity and presumably protect other lens proteins from the adverse effects of various cataractogenic insults. With aging, eye lens proteins undergo various post-translational modifications which mostly lead to their aggregation. Various physiological, environmental and genetic factors accelerate this aggregation and predispose the lens to cataract formation. Retinal degenerations (RD), a group of heterogeneous diseases of the retina, are characterized by photoreceptor degeneration and retinal pigment epithelium atrophy causing loss of visual field and acuities resulting in irreversible blindness. More than 200 genes have been implicated that cause RD (Retinal Information Network (RetNet), https://sph.uth.tmc.edu/ retnet/). Mutations leading to the altered conformation of proteins have been shown to be the underlying cause of several dominant neurodegenerative diseases including dominant retinitis degenerations. Protein misfolding leading to altered trafficking and aggregation are common features of these conditions.

Defects in the BRI2 gene on chromosome 13 leads to two autosomal dominant neurodegenerative disorders viz familial British and Danish dementias (FBD and FDD). While the onset of FBD usually occurs in the fifth decade of life with progressive dementia, spasticity, and ataxia, the onset of FDD ensues earlier, before 30 years of age with cataracts, a visual loss followed by impaired hearing, progressive cerebellar ataxia and late dementia. Histological signatures of FDD are cerebral amyloid angiopathy, parenchymal protein deposits, and neurofibrillary degeneration. In this regard, FDD is closely similar to Alzheimer's disease (AD), the major cause of dementia in aging populations. Also in both the cases amyloid deposits are made of short peptides generated in the brain by internal proteolysis of large transmembrane precursor proteins. These peptides of ~4 kDa, are amyloid β (Aβ) in AD, and ADan in FDD. In FDD, a 10-nucleotide duplication insertion causes a frame shift and the generation of the ADan sequence. FDD is also known as heredo-oto ophthalmo-encephalopathy (HOOE). HOOE/FDD is a dominantly inherited syndrome clinically characterized by a gradual loss of vision, deafness, progressive ataxia and dementia. Retinal neovascularization and posterior subscapular cataract in FDD cause visual loss in FDD. Cataracts seem to be the earliest manifestation of this disease,

starting at the age of 20 followed by hearing impairment developing at the age of 40. Interestingly, early-onset of cataract is also seen in Alzheimer's and Down syndrome patients.

Various studies now have shown a role for Aβ in retinal degenerations. Previously we also reported that the reduced form is more neurotoxic than that of the oxidized form (Cys5 and Cys22 linked by an intramolecular disulfide bond) (8). Further, we demonstrated the interaction between dementia associated peptides and α-crystallin which impaired the chaperone activity of α-crystallin (9). Interestingly, it was found that ADan reduced peptide (redADan) has both exquisite specificity and ability amongst several amyloidogenic peptides to compromise the chaperone function of α-crystallin. In the present study, we have investigated the effect of ADan (both reduced and oxidized) and Aβ (Aβ-40 and Aβ-42) peptides on lens and retina in animals upon intravitreal injection.

MATERIALS AND METHODS

Peptides: Danish dementia peptides (ADan-red and ADan-oxi), and Alzheimer's peptides (Aβ1-40 and Aβ1-42) were obtained from Bachem AG (Switzerland) and purified using a C-18 HPLC column and the purity and mass of the peptides were assessed by MALDI-TOF mass spectrometer. The lyophilized peptides thus obtained were dissolved in DMSO (dimethyl sulphoxide) under a flow of liquid nitrogen. While the sequence of ADan-red peptide is 'ASNCFAIRHFENKFAVETLICFNLFLNSQEKHY', there is a disulfide bond between Cys4 and Cys21 in ADan-oxi. The sequence of Aβ1-42 is 'DAEFRHDSGYEVHHQKLVFFAEDVGSNK-GAIIGLMVGGVVIA' and Aβ1-40 is devoid of last two C-terminal residues.

Animals and *intravitreal injection:* Twenty-eight 4-months old SD male rats *were fed ad libitum on rodent diet.* The animals were randomly divided into four groups. Peptides were injected into vitreous of the right (R) eye and the same volume of vehicle (DMSO) into the left (L) eye on day 0. Three more injections were given on every third day. Animal care and protocols were by and approved by the Institutional Animal Ethics Committee and conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Slit-lamp examination of eye lens: Eyes were examined every week using a slit lamp biomicroscope on dilated pupils. Initiation and progression of lenticular opacity were graded into four categories.

Collection of tissues: Rats were sacrificed by CO_2 asphyxiation after six weeks and eyes were enucleated. A set of eyeballs (n=4) were dissected using the posterior approach, lens and retina were frozen at −80°C until further analysis. Another set of eyeballs (n=3) were collected in 4% w/v paraformaldehyde fixative solution for histology and immuno-histochemistry.

Protein solubility, crystallin distribution, protein crosslinking, and protein aggregation: A 10% homogenate was made from the lenses in 50 mM phosphate buffer, pH 7.4. The homogenate was centrifuged at 10,000 ×g for 30 min at 4°C. The supernatant was referred to as the soluble fraction. Total and soluble protein content was estimated in homogenate and soluble fraction, and the percentage of soluble protein was calculated. An aliquot of soluble fraction was applied onto a 300×7.8 mm TSK-3000 SW-XL size exclusion chromatography (SEC) column connected to an HPLC system. The subunit profiles and cross-linking of lens soluble proteins were analyzed on 12% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) under reducing conditions.

Chaperone-like activity assay: The soluble fraction was applied onto a Sephacryl S-300 HR column equilibrated with the 25 mM Tris-Cl buffer, pH 8.0 containing 0.5 mM EDTA and 100 mM NaCl. The peak corresponding to αL-crystallin was pooled and dialyzed extensively against the buffer (8). The chaperone-like activity of α-crystallin was probed by measuring its ability to prevent the heat-induced aggregation of citrate synthase at 45°C (8).

Histology and immunohistochemistry: Eyeballs were collected from the animals and fixed in 4% paraformaldehyde in phosphate buffer (pH 7.2), followed by embedding and sectioning using standard protocols, as described previously. Three eyes were used for each age group. Paraffin sections were stained with hematoxylin and eosin. Immunohistochemistry was performed on sections using a respective primary antibody, as described previously. The slides were mounted in the antifade reagent containing DAPI and visualized using a Leica laser microscope.

RESULTS

Onset and progression of cataract: The onset of cataract due to peptide injection was observed in rats after one week of intravitreal injection and progressed to mature cataract by 2-6 weeks (Fig 1). The averaged stage of cataract at the given time in a given group is shown in Fig 1B. The onset of cataract was seen one week after intravitreal injection of ADan-red and ADan-oxi peptides. While the cataract matured by 2-weeks in the case of Adan-red, it took 5-weeks to mature in the case of ADan-oxi peptide. In the case of Aβ1-40, the onset of cataract was observed after two weeks which progressed to maturation by 6 weeks. However, with Aβ1-42, the onset of cataract occurred after four weeks, and it progressed to only stage 1.5 of cataract by six weeks. All the contra-lateral lenses injected with DMSO as a vehicle appeared to be normal and free of opacities during the experimental period. The potential of peptides inducing cataract was found in the order of ADan-red, ADan-oxi, Aβ1-40, and Aβ1-42.

Protein content: We analyzed the total, and soluble protein content in the lens as insolubilization and aggregation of soluble lens proteins lead to cataractogenesis. The percentage of soluble protein decreased to 58-70% in the lenses injected with peptides as compared to 80% of soluble protein in vehicle-injected lenses (Table 1). The decline in the percentage of soluble protein was highest in lenses injected with ADan-red and modest with Aβ1-42. These results indicate increased insolubilization (decreased soluble protein) is associated with the severity of cataract in dementia injected lenses.

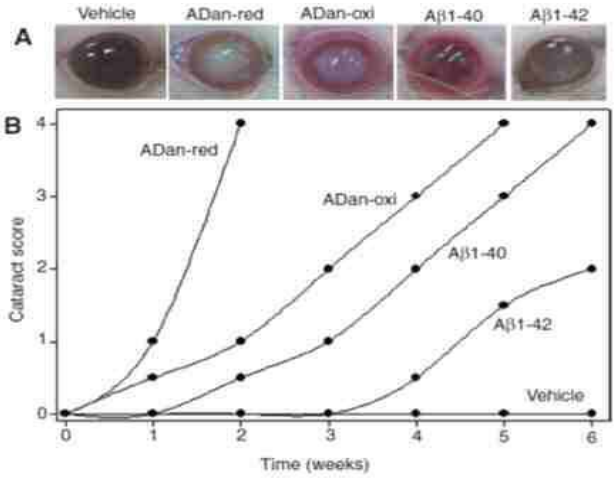
Crystallin distribution and protein cross-links: The soluble protein fractions were analyzed by HPLC on an SEC-column and SDS–PAGE. The lens-soluble fraction was clearly resolved into α-, β-, and γ-crystallin peaks on a SEC column (Fig 2).

Table 1. Protein content of the lens. The total and soluble protein in the lens of different groups was estimated by the Lowry's method, and the percentage soluble protein content was derived from the estimated values

Peptides		Total protein (mg/ g lens)	Soluble protein (mg/ g lens)	% Soluble protein
ADan-red	DMSO	615 ± 43	491 ± 30	80
	Peptide	524 ± 38	305 ± 28*	58
ADan-oxi	DMSO	605 ± 42	481 ± 31	79
	Peptide	505 ± 34	313 ± 30*	62
Aβ 1-40	DMSO	602 ± 46	475 ± 33	79
	Peptide	519 ± 40	345 ± 26*	66
Aβ 1-42	DMSO	590 ± 37	478 ± 29	81
	Peptide	495 ± 32	347 ± 26*	70

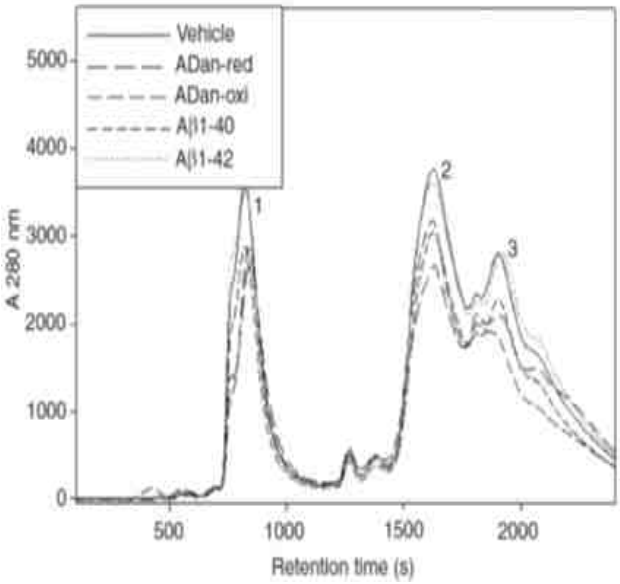
The data are mean ± SE (n=4). The superscript denotes that data are significantly different from the respective control (DMSO) group.

Fig 1. Induction of cataract upon intravitreal injection of dementia peptides



A: Representative photographs of the lens from each group. B: Quantitative representation of cataract progression in each group with time.

Fig 2. Crystallin profile of total soluble lens protein on gel filtration

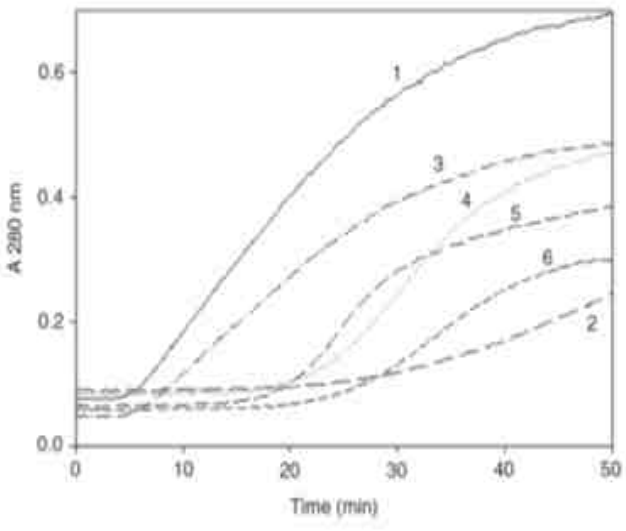


While there were alterations in crystallin peaks in ADan-red, ADan-oxi and Aβ1-40 injected lenses compared to vehicle-injected lenses (Fig 2), the crystallin distribution profile of Aβ1-42 injected lenses was similar to that of vehicle-injected lenses (Fig 2). The decrease in β- and γ-crystallin peak, particularly in ADan-red and ADan-oxi lenses, suggests that the oligomerization of α- crystallins in peptide-induced cataract lens may be involved in the formation of aggregates due to cross-linking which leads to their insolubilization. The SDS-PAGE pattern of soluble protein showing an increased proportion of cross-linked and aggregated proteins in the peptide-injected lenses compared with vehicle-injected lenses substantiates the HPLC data.

Chaperone activity of the α-crystallin: While α-crystallin isolated from the rat lenses treated with ADan-red showed significantly decreased (58%) chaperone activity as compared to α-crystallin isolated from vehicle-injected lenses in citrate synthase aggregation assay (Fig 3). In comparison, the reduction in chaperone activity of α-crystallin isolated from lenses treated with ADan-oxi, Aβ-40 peptide, and Aβ1-42 was 54%, 43%, and 31%, respectively (Fig 3).

Changes in retinal morphology: We evaluated the retinal morphology by H&E staining, and the retinas from rats injected with peptides showed significant alterations (Fig 4). The integrity of retina from ADan-red peptide injected rat was severely comprised. ADan-red peptide led to a reduced thickness of the

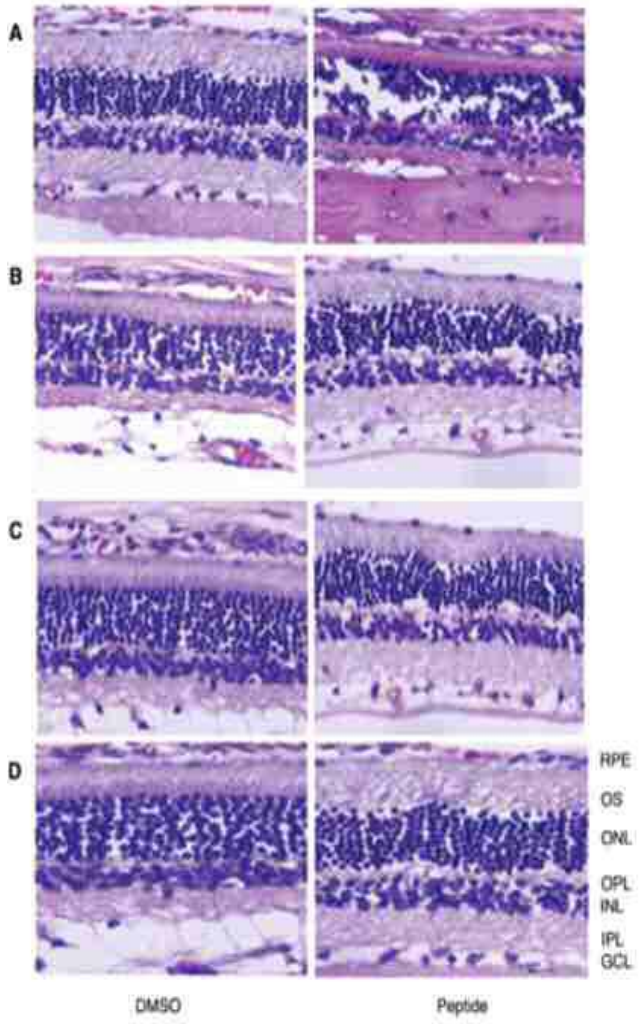
Fig 3. Chaperone activity of α-crystallin



As assessed by the suppression of heat-induced aggregation of citrate synthase at 45°C in the absence (trace 1) and presence of α-crystallin isolated from the lenses injected with vehicle (trace 2), ADan-red (trace 3), ADan-oxi (trace 4), Aβ40 (trace 5) and Aβ42 (trace 6). The traces are an average of four samples in each group.

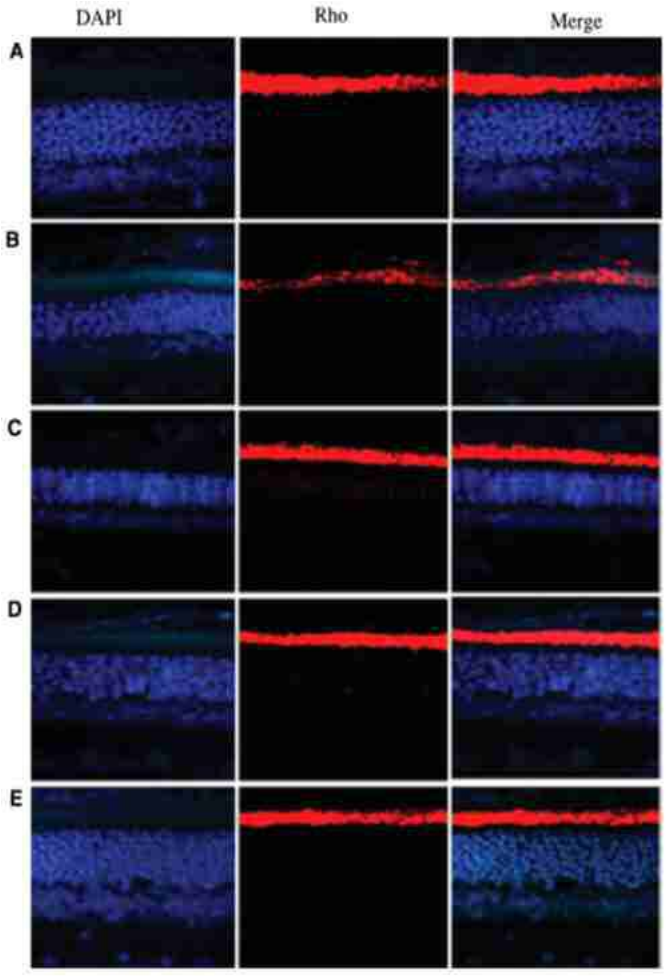
outer segment (OS), the outer nuclear layer (ONL) and the inner nuclear layer (INL). Further, there was a disruption of ONL, and no clear demarcation between ONL and INL in the retina of ADan-red treated animals. Also, the thickness of the outer plexiform layer (OPL) and inner plexiform layers (IPL) was observed to be decreased in peptide-injected retina compared to controls. While similar alterations were observed in ADan-oxi and A1-40 retinas, the retinal pattern of A1-42 was not much different from that of the respective DMSO control. These results suggest that dementia peptides, particularly ADan-red peptide, disrupt the integrity of retina in a profound manner. Immunohistochemical analysis of rat retinas was carried out using the antibodies to major retinal photoreceptor protein rhodopsin. Immunostaining with rhodopsin antibodies showed a significant reduction in the intensity of the rhodopsin signal in the retina of rats injected with dementia peptides compared with that of DMSO controls (Fig 5). The decrease in rhodopsin intensity was highest with ADan-red followed by ADan-oxi and A1-40. The rhodopsin staining of AB1-42 sections was similar to that DMSO control. To further confirm alterations in the retina, we evaluated and compared the immunoreactivity by labeling the retina with glial marker GFAP. Gliosis in retinal Muller cells is characterized by the increased expression of immuno-reactivity for the GFAP.

Fig 4. Morphology of the retina



Representative histology images of rat retina. Panel A-ADan-red; Panel B-ADan-oxi; Panel C-Aβ40 and Panel D-Aβ42

Fig 5. Immunohistochemical evaluation of rod photoreceptor marker rhodopsin



Representative retinal sections of control (A), ADan-red (B), ADan-oxi (C), Aβ40 (D) and Aβ42 (E) injected eye were labeled with rhodopsin (red). Nuclei are labeled with DAPI (blue). Scale bar, 50 µm.

This increased immunoreactivity of GFAP suggests the induction of retinal reactive gliosis in dementia-peptide injected eyes. In the present study, the observed increased expression of GFAP is suggestive of local inflammation. In concurrence with histological changes, injection of ADan-red peptide caused greater changes in rhodopsin and GFAP followed by ADan-oxi and A1-40, whereas the impact was least with A1-42.

CONCLUSIONS

In summary, we provided a molecular basis for the role of ADan and Aβ peptide under *in vivo* conditions upon intra-vitreal injection into rat eyes. Interestingly the ability of peptides to induce cataract *in vivo* in these studies correlated with their potential to impair chaperone activity of the α-crystallins *in vitro* and loss of lens transparency in *ex vivo* conditions reported earlier. Interestingly, it was observed that the integrity of retina upon injection of ADan peptides was relatively more affected in ADan-peptides compared to Aβ peptides. On the whole, the thickness of various layers of the retina was reduced. These results suggest that dementia peptides, particularly ADan-red peptide, disrupt the integrity of retina in a profound manner. Together, with our earlier studies that reported the neurotoxicity of ADan peptid, these results highlight that ADan peptides not only contribute to cataract but also to retinal dystrophies through their neurotoxic effects. Further, these observations suggest that ADan peptides are more potent than Aβ peptides regarding visual impairment.

6. HSP90 REGULATION OF FIBROBLAST ACTIVATION IN PULMONARY FIBROSIS

Pulmonary fibrosis is the final molecular-cellular process in adult and pediatric interstitial lung diseases in which the lung becomes irreversibly damaged and scarred due to repetitive injury and progressive interstitial expansion. Idiopathic pulmonary fibrosis (IPF) is an age-associated fatal fibrotic lung disease, wherein fibroblasts exhibit excessive proliferation, invasiveness, myofibroblast transformation and production of collagen and other ECM proteins in the parenchyma and subpleural areas of the lung. The lack of knowledge of the molecular regulators that initiate and maintain fibroblast activation is a major obstacle to the development of effective therapies for IPF patients. Studies over the past decade have suggested that chronic injury and multiple pro-fibrotic signaling pathways are responsible for fibroblast activation in the formation of scar tissue in IPF. Multiple signaling pathways and their downstream targets have been implicated in the persistent activation of fibroblasts that occurs in IPF. It has been demonstrated that combinatorial inhibition of two or more signaling pathways could result in a synergistic dampening of fibroblast activation and attenuation of pulmonary fibrosis (1,2). In a recent study, we found that inhibition of PI3 kinase and MAPK pathways together is more effective than that of either alone in pulmonary fibrosis (2). Furthermore, nintedanib, an inhibitor of multiple tyrosine kinases that is FDA-approved for the treatment of IPF, has been shown to simultaneously target multiple signaling pathways involved in fibroblast activation and pulmonary fibrosis. Therefore, identification of a single drug or therapeutic target that impacts more than one pathway or cellular process of fibroblast activation could both prevent and reverse fibrosis, leading to improved lung function.

Global gene-expression data are useful for classification and characterization of IPF-specific gene sets that regulate the fibroblast processes (e.g., proliferation, migration, invasiveness, and ECM production) involved in IPF pathogenesis. Here, we have taken an unbiased chemical genomics approach to identify drug targets that can alter multiple gene networks involved in IPF-related fibroblast activation. By prescreening more than one million diverse small-molecule-driven gene profiles in cells with IPF gene signatures, we identified Hsp90 as a potential therapeutic candidate gene for IPF. Hsp90 is a highly conserved molecular chaperone involved in protein folding and stabilization of a variety of client proteins associated with the activation of key signaling pathways and regulatory systems. The biological activity of Hsp90 depends on its capacity to bind and hydrolyze ATP, which drives the closed conformation of Hsp90 chaperone which represents its active form. Studies have demonstrated that inhibition of Hsp90 activity significantly impairs migration and invasion of cancer cells. An inhibitor of Hsp90 ATPase activity, 17-AAG exerts anti-tumor effects by selectively binding to a conserved binding pocket in the amino-terminal domain of Hsp90, inhibiting both ATP binding and ATP-dependent Hsp90 chaperone activity. In particular, cytoplasmic Hsp90 interacts with actin and tubulin to stabilize the cytoskeleton and regulate cell motility, a key cellular process in the pathogenesis of fibrosis in multiple tissues. Pharmacological inhibition of Hsp90 ATPase activity was sufficient to reduce bleomycin-induced dermal fibrosis in Tsk-1 mice, with no toxic effects observed. Further, inhibition of Hsp90 by 17-AAG repressed ECM production in murine renal and liver fibrosis models; however, the molecular basis for this remains unknown.

Here, we demonstrate that the levels of Hsp90 and associated ATPase activity are elevated in IPF lungs and a mouse model of TGFα-induced pulmonary fibrosis. Our findings describe a potential role of Hsp90 in pulmonary fibrosis via activation of proliferation, motility, fibroblast to myofibroblast transformation and ECM production. Functional enrichment analysis of gene networks was used to identify the gene targets regulated by Hsp90 in IPF. Further, our in vivo studies established the therapeutic efficacy of 17-AAG as an anti-fibrotic agent in attenuating established and ongoing pulmonary fibrosis. These data suggest unique mechanisms for the generation of pulmonary fibrosis by Hsp90 and use of 17-AAG in the treatment of pulmonary fibrosis.

METHODOLOGY

TGFα–transgenic mice and pharmacological treatment: TGF-α–overexpressing mice were generated in an FVB/NJ inbred strain background as described previously (3). Homozygous Club cell (Clara cell)–specific protein-rtTA1/2 (CCSP) mice were mated with heterozygous (TetO) 7-cmv TGF-α–Tg mice to generate transgenic TGF-α (CCSP/TGF-α) mice. Female mice, 8–14 weeks old, were used in all experiments. TGF-α expression was induced by administering food containing Dox (62.5 mg/kg) to CCSP/TGF-α–Tg mice for 6 weeks. CCSP/– mice fed with Dox food were used as nonfibrotic controls. A stock solution of 17-AAG (Tocris) was prepared in 20% DMSO + 20% Cremophor + 60% PBS solution to make a 1 mg/ml drug solution. Mice were treated with 17-AAG (15 mg/kg once daily, 7 d/wk) i.p. for 3 weeks. Control mice were treated with similar volumes of the vehicle. Mice were weighed at the beginning of the study at and weekly intervals.

αSMA reporter mice: The SMMHCCreERT2/+ROSAmTmG/+ heterozygous male transgenic mouse strain was generated by crossing male SMMHCCreERT2/+ (Jackson Laboratory, stock 019079) mice with ROSAmTmG mice (4). Heterozygous SMMHCCreERT2/+ mice (knock-in allele) are phenotypically normal and can be used to track the genetic lineage of αSMA-expressing cells. Heterozygous SMMHCCreERT2/+ROSAmTmG/+ mice (knock-in allele) express CreERT2 under the control of the promoter for mouse smooth muscle myosin, heavy polypeptide.

Human tissue samples: Human IPF and control non-IPF biopsies were obtained from Eric White, Department of Internal Medicine, University of Michigan Health System (UMHS). The biopsy material consisted of archived, paraffin-embedded, pathological specimens previously acquired from adult patients who had undergone lung transplantation for IPF. IPF was diagnosed according to the American Thoracic Society consensus criteria (93). Lung samples from donors' lungs with no lung disease were used as normal lung biopsies.

Computational analysis: A previously published transcriptomic data set (GSE53845) derived from analysis of the lung biopsies of 40 IPF patients and 8 healthy controls and available in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) was used to identify DEGs. Differential analysis for genes was performed using the R package limma or GEO2R (http://www.ncbi.nlm. nih.gov/geo/geo2r/) with *P*-value and FDR threshold at 0.05 and the fold-change threshold at 1.5. This IPF gene signature was queried against the LINCS database (http://www.lincscloud.org/), a massive catalog of gene-expression profiles collected from human cells treated with chemical and genetic perturbagens, using a connectivity-map approach as described previously. Briefly, the IPF DEG signature is queried against the 1.4 million gene-expression profiles in the LINCS L1000 dataset. The pattern-matching software in the LINCS cloud searches for 2-directional matches, taking into account both the up and down gene sets, in comparing IPF query against L1000 compound signatures (*Z*-scored differential expressions). The LINCScloud query app (http://apps.lincscloud.org/) then generates a list of rank-ordered signatures based on their strength of the match to the query, from highest to lowest. Functional enrichment analysis of the negatively correlated gene sets between IPF lungs and 17 AAG–treated cells from the LINCS database was conducted using the ToppFun application of the ToppGene Suite. For network representation of select significantly enriched biological processes and pathways, we used Cytoscape.

Immunohistochemistry: Formalin- or OCT-fixed human lung tissue sections from non-IPF and IPF patients were prepared and immunostained with antibodies against Hsp90 and vimentin as described previously (4). All images were obtained using a Leica DM2700 M bright-field microscope.

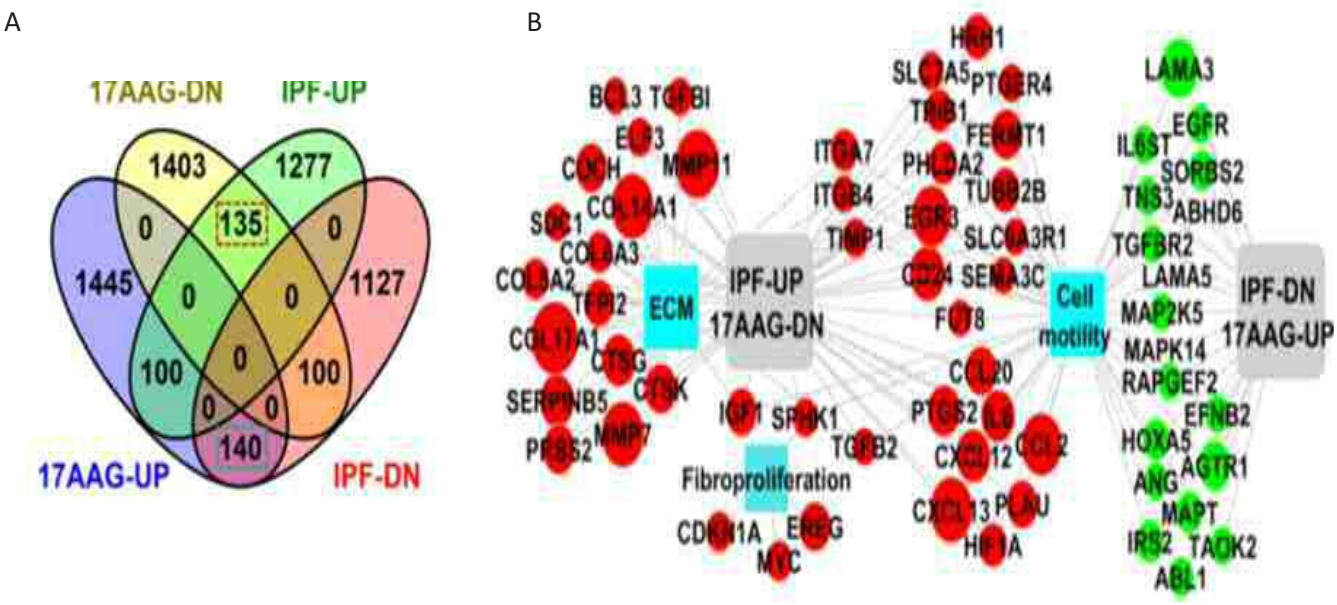
Histology, pleural thickness, and chord length measurement: Lungs were inflation fixed using 10% formalin and stained with Masson's trichrome or H&E as previously described (4). For pleural thickness measurement, 5 random measures per lung section were obtained for each animal using a brightfield microscope. For chord length measurements, 7 randomly selected uniform fields per lung tissue section in the alveolar regions were obtained for each animal. The pleural thickness and mean chord length were measured.

Hydroxyproline assay: Lung fibrosis was assessed by measuring hydroxyproline levels and normalizing to lung weight, using a colorimetric assay as previously described.

RESULTS

***Upregulation of Hsp90-driven gene networks in IPF*:** Among the top-ranked small molecules whose gene-expression profiles were inversely correlated to the IPF DEG profile was 17-AAG (tanespimycin), a derivative of the antibiotic geldanamycin and a known inhibitor of Hsp90 ATPase activity. Other major compound groups identified through a connectivity-map approach include corticosteroids, tyrosine kinase inhibitors, MEK inhibitors, and an inositol monophosphatase inhibitor. To elucidate the potential mechanism of action of 17-AAG in IPF, we compared the respective gene-expression profiles of 17-AAG (from LINCS) to that of IPF and identified multiple transcripts that were either up- (135 genes) or downregulated (140 genes) in IPF lungs (Fig 1A). We performed an enrichment analysis of these negatively correlated gene sets (i.e., genes upregulated in IPF, but downregulated by 17-AAG and vice versa) (Fig 1B). Notably, among the top enriched biological processes were fibroblast motility, proliferation, growth, and ECM production (Fig 1B).

Fig 1. Hsp90 regulated gene networks in IPF

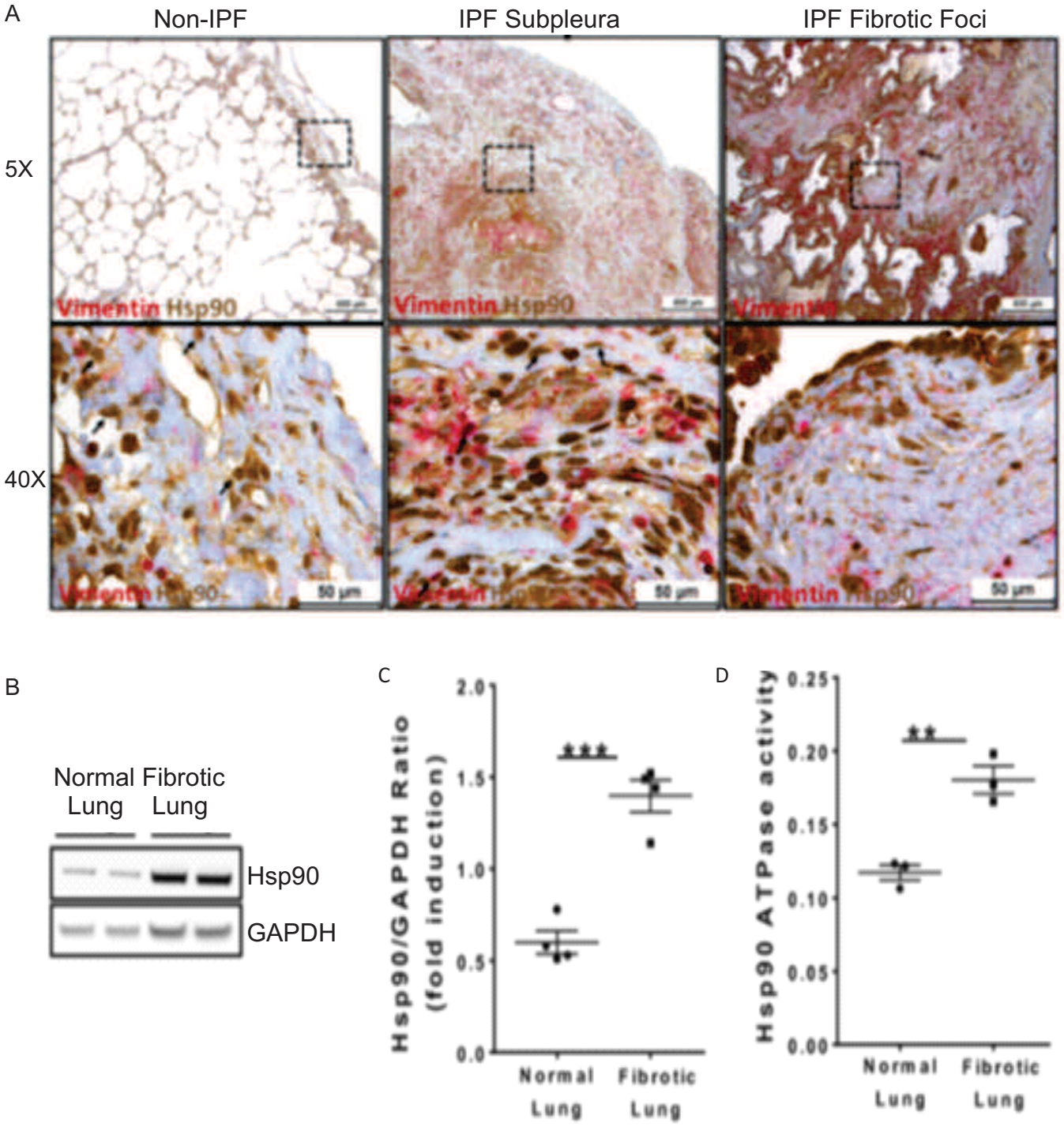


(A) Venn diagram depicting the overlap of differentially expressed genes in IPF lungs that are significantly reversed upon inhibition of Hsp90 activity with 17 AAG. Of the 2879 genes differentially expressed in IPF lungs, 475 genes are overlapped with 17AAG treatment. The box indicates 275 genes that are up- or down-regulated in IPF lungs compared with 17AAG treatment. (B) Hsp90-driven gene networks that are activated in IPF were analyzed using Cytoscape. Red and green colored circles are used to represent genes that up and down regulated in IPF lungs. The blue color squares indicate Hsp90 regulated genes in IPF shown to augment fibroblast activation including proliferation, motility and ECM production.

Increased Immunostaining and Activity of Hsp90 in Fibrotic Lungs*:* To validate Hsp90 as a candidate gene in pulmonary fibrosis, we assessed the levels of Hsp90 by co-immunostaining IPF and normal lung tissue sections with antibodies against Hsp90 and vimentin. The expression of Hsp90 was detected in human lung cells, including in vimentin-positive fibroblasts of normal and IPF lungs (Fig 2A). Notably, the staining of Hsp90 was increased in the lung sections of human IPF compared to normal lungs. Further, images taken at high magnification demonstrated that Hsp90 was localized in both the cytosol and nucleus of fibroblasts in IPF and non-IPF lungs. Also, we observed marked Hsp90 immunoreactivity in pneumocytes, airway epithelium and fibroblast foci in areas of active fibrosis in IPF lungs (Fig 2A).

To determine the levels of Hsp90 in pulmonary fibrosis, the lung lysates of control mice and TGFα-transgenic mice induced on doxycycline (Dox) for six wk were immunoblotted with antibodies against Hsp90. The levels of Hsp90 were heightened in the fibrotic lungs of the TGFα mice compared to the lungs of control mice (Fig 2B & C). Further, Hsp90-specific ATPase activity was quantified in the lung lysates of control mice and TGFα mice induced on Dox for six wk. We observed a two-fold increase in Hsp90 ATPase activity in the lung lysates of fibrotic mice compared to normal mice (Fig 2D). Notably, we observed a significant increase in Hsp90 ATPase activity in the lysates of fibroblasts from IPF lungs compared to fibroblasts from normal lungs. To determine whether Hsp90 isolated from fibrotic lungs had a higher binding affinity to 17-AAG than that from normal lungs, we incubated Hsp90 from the lysates of lung fibroblasts with increasing concentrations of 17-AAG in the presence of biotinylated-geldanamycin. Increasing doses of 17-AAG resulted in a decrease in binding of Hsp90 to the geldanamycin, which was greatest with Hsp90 of fibroblasts isolated from the fibrotic lungs of TGFα mice compared to normal mice.

Fig 2. Hsp90 and its ATPase activity elevated in fibrotic lungs

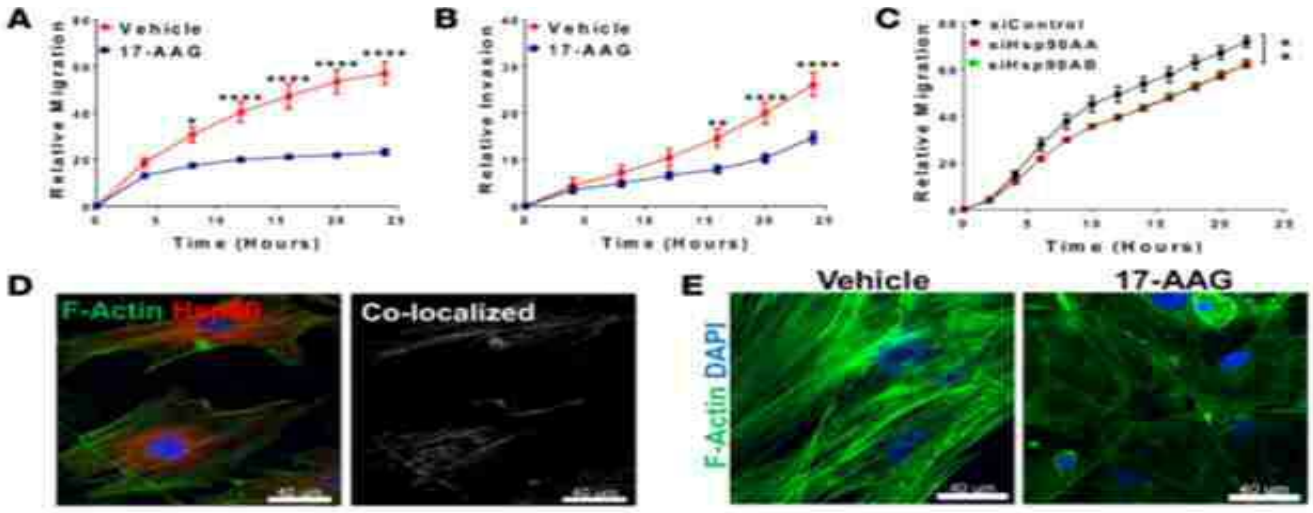


(A) Idiopathic pulmonary fibrosis and non-IPF lung tissue sections immunostained with antibodies against Hsp90 (brown) and vimentin (purple). Arrows indicate Hsp90 staining in the cytoplasm and nucleus of vimentin positive lung cells. Hsp90 localization was observed in epithelial cells and vimentin-positive cells of fibrotic foci. (B) The lung lysates immunoblotted with antibodies against Hsp90 to measure increases in Hsp90 in the fibrotic lungs of TGF-α–transgenic (TGF-α–Tg) mice compared with control mice on doxycycline (Dox) for 6 weeks. (C) Quantification of Hsp90 levels using Phosphor Imager software, and the amount of Hsp90 normalized with GAPDH levels in the lung lysates of TGF-α–Tg mice compared with control mice on Dox for 6 weeks. (D) Hsp90 ATPase activity measured in the lung lysates of control or TGF-α–Tg mice on Dox for 6 weeks.

Inhibition of Hsp90 attenuates fibroblast migration and invasiveness

Excessive migration and invasiveness are hallmarks of fibroblast activation that contribute to severe fibrotic lung disease. To identify whether inhibition of Hsp90 ATPase activity impedes migration and invasiveness of IPF fibroblasts, we performed real-time 3D scratch assays in the presence and absence of 17-AAG. Upon treatment with 17-AAG, resident lung fibroblasts showed a significant reduction in the ability to migrate through Matrigel (invasiveness) (Fig 3A & B). Fibroblast migration was significantly attenuated with the deficiency of either Hsp90AA or Hsp90AB isoform compared to control siRNA treatment (Fig 3C). Together these data demonstrate the migration capacity of fibroblasts was regulated by both Hsp90AA and Hsp90AB isoforms. To investigate the mechanisms of Hsp90-driven migration and invasion, lung fibroblasts were co-stained for F-actin and Hsp90. Notably, we observed a strong co-localization of cytoplasmic Hsp90 with F-actin filaments in fibroblasts (Fig 3D). Further, the formation of F-actin filaments was attenuated in fibroblasts treated with 17-AAG compared to vehicle treatment (Fig 3E). To determine whether the formation of F-actin filaments by Hsp90 was due to altered signaling via pathways involved in the F-actin formation, we assessed focal adhesion kinase (FAK), Cdc42 and phosphorylation of ERK. Inhibition of Hsp90 ATPase activity resulted in a significant decrease in the protein levels of total FAK and Cdc42, and also phosphorylation of ERK, but had no effect on phosphorylated FAK/FAK ratio, total ERK, and RhoA protein levels. To address whether Hsp90 inhibition alters the expression of genes involved fibroblast invasiveness, resident lung fibroblasts were treated with 17-AAG for 24h and transcripts were analyzed using RT-PCR. Treatment with 17-AAG resulted in a significant decrease in the levels of chemokines and cytokines (CCL3, CCL20, CCR5, and IL-10), as well as genes involved in the invasiveness of fibroblasts (HAS2, CD44, MMP12, and TIMP1), while treatment with 17-AAG had no effect on expression of MMP2, MMP9, and TIMP2. Thus, the data suggest that Hsp90 induces signaling and the expression of genes involved in migration and invasiveness of fibroblasts in IPF.

Fig 3. Mechanisms of Hsp90-driven migration and invasion in fibroblasts



Primary lung fibroblasts (CD45–Col1+) isolated from lung fibroblast cultures of human idiopathic pulmonary fibrosis (IPF) or TGF-α–transgenic (TGF-α–Tg) mice on doxycycline (Dox) for 4 weeks by negative selection with anti-CD45 magnetic beads. (A) Quantification of migration of IPF fibroblasts treated with vehicle or 17-AAG (1 μM) for 24 hours. (B) Quantification of invasiveness of IPF fibroblasts treated with vehicle or 17-AAG (1 μM) for 24 hours. (C) Fibroblasts of TGF-α–Tg mice on Dox for 4 weeks were transiently transfected with control, Hsp90AA-specific, or Hsp90AB-specific siRNA for 48 hours and the migration was quantified for 24 hours. (D) Costaining of F-actin and Hsp90 in lung fibroblasts of TGF-α–Tg mice on Dox for 4 weeks. Hsp90 colocalized (white signal) with F-actin in cytoplasmic fibrillary adhesions or focal complexes. (E) F-actin stained using phalloidin in lung fibroblasts of TGF-α–Tg mice on Dox for 4 weeks treated with vehicle or 17-AAG (1 μM) for 24 hours.

Inhibition of Hsp90 attenuates fibroproliferation

To identify the effect of Hsp90 inhibition on fibroproliferation, we treated lung fibroblasts of human IPF with 17-AAG or vehicle for 24h. Inhibition of Hsp90 ATPase activity resulted in a significant decrease in the percentage of proliferating cell nuclear antigen (PCNA)-positive fibroblasts compared to vehicle treatment. Similarly, the proliferation of lung fibroblasts isolated from the fibrotic lungs of TGFα mice was inhibited in a dose-dependent manner, as the concentration of 17-AAG was increased from 0.01 μM to 1 μM at 48h.

Inhibition of Hsp90 attenuates ECM production

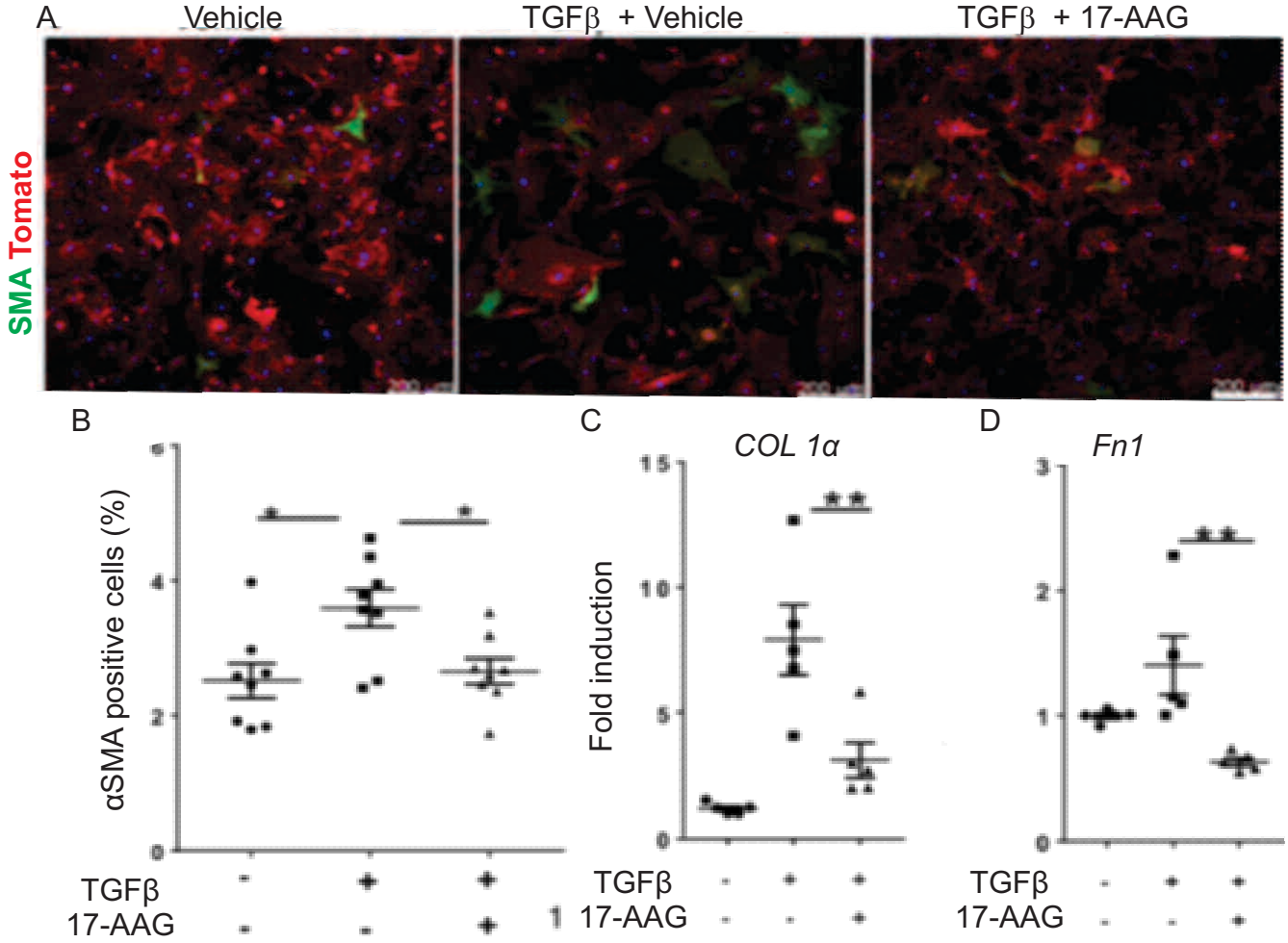
Myofibroblasts are the major effector cells responsible for the excessive ECM deposition in the fibrotic lesions of human IPF. To investigate whether inhibition of Hsp90 downregulates αSMA and ECM gene expression, lung fibroblasts were treated with 17-AAG or vehicle for 24h and transcript levels of αSMA, fibronectin (FN1), Col1α, and Col5α were quantified by RT-PCR. Inhibition of Hsp90 ATPase activity resulted in a significant downregulation of ECM gene. To determine the role of Hsp90AA and Hsp90AB in ECM production, lung fibroblasts were treated with Hsp90AA- or Hsp90AB-specific siRNA or control siRNA for 72h. Notably, loss of Hsp90AB was sufficient to attenuate the expression of ECM genes, such as αSMA, Col1α, and Col5α, but had no effect on FN1 compared to control siRNA. Hsp90AA siRNA treatment had no effect on the ECM genes. Furthermore, western blot analysis demonstrated that 17-AAG inhibits protein levels of αSMA and FN1 in fibroblasts isolated from the fibrotic lungs of TGFα mice on Dox for four weeks.

TGFβ is a master regulator of fibroblast to myofibroblast transformation and expression of ECM genes in pulmonary fibrosis. To determine whether inhibition of Hsp90 ATPase activity is sufficient to attenuate TGFβ-induced myofibroblast transformation, we performed lineage tracing studies using lung resident fibroblasts isolated from αSMA reporter mice ($\alpha SMA^{CreER}ROSA^{mTmG}$ mice). Primary fibroblasts were cultured in the presence of tamoxifen and 17-AAG or vehicle and treated with TGFβ for 72h to visualize and quantify transformation of fibroblast to myofibroblasts. In this system, upon activation of αSMA, fibroblasts change the expression of membrane tomato *(Red; mT)* to membrane GFP *(Green; mG)*. As anticipated, we observed a significant increase in αSMA expressing cells (green) by TGFβ and treatment with 17-AAG was sufficient to attenuate the number of *mG* expressing cells (Fig 4A-B). To determine whether 17-AAG attenuates TGFβ-induced ECM gene expression, human fibroblasts were treated with TGFβ in the presence and absence of 17-AAG and transcripts of ECM genes were quantified by RT-PCR. As predicted, TGFβ induced the expression of Col1α and FN1 (Fig 4C & 4D). Notably, treatment with 17-AAG was sufficient to attenuate TGFβ-driven expression of Col1α and FN1. Together, our *in vitro* results establish that either inhibition of Hsp90 ATPase activity or Hsp90AB expression was sufficient to attenuate TGFβ-driven myofibroblast transformation and ECM gene expression.

Inhibition of Hsp90 ATPase activity attenuates pulmonary fibrosis *in vivo*

The potential benefits of Hsp90 inhibition in pulmonary fibrosis using a mouse model of TGFα-induced pulmonary fibrosis was evaluated. At three wk on Dox, TGFα mice developed detectable fibrosis in subpleural and adventitial areas of the lung and also modest increases in lung weights and hydroxyproline levels. These changes progress and increased significantly in TGFα mice continued on Dox for an additional three wk compared to control mice or mice on Dox for 3 weeks. To determine whether Hsp90 inhibition influences the progression of established fibrosis, following three wk of Dox treatment when fibrosis is already manifest, TGF mice were administered 17-AAG while remaining on Dox for an additional three wk (6 wks total) (Fig 5A). The right lung weights were increased in TGFα mice compared to control mice on Dox for six wk. This increase in lung weights was attenuated in TGFα mice treated with 17-AAG when compared to vehicle-treated TGFα mice (Fig 5B). Similarly, the total lung hydroxyproline levels were

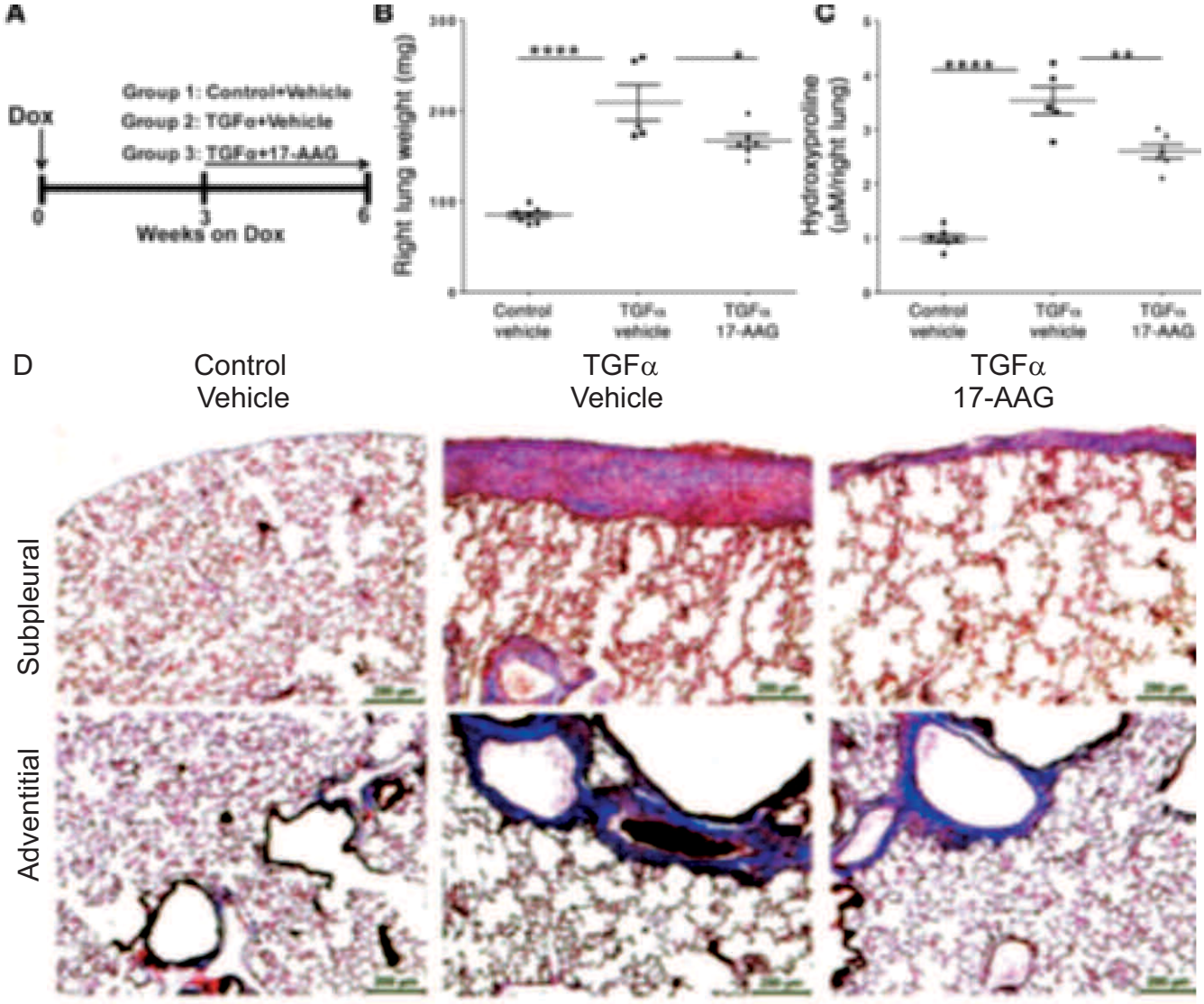
Fig 4. Hsp90 inhibition attenuates TGFβ induced myofibroblast transformation and ECM gene expression



Primary lung resident fibroblasts (CD45$^-$Col1$^+$) were isolated from lung cell cultures of αSMA^{creER}ROSA^{mTmG} mice by negative selection with anti-CD45 magnetic beads. (A) Fibroblasts were treated with TGFβ and 4-hydroxy tamoxifen in presence and absence of 17-AAG for 72h. Immunofluorescence images were collected at original magnification x10. (B) The number of green positive and total DAPI-positive cells were quantified using Metamorph image analysis software and were indicated as the percent αSMA positive cells in total DAPI-positive cells. (C-D) Human lung resident fibroblasts (CD45$^-$Col1$^+$) were isolated from non-IPF lung fibroblast cultures by negative selection with anti-CD45 magnetic beads. Fibroblasts were treated with TGFβ in the presence and absence of 17-AAG for 24h Quantified transcripts of ECM genes, Col1α and FN1 using qRT-PCR and shown as the fold induced gene transcripts relative to HPRT.

increased in TGFα mice treated with either vehicle or 17-AAG (Fig 5C). However, this increase in the lung hydroxyproline levels was attenuated in TGFα mice treated with 17-AAG compared to vehicle-treated TGFα mice (Fig 5C). Masson trichrome staining of lung sections revealed extensive subpleural thickening and adventitial lung fibrosis in TGFα mice compared with vehicle-treated control mice (Fig 5D). However, the therapy with 17-AAG resulted in a significant decrease in subpleural thickening, as well as reduced adventitial fibrosis compared with vehicle-treated TGFα mice. Increased αSMA and Ki67 immunostaining were detected in the subpleural fibrotic lesions in TGFα mice compared with vehicle-treated control mice, which was significantly diminished by 17-AAG treatment (Fig 5E & 5F).

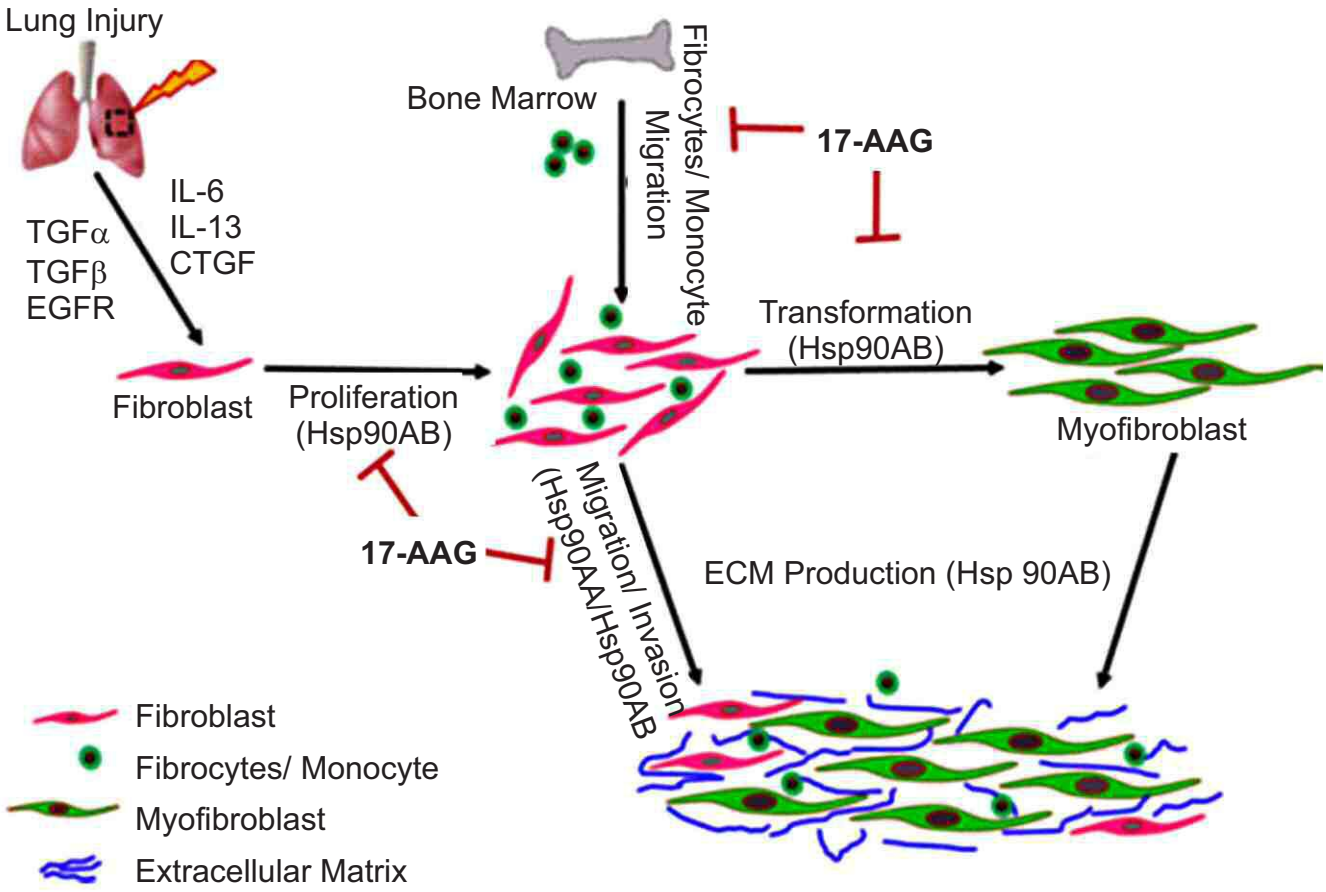
Fig 5. Therapeutic administration of 17AAG attenuates pulmonary fibrosis in vivo



(A) Schematic representation of 17-AAG treatment protocol. Control and TGFα mice were treated with vehicle or 17-AAG for the last 3 weeks while remaining on Dox for a total of 6 weeks. (B) Quantitation of the right lung weight of mice treated with vehicle or 17-AAG. (C) Quantitation of total lung hydroxyproline levels in mice treated with vehicle or 17-AAG. (D) Images of Masson's trichrome stained lung sections from all the groups. Top, subpleural regions of the lung; bottom, adventitia.

Further, the changes in fibrosis quantified specifically in the subpleural region by measuring the pleural thickness in the lung sections from all groups. Not surprisingly, TGFα mice show a significant increase in subpleural thickness upon six wk on Dox treatment compared to non-transgenic control mice. This increase in subpleural thickness was attenuated in TGFα mice administered 17-AAG compared with vehicle-treated TGFα mice. We evaluated mean alveolar chord length to determine the potential benefit or any toxic effect of Hsp90 inhibition on lung alveolar integrity during TGFα-induced pulmonary fibrosis. Thus, our *in vivo* data suggest that Hsp90 plays a critical role in pulmonary fibrosis and inhibition of Hsp90 using 17-AAG attenuates TGFα-induced progressive subpleural thickening and pulmonary fibrosis. Together, these studies demonstrate that Hsp90 inhibition at the time of extensive and established fibrosis modulates the progression of the disease based on biochemical and histologic parameters.

Fig 6. Schematic representation of Hsp90 regulated fibroblast functions in the pathogenesis of pulmonary fibrosis.



CONCLUSION

Increased Hsp90 ATPase activity plays a critical role in fibroblast activation in severe fibrotic lung disease was demonstrated. Gene-knockdown studies have demonstrated in vitro that Hsp90AB, but not Hsp90AA, is required for fibroblast proliferation, myofibroblast transformation, and ECM production, while the expression of both Hsp90AA and Hsp90AB isoforms was required in fibroblast migration. The findings from this study indicate that both Hsp90AA and Hsp90AB isoforms must be targeted to inhibit both overlapping and unique cellular properties by the isoforms (Fig 6). Our pre-clinical findings support a pathogenic role for Hsp90 in IPF and demonstrate that Hsp90 is a plausible therapeutic target at the molecular, cellular and whole animal levels. Our observations that Hsp90 inhibition attenuated fibroblast activation and myofibroblast accumulation and collagen deposition without any detectable toxic effects serve as proof of principle for a future interventional role of Hsp90 in the treatment of fibrotic lung disease.

7. EXPLORING THE POTENTIAL OF ISLET LIKE CELL-AGGREGATES GENERATED FROM MESENCHYMAL STEM CELLS OF HUMAN PLACENTA FOR TREATING TYPE1 DIABETES IN NOD MICE BY IMMUNOISOLATION APPROACH

Therapy for type 1 diabetes (T1D) is an open challenging problem. Restoration of normoglycemia and insulin independence in immunosuppressed type 1 diabetic recipients of islet allotransplantation (alloTx) has shown the potential of a cell-based diabetes therapy. Even if successful, this approach poses a problem of scarce islet tissue supply. Obtaining human islets from cadaveric pancreas is almost impossible in India. The islets generated from rodent or other mammalian species vary in architecture when compared to the human counterparts. Thus there is a dire need to generate human islets or islet equivalents from postnatal stem cells. The proposed research proposal deals with generation of Islet like cell Aggregates (ICAs) from mesenchymal stem cells (MSCs) of human placenta ,and working with T1D mice model ,to assess Frank diabetes, and establishment of optimal age for Tx studies.

Hypothesis: We hypothesize that hPMSCs could be the ideal source of MSCs to generate adequate number of Islet like clusters (ILCCs), and can be Tx in NOD mice – portraying Type 1 diabetic like changes.

OBJECTIVES

- To isolate MSCs from hPMSCs using the well established method.
- Characterization of the MSCs using specific markers.
- Working with the NOD mice to arrive at autoimmune response and co-precipitating with Frank diabetes.

METHODOLOGY

Isolation of human placetal derived from Mesenchymal stem cells (hPMSCs): The methodology was similar to the protocol briefly, the tissue was digested with collagenase 1,and subjected to washings and centrifugations. The mononuclear cells obtained in the pellet was seeded, and put for culture supplemented with DMEM knock out medium with 10% FCS and Antibiotics.The MSC phenotype was visible by 5th day and by 6-7 days attained confluence (70-80%). The cells were trypsinized, passaged (4-5) and characterized for MSCs phenotype.

***Animal Experiments:/NOD mice*:** Both male and female age groups were subjected to initial screening as a function of age (2 months, 6 months and 8 months). Based on these screening, it was found that the male NODmice at 8 months of age demonstrated hall markers of T!D such as GAD 65. hence, to exaggerate the frank diabetic response, we selected the same group (8 months old male mice) and injected with multiple injections (2, 3, 4 or 5) of low dose (40 mg/Kg body weight) STZ (Sigma, MO) prepared in 250ul chilled citrate buffer (pH 4.5) administered intraperitoneally. Blood glucose concentrations were monitored in the venous blood with a glucometer and test strips (One touch Horizon, Johnson & Johnson Ltd, USA) at frequent intervals by tail prick method. STZ injected animals acquired hyperglycemia (>250mg/dL) by 2 days. The treated mice were bled for serum glucose estimations at 5-day intervals for 30 days and then sacrificed and their pancreata removed for histological examination. On day three, high dose treatment produced hyperglycemia and body weight loss in comparison to mice without STZ.

***Physiologic and Biochemical Analysis*:** The body weights were monitored during the experimental duration. The blood glucose was measured by tail prick method with a glucometer and values were expressed in mg/dl (One touch horizon, Jhonson ans Jhonson Ltd, USA). Serum insulin was estimated using mouse insulin (Mercodia AB, Mouse Insulin ELIS, Swedan and Ray Biotech, Inc, GA, USA).

Biochemical analysis: Serum glucose levels were estimated using glucometer (One touch horizon, Jhonson and Jhonson Ltd, USA). The values were expressed in mg/dl every week to ascertain the glycemic status, simultaneously body weight also recorded. The animals were deprived of food before autopsy and sacrificed. The blood sample was collected by retro-orbital method at the time of autopsy, centrifuged at 4ºC at 10,000 rpm for 10 minutes; the separated serum was used for various biochemical analyses as given below. All parameters were estimated using fully automated bioauto analyzer (ACE Alera, USA).

Estimation of insulin content

Isolation of islets and culture for insulin secretion assay: The pancreas was collected aseptically from the adult mouse. After the animal was sacrificed by CO_2 asphyxiation, it was dissected in a sterile laminar air flow to collect the pancreas along with spleen. The tissue was collected in HBSS buffer. The pancreatic tissue was processed by removing the spleen and surrounding fat tissue followed by mincing into fine pieces. The tissue was not allowed to dry during the overall procedure using HBSS buffer. The minced pieces were digested using the enzyme type V collagenase for 10-15 minutes at 37ºC. The enzymatic reaction was stopped by the addition of cold medium. The collagenase digest of pancreas was washed twice with RPMI 1640 by centrifugation and the pellet was re-suspended in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum on uncoated culture plates (1). Bright filed images of the pancreatic progenitor cultures were obtained at regular time periods on Nikon Inverted microscope TE-2000S (Nikon, Tokyo, Japan) attached with digital CCD camera and ACT-2U software (version 1.2).

Serum insulin content

Blood was collected by retro-orbital bleeding of mice into the serum vaccutainer. Serum was prepared by centrifuging the blood samples at 1500 rpm for 20 minutes at RT and stored at -80^0 C till use. Serum insulin concentration was estimated using the mouse insulin ELISA kit (Mercodia AB, Mouse Insulin ELIS, and Swedan) as per the manufacturer's protocol.

Insulin secretion assay

To test whether the insulin release of islets (200) was glucose–dependent from all the groups, two glucose concentrations (basal 5.5mM and stimulated 16.5 mM) on islets were used. After pre-incubation in KRBH buffer at 37ºC for 1 hour the islets were incubated with KRBH buffer containing 5.5 mM glucose at 37ºC for 1 hour. To induce insulin release, the supernatant was collected and the same islets were incubated with 16.5mM glucose for another 1hour. The cells were tested for the content of released insulin with mouse insulin ELISA kit (Biosource, Nivelles, Belgium) as per the manufacturer's instructions and the values expressed as µIU/ml. Each sample was assayed in triplicates.

Gene expression studies

Total RNA extraction: For RNA isolation islets were prepared from the pancreatic tissues using the collagenase digestion protocol. The total RNA from treated and untreated islets was extracted with TRIzol reagent according to the manufacturer's instructions.

One-step PCR reactions

cDNA was synthesized from total RNA using MMLV Reverse Transcriptase enzyme. cDNA was amplified with Platinum PCR Super Mix Polymerase kit according to the manufacturer's protocol. The amplicons were resolved electrophoretically on 1.2 % agarose gels pre-stained with ethidium bromide. The image was captured on a Bio-Rad Gel Doc system (Bio-Rad Laboratories, Hercules, CA, USA) and were quantities using Quantity One 1D analysis software (Bio-Rad, USA). Results have been expressed as ratio of intensities of the band of target gene to that of housekeeping -Actin gene. Also, For PCR Analysis the primers were procured and the standardization of primers was carried out in both early and later periods. The list of primers with its annealing and the amplicon size are given below.

ROS assay (DCFH-DA)

The intracellular formation of ROS was measured using 2', 7'-dichlorofluorescein diacetate (DCFH-DA). Fluorescien di acetate is acted upon by the esterase enzymes of metabolically active cells and is cleaved to give Fluorescein and diacetate molecules. Fluorescein is a fluorescent dye which fluoresces to give a bright green colour. Islets from all treatment groups were incubated in fresh media and incubated with 10 µM DCFH-DA at 37ºC for 30 minutes, washed twice with PBS and fluorescence intensity was measured using spectrofluorimeter (Spectramax M5, Molecular devices, California, USA) with excitation at 495 and emission at 538nm. All values were corrected by subtracting auto-fluorescence for respective wells and fluorescent intensities were standardized by the amount of total protein in each well.

RESULTS

Physiological Parameters: The female NOD mice (10 weeks age groups as control) were screened initially. Basic physiologic analysis and screening on NOD mice was done to see if there are any marked evidence of symptoms of type I diabetes with episodes of insulitis and other biochemical changes. There was no decrease in body weight, rather body weight increased due to its age factor (Table 1). However, there was an increase in glycemic level reaching a borderline of pre-diabetic state at later age group (Table 2). The detail observation of body weight and glucose parameters were illustrated (Fig 1 & 2). However, STZ induced NOD mice showed a drastic changes in physiological parameters as specified in tables and graphs. High-dose STZ (single dose) treatment causes a greater increase in blood glucose levels at early but not late time points compared to low dose STZ (multiple dose). Still, the both high and low-do STZ-treated mice lost weight, with the high dose group tending to lose more than the low-dose group as indicated in Table 1 & 2 and Fig 1 & 2. This is similar to the studies performed previously.

Table 1. Body weight

Parameters → Groups ↓	Initial body wt (g)	Final body wt (g)	Body wt (gain/loss %)
Control (10Weeks)	18.5-20	22-23.5	+1.89
STZ induced single dose	18.2-19.5	16.5-17.0	-09.34
STZ induced multiple dose	18.4-22	17-17.5	-08.23

Table 2. Serum glucose level

Parameters Groups	Initial blood glucose (mg/dl)	In-between blood glucose (mg/dl)	Final blood glucose (mg/dl)
Control (10Weeks)	125-150	140-160	160-190
STZ induced single dose	134-160	300-350	556-560
STZ induced multiple dose	113-175	284-300	515-550

Fig 1. Body weight

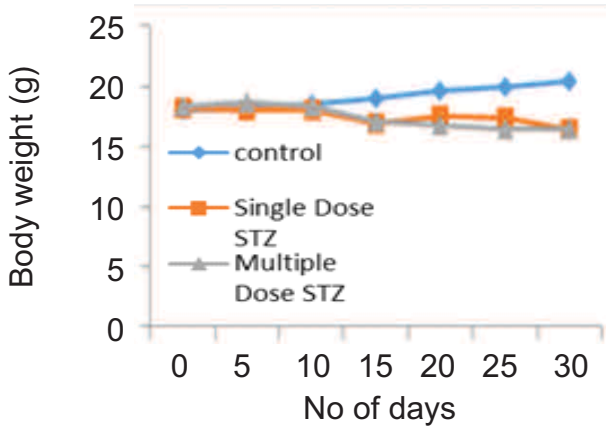
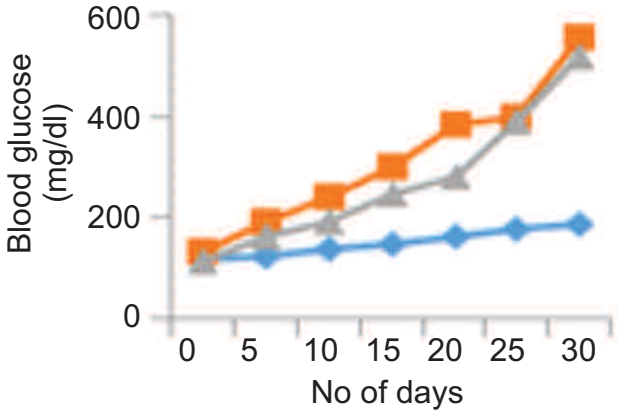


Fig 2. Serum glucose level



Biochemical analysis

From the biochemical analysis carried out in Automated Analyser, it was demonstrated that there was no marked significant changes until 10 weeks of age (control). However, upon induction, the parameters showed drastic changes in the biochemical parameter as specified (Table 3).The SGPT (ALT) and SGOT(AST) values for multiple dose STZ are higher compared to single dose STZ treatment which represent high percentage of Liver injury and chronic liver disease as previous report. Elevated Bilirubin total shows type 1diabetes.

Table 3. Auto analyser of SD MD and Control group

Parameters	control	Single dose	multiple dose
Blood urea (mgs/dl)	25	19	101
S.creatinine (mgs/dl)	0.4	0.5	1
Glucose (mgs/dl)	156	260	172
BIL (Total)(mgs/dl)	0.5	0.7	0.8
BIL (Direct)(mgs/dl)	0.1	0.2	0.2
BIL (Indirect)(mgs/dl)	0.4	0.6	0.5
ALK Po4 (IU/L)	240	265	305
SGPT (U/L)	46	55	175
SGOT (U/L)	120	210	200
T.Proteins(gm/dl)	4	3	4.5
S.Albumin (gm/dl)	3	2	3
S.Globulin (gm/dl)	1	1	1.5
A/G Ratio	3	2	2

Estimation of insulin content

Serum insulin level of 10 week mice (control) group was increased from the early age group due to age factor, indicating that serum levels are not affected. However, upon induction there was a drastic reduction in the serum insulin indicating the hypoinsulinemia condition of type I diabetic state (Fig 3). At insulin level, we found that multiple dose of injections were hypoinsulinemic in contrast that of hyperinsulinemic condition observed in single dose injected mice (Fig 3 & 4). Further, the induced diabetic mice showed a very less expression levels of insulin secretion, indicating the development of diabetes and loss of insulin secretion at induced level.

The primary islet cell cultures from both control and diabetic groups were subjected to insulin secretion assay both at basal and with glucose challenge (5). The insulin secretion capacity of 10weeks mice was less than early groups indicating that it reduced its insulin secretion capacity at later age group, which is a marked characteristic feature of NOD mice.

At later age group, the secretion levels was not that high and there was a slight decrease in its secretion at higher glucose challenge as compared to basal level. 3 weeks mice (early group) responded well to challenge assay as compared to 10 weeks, this could probably be attributed to insulitis stage as specified below. Further, the induced diabetic mice showed a very less expression levels of insulin secretion, indicating the development of diabetes and loss of insulin secretion at induced level.

Gene expression studies : Expression of Pdx1, Nestin and Ngn3 were up regulated in the case of Single dose STZ treatment whereas the expression of pax6 was been unregulated in the case of multiple dose STZ. The inflammatory markers in NOD multiple doses were down regulated in the case of TNF, IRS2, IRS1 and IL1b whereas the inflammatory marker IL6 was up regulated as shown in the fig 5.

Fig 3. Concentration of serum insulin (ng/ml)

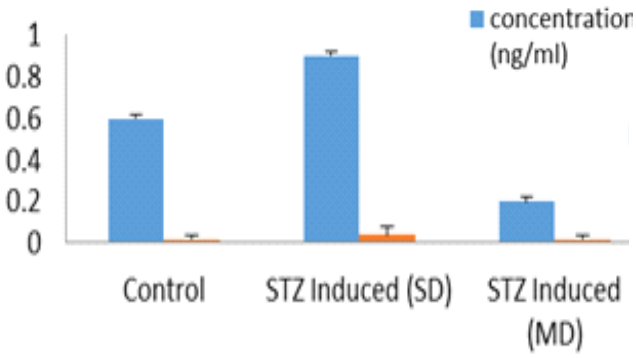


Fig 4. Insulin secretion assay (ng/mg protein)

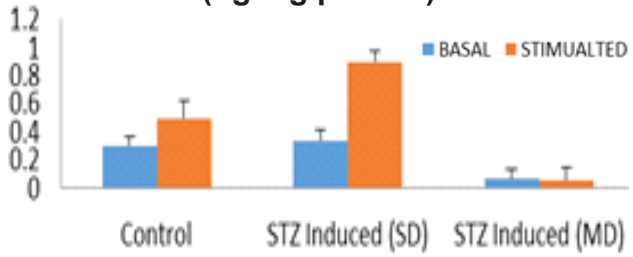
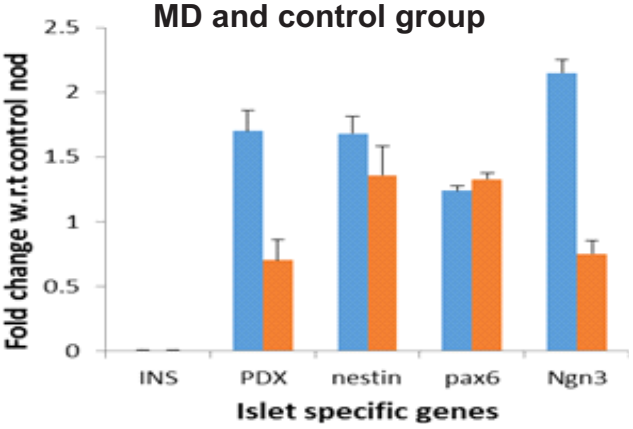
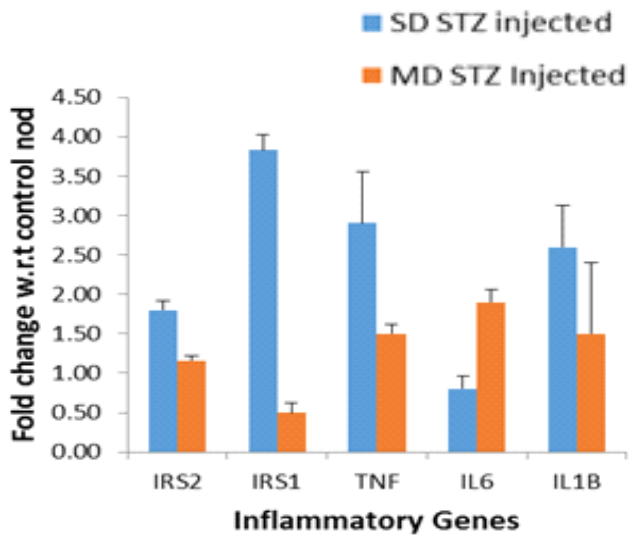


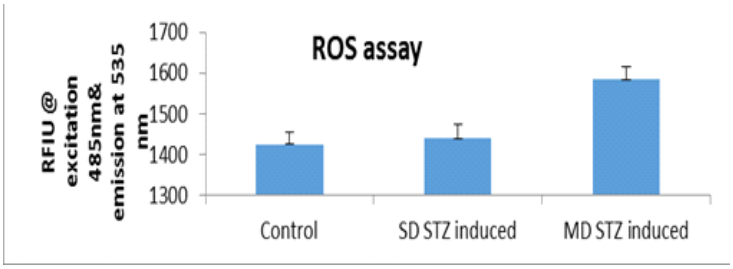
Fig 5. gene expression analysis of SD, MD and control group



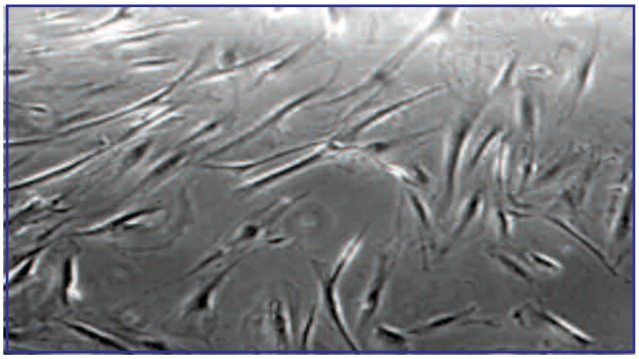


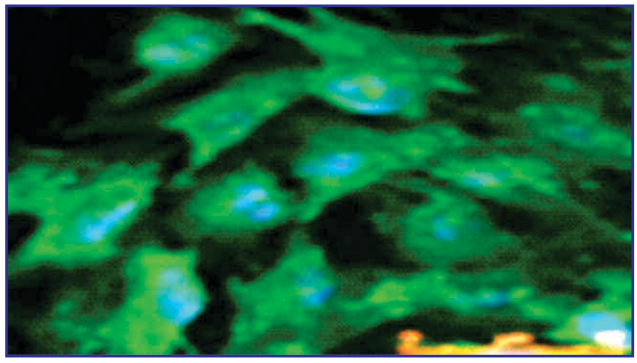
***ROS assay (DCFH-DA)*:** Streptozotocin (STZ) is widely used in studies of experimental type-1 diabetes because it selectively destroys pancreatic β cells through the generation of ROS and alkylation of deoxyribonucleic acid (DNA). In the current literature there are numerous studies indicating that diabetic subjects tend to have more oxidative internal environments than those of healthy normal subjects. From these studies it is clear that diabetic subjects (multiple doses) show an increase in ROS generation and oxidative stress markers. ROS activities were decreased in mice subjects with single dose and early hyperglycemia as depicted in type 2 DM patients (Fig 6).

Fig 6. ROS Assay of SD, MD and control group



Characterization of hPMSCs: We characterized the MSCS at passage 4-5 using the immuno-loclization approach using confocal mocroscopy.Interestingly,we were able to show > 96 % MSCs phenotype evidenced by Stro-I +ve cells ,CD 90 ve+,and CD 105+,with >90% viability (Fig 7).





CONCLUSIONS

- It is evident that Multiple dose STZ induced NOD has developed an autoimmune mediated diabetes with a triggering mechanism of insulitis.
- This was evidential from the similar data reported (10-11); and infers that multiple dose STZ injected NOD mice are more autoimmune mediated diabetes as compared to other animal models.

8. EFFECT OF TEST SUBSTANCES ON BETA CELL BIOLOGY AND FUNCTION WITH RESPECT TO NUTRITION AND DIABETES

The adult pancreas is composed of three major tissue types: exocrine cells that produce digestive enzymes, ducts that transport the digestive enzymes, and endocrine islets consisting of cells producing insulin (β cells), glucagon (α cells), somatostatin (δ cells), and pancreatic polypeptide (PP cells). Pancreatic organogenesis is a highly regulated and complex process orchestrated by various growth factors produced in the local microenvironment and key

transcriptional regulators responsible for specifying different cell types present within the pancreas. The beta cell mass in the pancreas is under dynamic balance by neogenesis, proliferation and apoptosis various strategies have been used to derive insulin-producing beta cells from adult pancreatic stem cells. Testing of the origins of cells that give rise to new islet cells *in vivo* is complicated by the lack of specific markers for the pancreatic stem cells and by the difficulty of following individual cells' fate in the pancreas. An *in vitro* differentiation system allowing generation of new pancreatic endocrine cells would greatly benefit this work Our published data shows that niche/ microenvironment plays an important role to maintain the dynamics and functional integrity of the islets, and supplementation with with nutrients and growth factors upregulated the neogenesis process to generate large number of glucose sensitive ILCC. Hence, the present study would address cellular, molecular and functional approaches using the test compounds, and their efficacy in the management of diabetes would be explored.

Hypothesis

The Feasibility of using test compounds as cytoprotective and Insulinotrophic and towards beta cell protection.

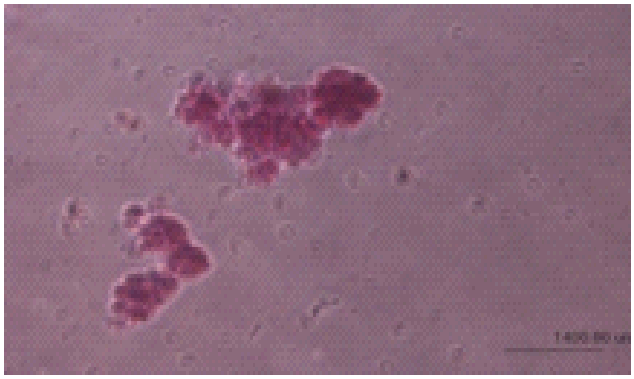
OBJECTIVES

- Isolation and maintenance of primary islet cell cultures to be processed from mice.
- To test the cytoprotective effects of the test compounds on the beta cells (viability, MTT assay, Dithiozone (DTZ) staining) in the diabetic model system (treated with and without STZ).
- Functional (Glucose stimulated insulin secretion) and gene expression (Insulin specific) studies using with and without STZ.

Brief methodology

The isolated islets were determined by using DTZ staining method. The dye binds to the zinc complexes of the insulin in the Beta cells of islets langerhans. In this insulin exists in hexameric form as zinc complexes, the islets upon bounds to DTZ dye stain and appears crimson red in color. DTZ staining was carried out by adding 10µl of DTZ to islet suspended in 1ml. of KRBH buffer, the islets were incubated at 37^0 C for 30 minutes. This staining helps to differentiate islets from other cells.

DTZ Stained islets



Experimental Approach:

a) ***Solubility test of test substance*:** The test compounds (H1 and Y1) provided by ITC were taken at1mg/1ml concentration. As per the data sheet issued to us, stock solutions were prepared in DMSO (Company/ Grade), filtered using Millex syringe driven filter units (0.22um) and stored at -20^0C.

b) ***Isolation of islets and maintenance of primary Islet cell cultures:*** The adult mouse (swiss albino mice) aged 6-8 weeks old were maintained at the NCLAS centre, housed at NIN. The animals were maintained under standard conditions of light (12hr light/ dark cycles) temperature (20±2^{O}C) and humidity. They were fed a regular diet (chow diet) and water *ad libidum*, till sacrifice. They were scarificed by CO_2 asphyxiation as per the ethical norms. Islets were isolated under sterile conditions as per our published protocol. After a period of 24–48 hrs, the islets were harvested by handpicking for *in vitro* assays including viability by

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay and islet cell integrity by dithizone staining (Sigma, USA) and images were captured with a Nikon inverted microscope using ACT-2U software.

c) ***MTT assay*****:** Approximatley 250-300 islets/vial were assessed for their viability by MTT assay after incubation at various concentrations of test substance respectively (H1/0,10,20,40,60,80,100 ug/ml and Y1 0,25,50,100,150,200,250 ug/ml). After 24 hrs of incubation, cell suspensions were taken in an 96 well plate and viability was checked by MTT assay and measured formazone formation, measured at 532nm.

d) ***Oxidative stress***: ROS Activity, Approximatley 250-300 islets from Control / test compounds/ concentrations (H1/0,10,20,40,60,80,100 ug/ml and Y1 0,25,50,100,150,200,250 ug/ml) were treated with 10µM DCFH-DA at 37ºC for 30 minutes, the cellular ROS was assessed by measuring fluorescein with excitation at 495nm and emission at 538nm in spectrofluorimeter.

e) ***Functional Assay***: Insulin secretion/ Insulinotrophic effect of the test substance: Briefly, 250 islets in triplicates were incubated with either: H1/0,10,20,40 ug/ml and Y1 0,25,50,100 ug/ml) and were placed in 6 well plate (Corning), containing 1 mL of Krebs–Ringer bicarbonate HEPES (KRBH) buffer (P^H 7.4),10 mmol/L HEPES, 1 mg/mL BSA with 5.5 mM glucose (basal level), 16.5 mM glucose (high glucose challenge) followed by 1 hr incubation at 37 ^{0}C. The supernatants were collected and stored at -80^{0}C till the assay.

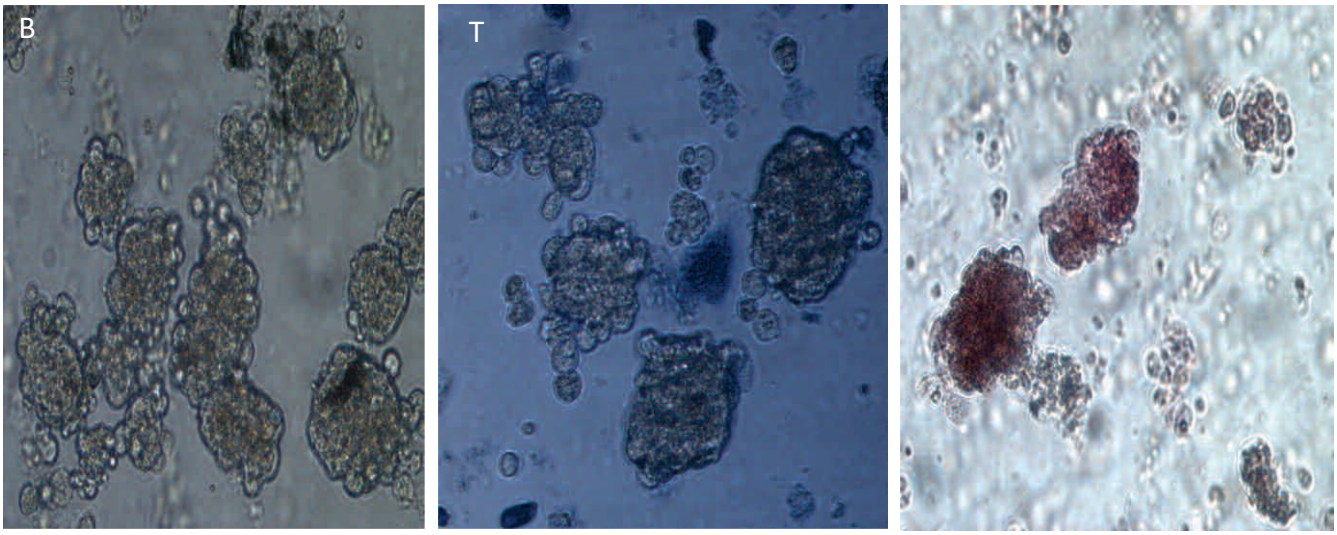
Statistical Approach: The data have been analysed by ANOVA as well as used students T test to compare between the two groups.

RESULTS

a) ***Solubility test of test substance*****:** As indicated in fig 1, and observed that H1 contained two different ingredients: a) yellow and b) black/ gray and in addition of DMSO solubilized only the yellowish substance leaving the black/ gray substance as the residue (indicated as sediment). However, the test compound Y1 was soluble in DMSO. Hence, Y 1 has been reported.

b) Isolation of islets and maintenance of primary Islet cell cultures: The primary islet cell cultures showed >98% in viability and integrity as presented below. Images have been captured with a Nikon inverted microscope using ACT-2U software.

c) MTT Assay: Fig 2 A indicates the viability of the primary islet cell cultures incubated with various cons of Y1 test substance for a period of 24hrs.Data shows that viability was >95% for Control and 25ug/ml. Following concentrations such as 50,100,150 200 and 250 ug/ml showed significant decrease in viability as indicated.



d) *Oxidative Stress: ROS*

Fig 3A indicates the ROS values of the primary islet cell culturesincubated with various cons of Y1 test substance for a period of 24hrs. Data shows the fluroscence units. Control cells treated only with DMSO showed least production of ROS, followed by 25,50,100,150,200 and 250 ug/ml. The concentrations between 100-250ug/ml were statistically significant in the production of ROS as compared to controls.

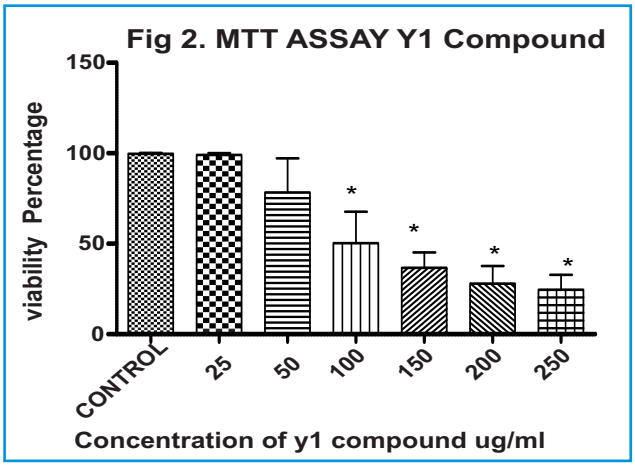
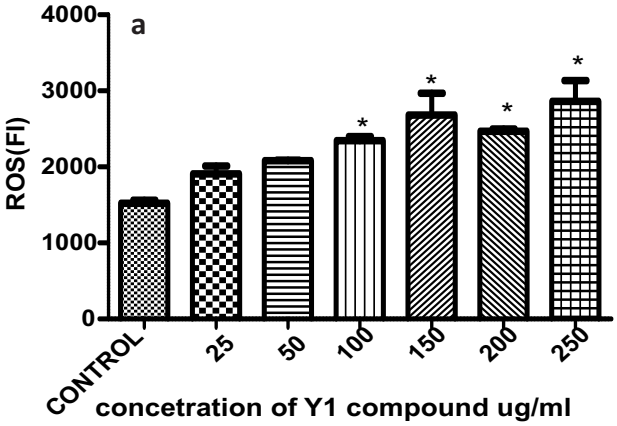
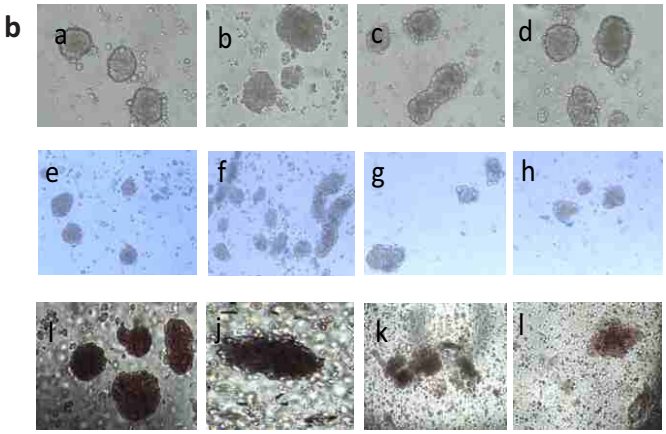


Fig 3. ROS levels for Y1 compound





A-D represents the BF images of the cultures,A) Control, B) 25ug /ml, c) 50 ug/ml and D)100ug /ml, E-H represents the TBE mages of the cultures, E) Control, F) 25ug/ml, G) 50 ug/ml and H)100ug/ml, I-L represents the DTZ staining with I) Control, J) 25ug /ml, K) 50 ug/ml and L)100 ug/ml. All the images have been captured in Leica DMI4000B, using the soft ware leica application suite 2.8.1 version.

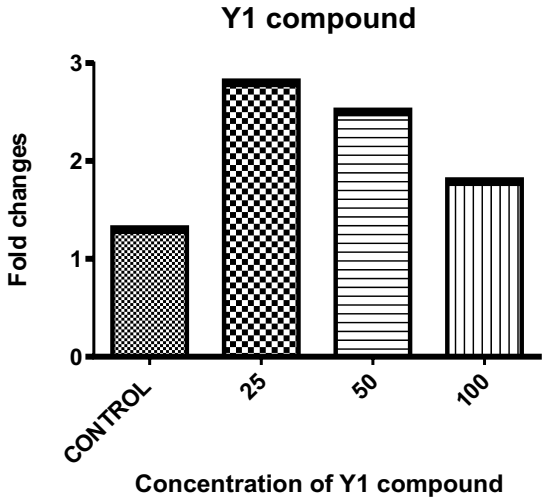
e) Insulin Secretion Assay: As given in Fig 5 (1) Test substanceY1 demonstrated insulinotrophic effects and there was significant stimulation of insulin (ELISA) at (16.5 mM, mol/L). The values represent the fold changes in the insulin stimulation as indicated by 16.5mm /5.5mm glucose.

The insulin secretory effects were >2 fold assessed at 25, 50 and 100 ug of the Y1 test substance as compared to control (only DMSO).

Concentration	ulu/mg protein (5.5mM)
Control	45.36±0.01
25	19.95±0.01
50	18.9±0.75
100	23.41±0.02

Concentration	ulu/mg protein (16.5mM)
Control	54.6±0.3
25	55.84±0.15
50	51.6±0.6
100	42.1±0.35

Fig 5(1)



CONCLUSIONS

- Using the primary islet cell cultures we have been able to recreate the physiological environment as close as possible to *in situ* conditions.
- The test test substances H1 and Y1 were beneficial at lower concentrations (H1,10, 20 & 40 - Y1, 25, 50 &100 ug/ml) as against the higher concentrations (H1 -60, 80 & 100 & Y1 100, 150, 200, 250 ug/ml) tested for islet cell viability, integrity, inflammatory, stress and insulin secretory effects.
- Interestingly, treatment with H1 and Y1 mitigated all the STZ induced diabetic response(s) such as reduction in stress, inflammatory responses, increased mRNA expression for Pdx-1 and Insulin (β cell specific genes),
- To arrive at most promising molecule between the two substances (H1 and Y1), we did the correlation analysis using the statistical tools. Our data showed the promises of Y1>H1for all the parameters tested advocating its efficacy to negate the STZ induced altered β cell functions.

9. IMPACT OF DIETARY FATTY ACIDS ON THE PROGRESSION OF NONALCOHOLIC FATTY LIVER DISEASE IN FRUCTOSE INDUCED MODEL OF STEATOSIS – ROLE OF ADIPOSE TISSUE INSULIN SENSITIVITY AND SECRETORY FUNCTION

Nonalcoholic fatty liver disease (NAFLD) covers a spectrum ranging from simple steatosis to advanced nonalcoholic steatohepatitis (NASH) with some patients ultimately progress to fibrosis, cirrhosis and liver failure. The pathophysiology of NAFLD is not fully understood and little is known about the factors that are responsible for the transition from benign steatosis to steatohepatitis. Lipotoxicity, insulin resistance, oxidative stress and imbalance in pro and antiinflammatory cytokines appear to play a role in the progression of NAFLD.

The recent phenomenal increase in prevalence of NAFLD may be associated with significant modification of dietary habits due to “Westernization” of the diet. The changes in dietary habit have made fast food as an important component of today's diet. In addition to high calorie intake, several components of the fast food based diets could play a major role in the pathogenesis of NAFLD. Increased consumption of soft drinks which contain high levels of fructose along with high intake of saturated fat, *trans* fat, n-6 PUFA and low intake of n-3 PUFA may be the possible cause of increased prevalence of NAFLD. An excessive amount of n-6 PUFA and high n-6/n-3 ratio in the diet was observed in patients with NASH compared to controls with hepatic steatosis. Dietary fructose is known to stimulate lipogenesis and causes obesity and hepatic steatosis. Adipose tissue dysfunction, characterized by insulin resistance and dysregulated adipocytokine production is considered to be the central mechanism involved in the transitional process to NASH in NAFLD.

Hypothesis: The type of dietary fatty acid determines the progression of NAFLD in the setting of hepatic steatosis by modulating adipose tissue insulin sensitivity and secretory function.

OBJECTIVES

1. To investigate the effects of different kinds of dietary fatty acids on progression of NAFLD in fructose-induced model of steatosis.

2. To evaluate the role of adipose tissue insulin sensitivity and adipocytokines in the progression of NAFLD.
3. To understand the underlying mechanisms involved.

METHODOLOGY

Animal experiment – I

To investigate the effects of dietary saturated (palmolein, PO; ghee, GE and coconut oil, CO) and trans fatty acids (vanaspati, VA) on the development of NAFLD in fructose induced model of steatosis, weanling Sprague Dawley rats (n=48) were divided equally into six groups and fed starch/fructose - casein based synthetic diet containing 20% fat. All the diets were prepared as per AIN-93 recommendations. In the first group starch (ST) was used as the source of carbohydrate whereas in the rest of the group, starch was replaced with fructose (FR) to induce hepatic steatosis. Groundnut oil (GNO) was used as the source of dietary fat in ST-GNO and FR-GNO groups. The various experimental groups are as follows:

Groups	Number of animals	Carbohydrate (50%)	Fat (20%)
ST-GNO	8	Starch	GNO
FR-GNO	8	Fructose	GNO
FR-PO	8	Fructose	PO
FR-GE	8	Fructose	GE
FR-CO	8	Fructose	CO
FR-VA	8	Fructose	VA

Animal experiment – II

To investigate the effects of substitution of n-6 PUFA with n-3 PUFA (α-linolenic acid or long chain n-3 PUFA) on the development of high fructose high cholesterol (HFHC) induced NASH, weanling Sprague-Dawley rats (n=32) were divided into four groups and fed starch-casein based synthetic diet containing 10% fat as per AIN-93 requirements. The n-6/n-3 PUFA ratio was altered by blending of groundnut oil, palm oil and linseed oil (source of ALA) to get the ratio of 200 and 2 or by blending of groundnut oil, palm oil and fish oil (source of EPA and DHA) to get the ratio of 5. The various experimental groups are as follows,

Groups	Number of animals	Carbohydrate (50%)	n-6:n-3 ratio
ST-200	8	Starch	200
HFHC-200	8	Fructose	200
HFHC-2	8	Fructose	5
HFHC-5	8	Fructose	2

All the animals were fed the respective diets for 24 weeks and at the end of the experimental period animals were sacrificed after overnight fasting.

The major findings of the study are summarized as follows:

- Ground nut oil prevents the development of fructose induced NAFLD
- Fructose in combination with saturated fatty acids/*trans* fatty acids induced hepatic steatosis of similar extent by upregulating the expression of lipogenic gene, SCD-1. In addition to the up regulation of SCD-1, FR-CO and FR-VA combination down regulate the expression of CPT-1 which is involved in fatty acid oxidation. Hepatic steatosis was further confirmed by increased liver triglyceride content and histopathological examination of liver (H & E and Oil red O staining).

- *Combination of fructose:* saturated/*trans* fatty acid induced insulin resistance as evidenced by increased HOMA-IR.
- *Fructose:* saturated/ *trans* fatty acid combination decreased plasma adiponectin level whereas plasma leptin level was increased only in fructose: trans fatty acid combination.
- *Fructose: trans* fatty acid combination increased visceral adiposity, increased the liver oxidative stress and upregulated the expression of proinflammatory cytokine and fibrogenic genes in liver.
- Histopathological evaluation of liver (H&E staining) showed lobular inflammation only in the liver of the rats fed diet with fructose: *trans* fatty acid combination. Masson trichrome staining of the liver revealed that the rats fed diet with fructose: *trans* fatty acid combination induced fibrosis. These results suggests that fructose: *trans* fatty acid combination is detrimental to the liver compared to fructose: saturated fatty acid combination.
- Substitution of n-6 PUFA with n-3 PUFA (α-linolenic acid and LC n-3 PUFA) prevented the development of HFHC induced NASH.
- n-3 PUFA supplementation efficiently prevented HFHC induced glucose intolerance, insulin resistance and dyslipidemia while reversing the steatosis which was associated with down regulation of hepatic lipogenic gene (SCD-1) and upregulation of gene involved in fatty acid oxidation (CPT-1).
- The protective effect of n-3 PUFA supplementation on HFHC induced NASH could be attributed to the suppression of proinflammatory cytokines and oxidative stress.

CONCLUSION

The results of the present study reinforce the current recommendations of restricting the intake of *trans* fats, moderate the intake of n-6 PUFA and increase the intake of n-3 PUFA for the prevention of diet related chronic diseases including NAFLD.

10. EFFECT OF RAW CARROT JUICE ON THE DEVELOPMENT OF NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD) AND ADIPOSE TISSUE INFLAMMATION; A CONTRIBUTORY FACTOR OF DISEASE PROGRESSION

Non-alcoholic fatty liver disease (NAFLD) is one of most prevalent health problems of developed countries and rising in developing countries as well. Obesity, diabetes and metabolic syndrome are considered as an important contributory/risk factors for NAFLD. In the context of disease prevention and control, there are wide variety of foods/dietary nutrients and factors (from both vegetables and fruits) have shown to be protective and beneficial against various life threatening diseases such as cancer, cardiovascular diseases etc. In this context, here assessed impact of raw carrot juice ingestion on the development NAFLD and its progression in a diet-induced rat model.

METHODS

Weanling male Wistar rats were given either control or high fructose diet with or without raw carrot juice (containing 0.3mg of β-carotene) for a period of 8 weeks. At the end, animals were killed and biological samples were analyzed for various parameters.

RESULTS

- Administration of carrot juice decreased the weight gain/adiposity and hypertriglyceridemia induced by HFr diet consumption.
- Although, glucose tolerance and insulin tolerance tests showed no significant changes, the observed hyperinsulinemia due to HFr diet was reversed by carrot juice administration, without altering the plasma glucose levels, which in turn reflected in improved HOMA-IR.
- In line with this, insulin-stimulated glucose uptake in muscle found significantly high in carrot juice-administered groups.

CONCLUSION

Carrot juice administration attenuates high fructose-induced hypertriglyceridemia, hepatic steatosis, inflammatory adipocytokines elevation and adiposity. Further, it reduced hyperinsulinemia and improved the insulin sensitivity.

11. EFFECT OF MATERNAL LIPIDS ON ANGIOGENIC FACTORS IN FIRST TRIMESTER PLACENTAL TROPHOBLASTS AND THEIR INVASIVE PROPERTIES

Placenta preferentially mediates the transfers of long chain polyunsaturated fatty acids (LCPUFA) from the mother to the fetus. Human fetal growth and development depends on the supplies of fatty acids especially LCPUFA for the fetal brain and retina development. The placenta, which considers as a “tree of life”, connects mother to the fetus. It is one of key pivotal organs that possibly script the genome-imprint codes of later life. Understanding its function and regulation becomes obligatory in order to prevent the cascade of prematurity, low birth weight and asphyxia and other cases of developmental deficiency at birth. Data shows that inadequate placentation process confers many of those cases. The primary aim of the project was to investigate effects of maternal fatty acids on placental angiogenesis and their invasive properties with reference to first trimester trophoblast development as an indicator of placentation processes.

METHODS

Finding suitable experimental model to mimic placentation is not easy as human placenta is distinct among mammals for trophoblast invasion and other determining processes such as placental permeability to the nutrients, net flux of fatty acid across the placenta and selectivity in the rate of transfer between different fatty acids. Therefore, present study employed in vitro and ex vivo approaches using model for the first trimester extravillous trophoblast cells HTR8/SVneo.

Findings

The initial data demonstrated that DHA (docosahexaenoic acid) stimulates the synthesis of angiogenic factor such as vascular endothelial growth factors (VEGF) with concomitant increase in the proliferation and tube formation (in vitro angiogenesis) of the first trimester trophoblast cells. Protective effects of DHA were observed *in vitro* where DHA rescued destabilization of tube formation induced by high glucose in HTR8/SVneo cells. Next we

investigated fatty acid (^{14}C) uptake in first trimester placental trophoblast cells, HTR8/SVneo. Uptake of ARA was greater compared with that of EPA and DHA in these cells (Fig 1). This is in contrast to what was observed in the case of last trimester placental trophoblast cells where uptake of DHA was preferred over ARA and other fatty acids. Use of triacsin C, an inhibitor of acylCoA synthetase, significantly inhibited fatty acid uptake as well as fatty acid-induced cell proliferation in these cells. Inhibition of tube formation and cellular proliferation with Triacsin C concluded that uptake of fatty acid into the first trimester trophoblast cells is the key energy driving factor in tube formation process.

The effects of trans fatty acid (TFA), its key isomeric form elaidic acid (ELA, t9) was tested in vitro to investigate its effects on ^{14}C uptake of ARA, EPA and DHA. Pre-incubation of HTR8/SVneo cells with ELA caused a decreased uptake of growth promoting ARA and EPA (Fig 2). This is the first report that shows TFA, those are mostly derived from industrially produced vegetable fats, may negatively affect the uptake of key long chain fatty acids such as arachidonic acid and eicosapentenoic acid in the first trimester trophoblast in vitro.

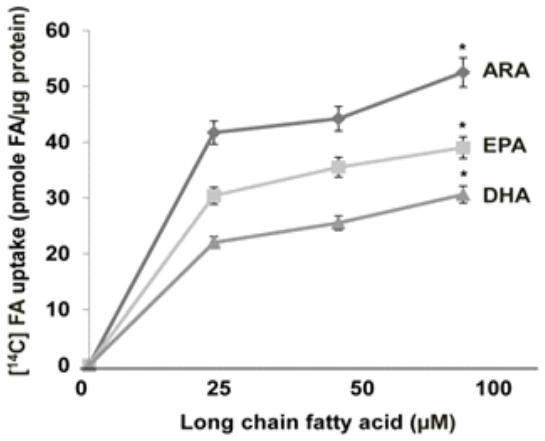


Fig. 1 : Uptake of long chain fatty acids by the first trimester placental trophoblast cells, HTR8/SVneo. Uptake of [14C] fatty acids was measured after incubating these cells with 100 µM of different fatty acids (arachidonic acid : ARA, eicosapentaenoic acid : EPA, docosahexaenoic acid : DHA) for 3 h. Uptake of [^{14}C] fatty acids was calculated as pmol of [^{14}C] fatty acid related to micrograms of protein per sample. The results represent means ± SEM of 3–6 experiments run in triplicate. *p < 0.05.

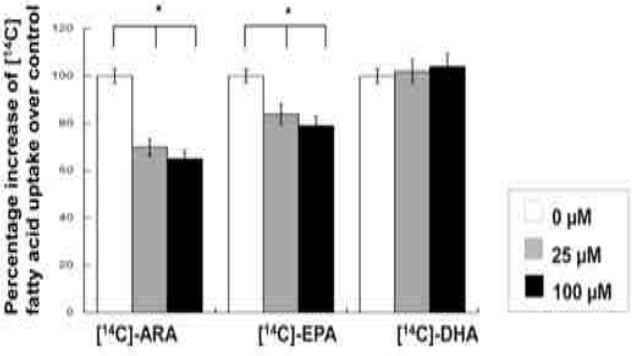


Fig.2 : Effect of trans fatty acid (*trans*-9-elaidic acid) on the uptake of long chain fatty acids in the first trimester placental trophoblast cells, HTR-8/SVneo . Uptake of [^{14}C] fatty acids was measured after incubating these cells with 25-100 µM of different fatty acids (ARA : Arachidonic acid, EPA : eicosapentaenoic acid; DHA : docosahexaenoic acid) for 3h. Uptake of [^{14}C] fatty acids was calculated as pmol of [^{14}C] fatty acid related to micrograms of protein per sample. The results represent means ± SEM of 3–6 experiments run in triplicate. *p < 0.05.

Interpretation

Placenta related disorders are serious causes of perinatal and maternal morbidity and mortality in India. There were few interesting leads that generated key knowledge in the public health and existing knowledge of early development biology. Optimum maternal nutrition especially role of fatty acids is crucial at least for the first trimester development of the pregnancy. Initial data reported for the first time that DHA may be equally important for early placental development activities as compared to its requirement at term placental development. As such LCPUFA intake during gestation is low in India as compared to its recommended intake. This data further elaborate that TFA intake could further down regulates the uptake of growth promoting fatty acids such as ARA and EPA.

IV. PUBLICATIONS, EXTENSION AND TRAINING

HEALTH SEEKING BEHAVIOUR, FOOD BELIEFS AND PRACTICES AMONG CHENCHU WOMEN DURING PHYSIOLOGICAL CHANGES – A GENDER BASED APPROACH

In India, tribal population makes up for 8.6% of the total population. Tribal people live in about 15% of the geographical area of the country. The places they live vary from plains, forests, hills, inaccessible areas etc. In 1975, the Government of India initiated to identify the most vulnerable tribal groups as a separate category called PTGs (Primitive Tribal Groups) and declared 52 such groups, while in 1993 an additional 23 groups were added to the category, making it a total of 75 PTGs out of 705 Scheduled Tribes, spread over 17 states and One Union Territory (UT), in the country (2001 census). In 2006, the Government of India renamed the PTGs as Particularly Vulnerable Tribal Groups (PVTGs) have some basic characteristics – they are mostly homogenous, with a small population, relatively physically isolated, social institutes caste in a simple mould, absence of written language, relatively simple technology and slower rate of change etc. PVTGs are scattered in different geographical areas of the country. According to the 2001 census, the PVTGs population is approximately 27,68,322. There are 12 PVTGS having a population above 50,000 and the remaining groups have a population of 1000 or less[1].

Tribal population in Andhra Pradesh (before state bifurcation) constitutes about 6.6% of the total population of the state. There are 12 PVTGs in Andhra Pradesh and Telangana. Chenchu is one of the PVTG recognized by Government of India. Nagarjunasagar Srisailam Tiger Reserve and Amrabad Tiger Reserve are inhabited by the primitive tribal group called “Chenchu”, who live in groups of huts called 'Gudem/Penta'. Chenchu literally means “a person who lives under chettu (Tree). They are one of the aboriginal tribes of Andhra Pradesh and Telangana [2]. In general, Chenchus are hunters and food gatherers. The predominant community is “Chenchu” followed by Lambada and Erukula. They are mainly found in the districts of Prakasam, Kurnool and Guntur (Andhra Pradesh), Mahaboobnagar, Nalgonda and Rangareddy (Telangana). The Chenchu tribal population in Andhra Pradesh and Telangana is 41,787 (Census of India, 2001) [3] presented in below Table-1.

Table 1. Chenchu Tribal population (Census of India, 2001)

Sl. No	State	District	No. of Mandals	No. of Villages	No. of Families	Population
1	Andhra Pradesh	Prakasam	7	81	3136	13321
2		Kurnool	13	40	2036	7915
3		Guntur	6	48	1643	6376
4	Telangana	Mahaboobnagar	10	123	2766	10406
5		Nalgonda	5	15	291	1069
6		Rangareddy	7	31	799	2700
Total			48	338	10671	41787

During adolescent age many physiological and biological changes occur which can be helpful when they become adult. Similarly, during pregnancy and after delivery, women need better nutrition to regain their health and to produce enough feed for the baby. Many

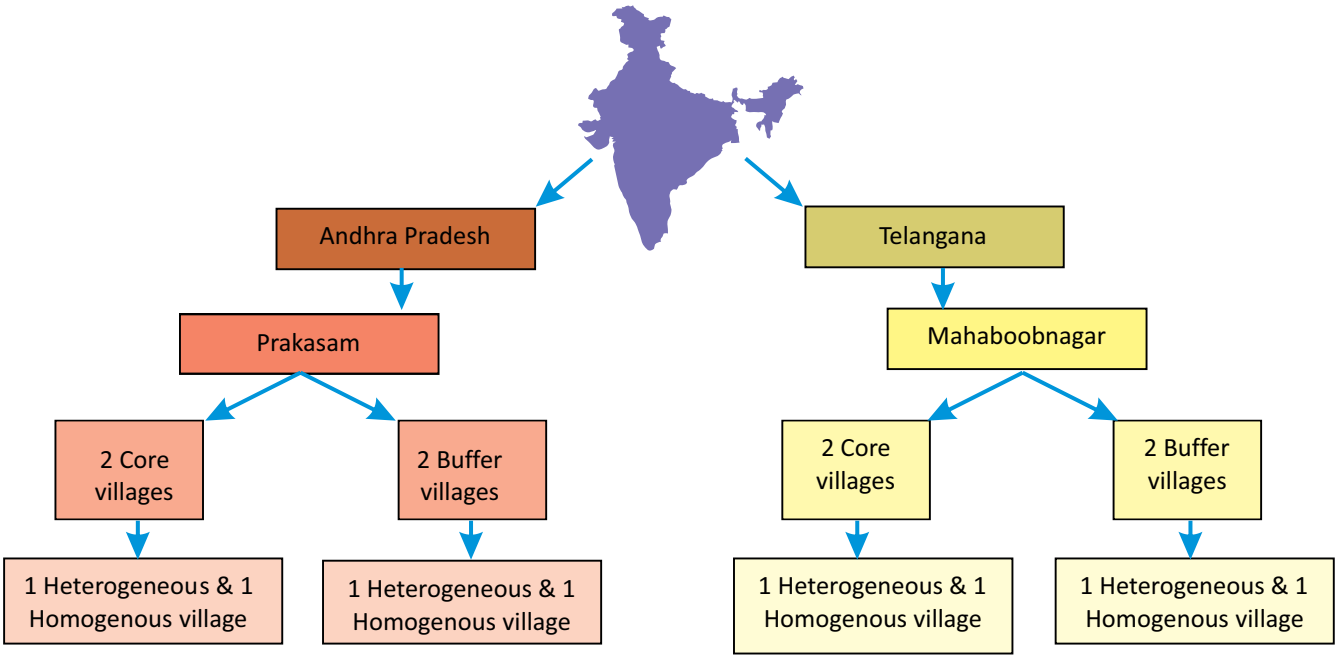
developmental programmes have been implemented to improve the nutritional status of tribal people were not found to be effective. Hence, this study has been planned to educate the Chenchus on nutrition and health.

OBJECTIVES

- ✓ To collect data on special food and dietary practices (if any) provided to girl children after menarche and to women during pregnancy and lactation.
- ✓ To asses food fads, myths and beliefs (if any) guiding such special food and dietary practices.
- ✓ To assess their health seeking behaviours.
- ✓ To develop a sustainable nutrition and health awareness / training programmes using multi-step flow of communication model.

METHODOLOGY

Stratified sampling technique was adopted to select research areas. From each of the two states of Andhra Pradesh and Telangana, one district with high proportion of Chenchus was selected and from each district four villages were selected. In four villages, 2 core zone villages (1 homogenous village & 1 heterogeneous village) and 2 Buffer zone villages (1 homogenous village & 1 heterogeneous village) were selected. Based on the sample frame, 8 villages were selected from the two districts. Sample size of 320 each from 40/8 villages was determined (Fig 1).



** [Core zone (critical tiger habitats): This is where tigers usually rest, reside, feed and breed. Therefore, Government should prevent any disturbance in such areas, including tourism.*

*** Buffer zones constitute the fringe areas (The outside boundary or surface of something) of tiger reserves up to 10 kms.]*

RESULTS

Data Collection was completed in Telangana and Andhra Pradesh. From each state 4 core villages (2 homogenous villages and 2 heterogeneous villages) and 4 buffer villages (2 homogenous villages & 2 heterogeneous villages) were selected for the research study (i.e. Total 16 villages). a total of 322 women were interviewed to assess food fads, myths and beliefs affecting the dietary practices during different physiological stages of chenchu women.

Major findings

1. During lactation (first six months) most of the foods were avoided except rice and spicy chilli powder. Hence, malnutrition of both mother and child was common.
2. No additional food intake was encouraged during pregnancy.
3. About 80% of women were getting married before the age of 18 years.
4. Consumption of millet was low due to the availability of subsidized cereals in the area.
5. A majority (76%) of women were using PHC to access health care.

At the end of the project, one day dissemination workshop on “Health and Nutrition awareness” was conducted for the Anganwadi workers, ASHA Workers, ICDS, CDPOs, ICDS supervisors, IKP staff, Balvadi teachers, NGO's who were working with Chenchu tribes. Project officer from ITDA, Primary Health Centre medical officers and NABCONS team also participated at the workshop. IEC material (developed based on information collected from chenchu tribe such as folders, charts) were distributed among the participants to educate the women.

V. FOOD CHEMISTRY

1. STUDIES ON BIODIVERSITY OF FOOD RESOURCES IN MEGHALAYA

Food biodiversity refers to the diversity of plants, animals and other organisms used for food, covering the genetic resources within species and between species provided by the ecosystem. In the past traditional societies used a wide variety of foods and maintained practices that permitted adequate nutritional status long before nutrition intervention programme. Central to the success of such practices was the exploitation of wild food resources, an approach used not only by traditional hunter-gatherer societies, but also by pastoral and agricultural societies as well. It is now well recognized that wild foods are part of local and regional agriculture as well as food procurement systems and wild species are important genetic resources in global efforts to maintain biodiversity. However, in economic development efforts, dietary utilization of non-domesticated plants or less familiar foods has received little attention. Along with this omission has been the revelation that despite increased food production in some sectors, a drastic narrowing of the food base has occurred in many traditional societies. The global food system is becoming increasingly homogenized in a way that's unhealthy for people and the environment, disconnecting us from our cultural food traditions, and presenting a serious threat to the future of our food supply. Today around 15 species contributes as the main source of human food energy with only three crops corn, wheat and rice accounting for more than 60% of the world's caloric intake.

Northeast India is a region where the land and climatic conditions are suitable for growth and survival of numerous plants, shrubs and trees. Forest cover in Northeast India accounts for 23.28% of the total forest cover in the country. This region is known for the wealth of its biodiversity and is known as one of the eighteen hot spots of biodiversity recognized across the globe. Meghalaya is one of the states in Northeast India mainly inhabited by three major indigenous tribes namely *Khasi, Jaintias* and *Garos*. The Khasi hill is one of the seven districts of Meghalaya comprising of 1033 villages in East Khasi Hill and 1126 villages in west Khasi hills with a population of 8,24,059 and 3,85,601 respectively. Over the ages the Khasis have developed ingenious uses of many wild plants within their environment as food sources, perhaps after a good deal of trial and error. They are grown for food within the farming system operating in any particular locality or gathered as wild or semi wild products. The nutritional composition of wild food resources, suggest that they are comparable or even superior in some instance to more recently introduced cultivars. Many such plants have been identified but the lack of data on their dietary use, preference and chemical composition has limited the prospects for their utilization. Most report on some lesser known and unconventional crops indicate that they could be good sources of nutrients, and many have the potential of broadening the present narrow food base. Therefore, dietary incorporation or maintenance of non-cultivated plant resources could be highly advantageous to nutritionally marginal populations or to specific vulnerable groups within populations.

Our food traditions are not only a big part of our identity but also provide the diversity that is integral part of healthy and resilient ecosystem (Slow Food, USA). Despite rapid urbanization, many of the less familiar foods still forms an integral part of the Khasis diet throughout the year. Indigenous people's food practices and patterns are highly influenced by their tradition and the environment, therefore, understanding the food systems of the Khasis and improving these systems in the context of nutrition and health merits attention.

Work completed

1. Acquiring the indigenous foods of Meghalaya

In order to capture the diversity of foods in Meghalaya, a list of the commonly consumed and less familiar foods has to be acquired through a survey. Hence, ten villages (four villages from each block) of the West Khasi Hills, Meghalaya (Table-1) were identified for the study. The villages were selected statistically by random stratified sampling, thereby dividing all the villages of the west khasi hills into quartiles based on population ranging from least populated (<53) to the most populated (≥174).

Sl. No.	Village name	Block name	Total population	Total No. Household	Household For Dietary Diversity Study
1	Ladmiri	Mairang	51	11	3
2	Nongum		680	136	34
3	Mawkhyllung	Mawkyrwat	52	11	3
4	Peinlang	Mawthadraishan	330	66	17
5	Mawlan	Mawshynrut	80	16	4
6	Khlangrin		440	88	22
7	Mawthaw	Nongstoin	98	20	5
8	Ramsngiwar		389	78	20
9	Kemsohsan	Ranikor	54	11	3
10	Balat		1295	259	65

An *informed consent* was sent to the Government of Meghalaya, Directorate of Social Welfare, Meghalaya, Shillong, requesting for local logistic help in the field study through their Integrated Child Development Services (ICDS) team. The interview was carried out with the help of an interdisciplinary team consisting of ladies supervisors of the ICDS team, a nutritionist, a village headman and the anganwadi in charge of the village block.

As per the guidelines, an informal interview with focus group and key informants was conducted in these villages whereby a group of ten to twenty elderly women (focus group) were gathered at the village community hall and were asked open ended questions (with probing and prompting) on the types of traditional or indigenous foods they consume, the availability and seasonality, the cultivars and varieties, the parts used, therapeutic used if any and the frequency of consumption. Key informants interview was conducted with community leaders familiar with the hunting, fishing and harvesting practices, Elders consistently resident in the community and familiar with the changes that have taken place during the last several decades, mothers and elderly like grandmothers. Representativeness of age, gender, ethnicity and geography was taken care in the selection of these Key informants. The information gathered was noted down in a pre-formulated sheet. The groups were asked to show the interviewers the foods that they had mentioned if it was available and pictures of certain foods were introduced to them by the interviewer to probe and prompt them if any other varieties similar to that are available and consumed. This helped in arriving at the varieties or cultivars of any particular food. Cameras and recording tools were used during the interview. Pictures of some foods that are available around were captured.

The foods were categorized into thirteen groups in the table namely cereal and grain, pulses and legumes, roots and tubers, green leafy vegetables, other vegetables, fruits, nuts and oilseeds, mushrooms, spices and condiments nuts, meat, fish and poultry, game meat and insects. Botanical identification of some of the foods were obtained from the Botanical Survey of India, Shillong.

A total of three seventy one plant and animal foods were captured through the FGD. These comprises of thirty two cereals and millets, thirteen pulses and legumes, twenty four roots and tubers, fourty five green leafy vegetables, nineteen other vegetables, seventy seven fruits and berries, five nuts and oilseeds, fourty two mushrooms, eleven spices and condiments, five types of meat and poultry, eighteen fishes and shell fish, fifty four game meat and twenty six varieties of insects (Table-1.1).

Food group	Food count		Currently consumed	
	Cultivated	Wild	Cultivated	Wild
Cereals & Millets	32	-	22	-
Pulses & legumes	13	-	13	-
Roots and Tubers	22	2	19	1
Green leafy vegetables	14	31	14	30
Other vegetables	16	3	16	2
Fruits and Berries	25	52	25	50
Nuts and Oilseeds	3	2	3	2
Mushrooms	-	42	-	33
Spices and Condiments	9	2	6	-
Meat and Poultry	5	-	5	-
Fishes and shell fish	9	9	6	9
Game meat	-	54	-	9
Insects	1	25	1	10

Table-1.1 listed down the number of foods that are cultivated/ domesticated and those gathered from the wild. All the cereals were cultivated and included the landraces rice, local maize, millets as well as the rice sold in the market and distributed through the Public distribution system (PDS). The pulses were either cultivated or bought from the market. Among the roots and tubers two varieties were wild i.e the lynniang (*Potentilla lineata)* and Soh kyrsu as it is locally known. Most of the green leafy vegetables are from the wild whereas the other vegetables were mostly cultivated. The maximum number of varieties was seen in the fruits and berries group with fifty two growing in the wild and twenty five either cultivated or sold in the market. Nuts and oilseeds are less in number all of which are cultivated. All the mushrooms recorded were collected from the wild. Khasis use very minimal spices and condiments in their preparation and seven very commonly used spices and condiments was recorded to be cultivated and sold in the market and two are wild. The khasi either domesticate animals like pig, cows, goats and hens for consumption or to sell them for their meat in the market. Usually all the parts of every animal are being consumed. Apart from the domesticated animals, the Khasis also hunted few game meat

and fifty four game meats were recorded during this study which are rarely consumed. There were twenty six insects recorded of which only one was domesticated. The seasonality of each food specie was recorded and presented in Table-3. All these food recorded are available in different season of the year except for the rice which were harvested during season and may be stored to be available throughout the year.

2. Nutritive content of indigeneous foods collected

Out of all the foods recorded in, one twenty seven indigeneous plant foods (mostly consumed) were collected for nutrient analysis. Sampling of each food was done following the prime objectives set by FAO. One twenty seven key indigeneous plant foods of the Khasis consisting of six varieties of rice, twenty one roots and tubers, fifty green leafy vegetables, nine other vegetables, twenty six fruits, two each from nuts/ oilseeds, seven wild mushrooms and four spices/ condiments were sampled. All the samples were collected separately from each selected villages and pooled together into one sample to make single composite samples. The composite samples were transported to laboratory with well-established package technique for nutrient analyses. The edible portion of food samples were separated cleaned and processed immediately for the nutrient analyses. Herbarium specimens of the samples were prepared and the botanical identification was done at the Botanical Survey of India, North Eastern Circle, Shillong.

Nutrient Analysis

Chemicals and standards

All the chemicals used in the study were of analytical grade and the solvents such as acetonitrile and methanol were HPLC grade. The chemical and solvents were procured from Merck (Merck, India). Standards of vitamins and minerals were purchased from Sigma chemicals (St. Louis, MO).

Determination of the proximate principle

The methods of the Association of Official Analytical Chemists (AOAC, 2006) were used for analysis of proximate composition i.e. moisture (2001.12) by calculating the loss in weight of samples dried in an oven at 60˚C for 48 hours; crude protein (984.13) by Kjehdahl method which gives the nitrogen content of the food that can be converted to total protein content using Jone's factor; ash (942.05) using a Muffle furnace; fat (2003.05) by solvent extraction method, using chloroform: methanol (2:1) as extractant and the dietary fibre (985.29) was assayed enzymatically. Carbohydrate content was calculated by difference. All the analytical determinations were done in triplicates and the results were expressed in g per 100 g of the edible portion. The proximate principles of some foods were given in Table-2.

Determination of minerals

Elemental analysis was carried out after doing wet digestion according to AOAC (968.08) method. Briefly the powdered samples was digested using Suprapure HNO_3(65%)and H_2O_2in the ratio of 2:1(v/v), filtered and used for the analysis of potassium (K), sodium (Na), iron (Fe), calcium (Ca), copper (Cu), manganese (Mn), magnesium(Mg) and zinc (Zn) by flame atomic adsorption spectroscopy (Varian- SpectrAA 220). The phosphorus content was estimated by modified as described in AOAC method (931.01) using a spectrophotometer (Analytikjena - U-2800 SPECORD S.600). The mineral content of few foods were given in Table-3.

Quantification of vitamins

Analysis of water soluble vitamins (Vitamin C, B_6, B_5 and B_9) was carried out using HPLC techniques in Ultra-High Performance Liquid Chromatography (U-HPLC Dionex ultimate 3000

Table 2. Proximate composition of Khasi indigeneous foods

LOCAL NAMES	BOTANICAL NAME	MOISTURE	ASH	PROTEIN	FAT	IDF	SDF	TDF	CHO	ENERGY (KCal)
		g/100g								
CEREALS										
Kba- Shulia	*Oryza Sativa*	10.6±0.40	0.68±0.02	6.53±0.12	0.43±0.02	6.36±0.34	1.27±0.57	6.13±0.11	78.02±0.10	345.76±0.51
Kba - Lwai	*Oryza Sativa*	10.35±1.45	0.73±0.06	8.34±0.09	0.45±0.01	6.60±0.62	1.38±0.36	7.98±0.43	71.65±0.21	339.99±1.27
kba bakut	*Oryza Sativa*	10.87±1.07	0.83±0.08	7.11±0.08	0.77±0.03	5.11±0.27	1.39±0.06	6.50±0.35	73.70±0.01	343.16±0.68
OVEG										
Nudkait	*Musa acuminata*	86.25±0.36	0.49±0.03	2.66±0.77	2.62±0.30	5.41±0.30	1.41±0.19	6.82±0.59	1.16±0.06	52.50±7.20
Soh ngang rit	*Solanum ferox*	69.33±0.23	2.54±0.48	4.20±0.01	2.83±1.58	1.23±0.23	0.99±0.21	2.22±0.22	13.33±0.25	99.23±15.70
Soh ngang	*Solanum gilo*	87.46±0.44	0.71±0.06	4.20±0.08	0.52±0.02	1.93±0.05	1.58±0.03	3.51±0.51	3.60±0.08	42.90±1.36
ROOTS & TUBERS										
Shriew dohnud	*Colocassia sp.*	72.16±0.93	1.43±0.41	2.25±0.31	0.20±0.05	2.65±0.13	1.10±0.13	3.75±0.70	20.21±0.16	99.14±0.35
Lynniang	*Potentilla polyphylla*	55.8±0.10	1.51±0.04	3.16±0.04	0.57±0.02	23.55±1.15	2.54±0.45	26.10±0.84	12.86±0.39	121.45±0.26
Phan san minit	*Solanum tuberosum*	71.08±0.91	1.45±0.1	2.89±0.01	0.17±0.02	1.10±0.05	7.10±0.21	8.20±0.09	16.21±0.66	94.33±2.60
Sying makhir	Zingiber zerumbet	80.49±1.87	0.75±0.04	1.64±0.55	0.92±0.42	3.78±0.33	0.50±0.07	4.27±0.61	11.53±1.16	72.10±7.44
GREEN LEAFY VEGETABLES										
Jarianglot	*Artemisia nilagirica*	83.34±1.51	1.45±0.44	2.23±0.12	0.41±0.02	6.47±0.33	1.34±0.32	7.81±0.03	4.76±0.02	39.27±0.33
Sla soh byrthit	*Bidens tripartita*	86.23±0.80	1.89±0.74	3.29±0.18	0.70±0.05	3.06±0.05	1.39±0.28	4.45±0.71	3.44±0.1	42.12±2.08
Jalei	*Chlorophytum khasianum*	83.08±0.23	1.69±0.13	2.24±0.21	0.88±0.02	5.75±0.63	2.22±0.07	7.97±0.74	4.14±0.12	49.38±2.62
Sla jaiaw	*Chrysanthemum* sp.	82.58±0.82	1.32±0.31	1.57±0.46	0.71±0.04	5.16±0.17	0.99±0.01	6.15±0.01	5.67±0.03	47.65±1.59
Jangew/ Jathang	*Emelia soncifolia*	90.95±2.93	1.10±0.02	1.72±0.03	0.12±0.04	2.81±0.06	1.15±0.40	3.96±0.05	2.15±0.09	24.48±0.50
Jarain	*Fagopyrum dibotrys*	84.17±0.48	1.60±0.56	3.71±0.57	0.37±0.03	6.26±0.06	2.08±0.28	8.35±0.23	1.8±0.24	42.07±3.97
Sla tiewjain	*Hibiscus syriacus*	73.36±1.77	3.21±0.18	3.10±0.23	1.61±0.05	5.88±0.32	1.71±0.05	2.8±0.44	7.14±0.09	73.04±0.04
Sla-sohkrot	*Smilax glaucophylla*	88.34±0.02	2.47±0.22	2.35±0.35	0.44±0.03	2.22±0.02	1.22±0.77	3.44±3.44	2.97±0.08	32.07±5.58
Jalynshir	*Sonchus asper*	77.21±0.78	1.74±0.03	2.4±0.10	0.76±0.05	11.46±0.08	1.40±0.63	12.86±1.39	5.04±0.02	62.28±3.67

LOCAL NAMES	BOTANICAL NAME	MOISTURE	ASH	PROTEIN	FAT	IDF	SDF	TDF	CHO	ENERGY (KCal)
		g/100g								
FRUITS										
Soh sat blang	*Elaeocarpus floribuBDLus*	71.6±0.15	1.50±0.06	1.96±0.04	0.81±0.06	2.02±0.54	0.92±0.03	2.94±0.06	21.19±0.18	105.77±0.09
Soh matan	*Myena spinosa*	80.75±0.62	0.81±0.07	0.65±0.01	0.82±0.06	3.71±0.66	1.60±0.13	5.31±0.20	11.66±0.02	67.24±0.10
Soh jhur	*Pyrus pashia*	85.56±0.54	0.38±0.02	0.40±0.05	0.78±0.02	3.08±0.70	1.12±0.86	4.20±0.67	8.68±1.79	51.74±5.80
Soh ma	*Rhus semialata*	13.91±0.22	2.25±0.44	7.48±0.03	12.34±0.21	24.87±0.31	11.56±0.24	36.43±0.32	27.59±0.14	324.2±3.21
Soh lang	*Viburnum foetidum*	78.15±1.3	0.69±0.03	1.47±0.03	0.94±0.01	2.66±0.29	1.05±0.32	3.71±0.03	15.04±0.62	81.91±2.64
Soh meilangkait		74.78±0.65	0.96±0.04	1.50±0.27	1.30±0.15	2.67±0.47	1.21±0.11	3.88±0.82	17.58±0.35	95.79±2.78
SPICES & CONDIMENTS										
rynsunkhasi	*Allium tuberosum*	80.19±0.8	0.89±0.13	1.20±0.24	0.88±0.10	5.6±0.38	1.23±0.21	6.29±0.05	10.3±0.58	79.23±4.18
Sla-Jaiur	*Zanthoxylum acanthopodium*	83.15±0.46	1.48±0.46	2.50±0.15	1.07±0.49	1.41±0.18	0.56±0.12	1.97±0.52	9.83±0.02	66.83±0.82
Sying Makhie	*Zingiber zerumbut*	80.49±0.73	0.75±0.04	1.64±0.03	1.12±0.01	3.78±0.34	0.50±0.26	4.27±0.72	11.73±0.18	78.76±1.90
Jaiur	*Zanthoxylum acanthopodium*	5.20±0.14	1.65±0.23	0.98±0.06	1.20±0.70	4.6±0.58	0.66±0.05	5.26±0.63	12.1±0.12	84.22±5.78
NUTS & OILSEEDS										
Kwai	*Areca nagensis*	56.22±1.33	0.88±0.02	3.66±0.54	4.42±0.30	10.13±0.13	2.58±0.46	12.7±0.19	22.12±0.24	168.30±3.52
Soh - Ot - Rit	*Castanopsis indica*	27.89±1.74	1.18±0.02	3.60±0.48	0.76±0.12	9.66±0.46	2.02±0.56	11.67±1.07	55.46±1.28	266.40±6.44
Sohliang	Gynocardia odorata	67.91±0.89	0.32±0.01	6.31±0.24	6.24±0.10	3.91±0.36	1.68±0.56	5.59±0.73	13.64±0.15	147.08±3.98
Nei - Lieh	*Perilla frustescens*	5.07±0.59	5.59±0.01	23.95±1.30	23.82±0.30	11.17±0.67	1.55±0.24	12.72±0.01	28.85±1.03	451.01±1.65
Nei - long	*Sesamum indicum*	5.06±0.04	5.35±0.08	37.80±0.11	28.16±1.50	10.55±0.59	1.68±0.35	12.23±0.27	11.4±0.14	474.72±15.02
MUSHROOMS										
Tit - kjat syiar	*Clavulina* sp.	4.13±0.05	11.8±0.78	29.39±0.24	3.26±0.93	15.14±0.98	2.13±0.61	17.27±0.04	36.69±4.78	317.24±26.93
Tit - tyndong	*Gomphus floccosus*	5.21±0.19	7.86±0.63	29.32±0.56	4.69±4.03	11.22±0.12	4.56±0.53	15.78±0.63	22.68±0.48	317.49±39.97
Tit sia	*Ramaria sp.*	4.11±0.09	6.98±0.02	24.26±0.11	2.96±0.84	14.43±0.20	4.12±0.01	18.55±0.32	43.14±0.03	342.90±7.84
Tit-Tap	*Suillus granulatus*	4.50±0.52	5.03±0.03	35.75±0.76	2.13±0.08	18.37±0.22	3.12±0.89	21.49±0.82	17.72±0.60	221.39±0.20
Tit snam		5.21±0.02	5.41±0.29	22.09±0.06	1.54±0.49	19.32±0.20	3.11±0.45	22.43±0.26	31.25±0.20	280.76±5.13

Table 3. Mineral content of Khasi indigeneous foods

LOCAL NAMES	BOTANICAL NAME	Fe	Zn	Cu	Mn	Mg	K	Ca	Na	P
		mg/100g								
Cereals										
kba bakut	*Oryza Sativa*	3.02	1.55	0.34	4.12	124.12	386.12	19.91	0.74	405.12
Kba Lwai	*Oryza Sativa*	2.84	1.09	0.36	3.98	166.12	333.21	10.86	1.13	444.52
Kba shulia	*Oryza Sativa*	3.12	1.22	0.52	3.46	140.3	206.32	11.47	0.62	369.56
Roots and tubers										
Shriew dohnud	*Colocassia* sp.	0.52	0.48	0.32	0.49	40.34	341.38	33.05	4.62	80.56
Lynniang	*Potentilla polyphylla*	9.1	0.5	0.09	0.13	24.3	106.01	194.92	11.12	52.12
phan san minit	*Solanum tuberosum*	0.59	1.2	0.1	0.11	24.3	489.36	8.55	4.22	46.56
Sying Makhir	*Zingiber zerumbut*	1.01	0.42	0.11	3.99	50.23	412.02	17.23	11.23	40.56
Green leafy vegetables										
Jarianglot	*Artemisia nilagirica*	4.06	1.19	0.89	2.52	41.38	510.56	164.38	26.88	74.12
Sla sohbyrthit	*Bidens tripartita*	2.48	1.74	0.47	0.9	47.84	773.65	115.91	14.23	86.02
Jalei	*Chlorophytum khasianum*	2.79	0.67	0.21	2.14	82.19	661.77	131.08	22.30	26.79
Sla jaiaw	*Chrysanthemum* sp.	2.27	0.8	0.32	2.78	47.41	379.71	122.59	13.89	88.21
Jangew/ Jathang	*Emelia soncifolia*	2.54	0.25	0.12	0.24	26.44	321.05	133.45	18.76	22.47
Sla Sohkrot (V2)	*Smilax glaucophylla*	2.91	0.51	0.18	1.49	17.36	451.36	139.41	10.23	40.85
Other vegetables										
Nudkait	*Musa acuminata*	1.24	0.49	0.14	1.22	42.28	1375.18	25.16	26.33	51.88
Soh Ngang Rit	*Solanum ferox*	0.91	0.21	0.10	0.32	35.29	282.45	139.02	3.25	37.43
Soh ngang	*Solanum gilo*	1.02	0.26	0.04	0.33	51.21	298.21	40.12	4.33	62.3
Fruits										
Soh sat blang	*Elaeocarpus floribundus*	0.92	0.23	0.98	0.91	51.53	293	86.42	1.12	15.23
Soh jhur	*Pyrus pashia*	0.12	0.09	0.05	0.08	12.2	100.21	23.39	0.63	12.2
Soh lang	*Viburnum foetidum*	1.99	0.95	0.01	0.05	8.51	185	58.83	1.05	15.6
Soh meilangkait		0.47	0.37	0.41	0.93	39.03	198.22	45.94	0.72	9.66

Table 3. Mineral content of Khasi indigeneous foods (Contd..)

LOCAL NAMES	*BOTANICAL NAME*	Fe	Zn	Cu	Mn	Mg	K	Ca	Na	P
		mg/100g								
Nuts and oil seeds										
Kwai	*Areca nagensis*	1.05	0.51	0.88	1.05	45.23	356.23	42.36	6.23	90.15
Soh Ot Rit	*Castanopsis indica*	1.16	0.52	0.05	0.17	92.29	68.69	9.85	0.95	93.5
Nei Lieh	*Perilla frustescens*	8.26	5.02	0.11	5.2	255.12	475.21	335.84	5.2	698.12
Nei Iong	*Sesamum indicum*	16.35	8.26	4.12	2.56	302.15	555.21	980.02	9.22	602.2
Mushrooms										
Tit kjat syiar	*Clavaria flava*	54	9.5	0.41	2.5	96	1619	36.84	36.93	0.46
Tit tyndong	*Gomphus floccosus*	42	9	1.07	3.6	41	1581	124.13	14.12	0.44
Tit Tap	*Pleutorus* sp.	38	5	1.78	1.4	58	654	173.78	19.92	0.59
Spices and condiments										
Rynsun Khasi	*Allium sativum*	1.28	0.9	0.21	0.26	27.64	751.29	8.59	3.43	45.12
Sying Smoh	*Kamferia galanga*	51.12	1.54	0.29	1.23	42.12	606.23	20.18	8.56	32.12
Sla Jaiur	*Zanthoxylum acanthopodium*	1.1	0.62	0.19	0.32	38.3	361.64	128.8	0.42	62.38
Jaiur	*Zanthoxylum acanthopodium*	2.26	1.9	0.3	1.3	48.65	615.2	356.23	32.22	168.23

RSLC). Vitamin C was quantified as describe. The extraction was performed using metaphosphoric acid, acetic acid and tris-2-carboxy ethyl phosphine hydrochloride for analysis of total ascorbic acid while only metaphosphoric acid and acetic acid were used as extraction buffers for analysis of ascorbic acid. The extracts were filtered and run into the HPLC through a C18 column (Thermo BDS HYPERSIL C18 column 250 x 4.6 mm, 5µ) using potassium phosphate buffer and acetonitrile as mobile phase. Vitamin B_6 was determined by using metaphosphoric acid for extraction, following analysis in the U-HPLC using phosphate and acetonitrile as mobile phase through a C18 column (Thermo, Hypersil BDS 250x4.6mm, 5µ). Quantification of B_5 was done as described. B5 was extracted from the food samples using 3% (v/v) acetic acid solution . The extract was then analyzed by HPLC with a DAD detector through a C18 column (µ Bondapak C18 column, 300 x 3.9 mm, 10µ) using phosphate buffer (0.1M, pH-2.25) as mobile phase. Vitamin B_9 was determined by method as described. B_9 was extracted using buffer containing dipotassium hydrogenphosphate, ascorbic acid, sodium azide and 2-mercapto ethanol at pH 7.2. then the extract was treated with tri-enzyme (α-amylase, protease and deconjugase) to convert folates into its different forms. Extract was purified through a strong anion exchange catridge (SEP-PAK catridge) and analysed by U-HPLC (Dionex ultimate 3000 RSLC). Content of water soluble vitamins of few foods are given in table-4.

Fat soluble vitamin content of few food is listed in table-5. Fat soluble vitamin D was determined as per method described. Vitamin D was saponified with alcoholic potassium hydroxide and then extracted using n-hexane. The determination of vitamin D is done by reversed-phase analytical HPLC-APCI-MS (Spectra Systems, Thermo Finnigan and LCQ Advantage max, Thermo). Vitamin D is detected by mass spectrometry and chromatographic peaks are identified based on retention times, mass-to-charge value and additionally by mass spectral profile.

Vitamin E was quantified with slight modifications. The tocopherols and tocotrienols were extracted after alkaline saponification using n-hexane and separated by normal phase U-HPLC (Dionex ultimate 3000 RSLC) through a silica column (Waters, Sperisorb silica 100 x 4.6 mm I.D., 3 µm).

Vitamin K was extracted using dichloromethane and methanol (2:1), further extracted solvent-to-solvent using n-hexane. The determination of vitamin K present in the sample extract solution was analyzed by reversed-phase analytical HPLC-APCI-MS (Spectra Systems, Thermo Finnigan and LCQ Advantage max, Thermo). Vitamin K was detected by mass spectrometry and chromatographic peaks were identified based on retention times, mass-to-charge value and additionally by mass spectral profile.

Caroteniods were extracted after saponifying the sample with petroleum ether in the presence of 12% (w/v) alcoholic potassium hydroxide. The total carotenoids was estimated by taking the optical density (OD) at 450 nm in Spectrophotometer (Analytikjena U-2800 SPECORD S.600).

3. Dietary diversity and nutritional status of the Khasis

Household Diet recall study to capture their nutritional status and nutrient intake were conducted in the earlier selected villages during the “Synrai” or Atumn season. One fourth of the total number of households (one hundred seventy four) was taken for the diet survey i.e. every fourth houses were selected in each villages and 174 households were studied in this season (Table-1). A dietary survey using 24 hour (based on the NNMB 24 hour recall scheduled with a modification to include the variety/ cultivar) and 7 day food frequency questionnaire to record the frequency of consumption of every food in every food group during the past seven days was used to elicit information on food / nutrient intake in order to link this to the nutritional status.

Table 4. Water soluble Vitamin content

LOCAL NAMES	*BOTANICAL NAME*	TOTAL ASCORBIC ACID (mg/100g)	TOTAL B5 (µg/100g)	TOTAL B6 (µg/100g)	TOTAL FOLATES (µg/100g)
Cereals					
kba bakut	*Oryza Sativa*	BDL	1350	82.81	120.26
Kba Lwai	*Oryza Sativa*	BDL	1750	135.66	125.35
Kba shulia	*Oryza Sativa*	BDL	1062	40.52	145.11
Roots and tubers					
Shriew dohnud	*Colocassia* sp.	1.2	278	167.60	20.63
Lynniang	*Potentilla polyphylla*	7.35	298	51.64	253.41
phan san minit	*Solanum tuberosum*	23.44	978	147.73	23.63
Sying Makhir	*Zingiber zerumbut*	5.04	242	254.53	12.36
Green leafy vegetables					
Jarianglot	*Artemisia nilagirica*	40.93	137	67.71	24.32
Sla sohbyrthit	*Bidens tripartita*	56.89	746	71.55	164.83
Jalei	*Chlorophytum khasianum*	14.92	605	51.12	45.89
Sla jaiaw	*Chrysanthemum* sp.	55.7	513	64.84	57.69
Jangew/ Jathang	*Emelia soncifolia*	43.62	229	117.02	42.36
Jarain	*Fagopyrum dibotrys*	123.69	734	83.54	28.15
Sla sohkrot	*Smilax glaucophylla*	77.24	610	134.89	157.99
Sla nalia		30.12	511	222.02	29.72
Other vegetables					
Nudkait	*Musa acuminata*	12.59	380	40.120	123.450
Soh Ngang Rit	*Solanum ferox*	2.88	107	79.358	131.44
Soh ngang	*Solanum gilo*	1.16	261	13.576	100.230
Fruits					
Soh sat blang	*Elaeocarpus floribundus*	2.63	853	135.15	116.62
Soh shang	*Eleagnus caudata*	4.62	73	76.97	87.15
Soh Matan	*Myena spinosa*	26.28	165	43.10	144.32
Soh jhur	*Pyrus pashia*	16.23	373	176.65	114.37
Soh ma	*Rhus semialata*	30.01	112	177.39	128.84
Soh lang	*Viburnum foetidum*	40.12	636	104.06	77.87
Soh meilangkait		25.58	550	107.00	18.67
Nuts and oil seeds					
Kwai	*Areca nagensis*	1.22	307	213.12	20.23
Soh Ot Rit	*Castanopsis indica*	BDL	170	184.36	35.23
Nei Lieh	*Perilla frustescens*	BDL	673	65.81	169.63
Nei long	*Sesamum indicum*	BDL	512	123.86	177.94
Mushrooms					
Tit kjat syiar	*Clavaria flava*	BDL	1040	147.78	157.04
Tit tyndong	*Gomphus floccosus*	BDL	1040	202.40	104.80
Tit Tap	*Pleutorus* sp.	BDL	1080	213.00	126.23
Spices and condiments					
Rynsun Khasi	*Allium sativum*	15.42	609	59.04	161.15
Sying Smoh	*Kamferia galanga*	1.05	1042	54.53	55.38
Sla Jaiur	*Zanthoxylum acanthopodium*	53.52	661	27.56	32.12
Jaiur	*Zanthoxylum acanthopodium*	BDL	566	144.1	35.12

Table 5. Fat soluble vitamin content of Khasi indigenous foods

LOCAL NAMES	*BOTANICAL NAME*	TOTAL CAROTENOIDS (µg/100g)	VIT-D2 (µg/100g)	TOTAL VIT E (mg/100g)	VIT-K (µg/100g)
CEREALS					
kba bakut	*Oryza Sativa*	1.69	BDL	0.45	1.4
Kba Lwai	*Oryza Sativa*	10.97	BDL	0.15	BDL
Kba shulia	*Oryza Sativa*	5.43	BDL	0.78	BDL
ROOTS AND TUBERS					
Shriew dohnud	*Colocassia* sp.	28.59	BDL	0.30	3.69
Lynniang	*Potentilla polyphylla*	31.25	1.12	4.45	5.41
phan san minit	*Solanum tuberosum*	338.07	0.98	0.08	1.95
Sying Makhir	*Zingiber zerumbut*	375.09	0.28	0.32	26.36
GREEN LEAFY VEGETABLES					
Jarianglot	*Artemisia nilagirica*	1860.78	BDL	3.88	302.45
Sla sohbyrthit	*Bidens tripartita*	7077.04	BDL	1.74	344.08
Jalei	*Chlorophytum khasianum*	4321.72	0.41	0.23	182.71
Sla jaiaw	*Chrysanthemum* sp.	1788.57	BDL	1.64	312.45
Jangew/ Jathang	*Emelia soncifolia*	2515.02	BDL	0.49	128.54
Sla soh pian	*Eriosems himalaicum*	1397.86	BDL	1.78	314.20
Jarain	*Fagopyrum dibotrys*	1897.56	6.36	1.27	354.23
Sla tiew jain	*Hibiscus syriacus*	6424.49	BDL	0.48	278.95
Sla sohkrot	*Smilax glaucophylla*	771.20	BDL	0.12	224.16
OTHER VEGETABLES					
Nudkait	*Musa acuminata*	38.81	BDL	0.03	23.98
Soh Ngang Rit	*Solanum ferox*	496.13	BDL	1.35	23.69
Soh ngang	*Solanum gilo*	89.19	BDL	0.14	33.56
FRUITS					
Soh sat blang	*Elaeocarpus floribundus*	699.71	BDL	0.88	10.25
Soh Matan	*Myena spinosa*	166.24	3.80	0.72	5.83
Soh jhur	*Pyrus pashia*	71.20	BDL	0.25	10.30
Soh ma	*Rhus semialata*	827.41	BDL	3.88	4.12
Soh lang	*Viburnum foetidum*	444.62	BDL	13.43	12.23
Soh meilangkait		184.91	BDL	1.44	3.01
NUTS And OIL SEEDS					
Kwai	*Areca nagensis*	47.78	12.36	0.06	2.22
Soh Ot Rit	*Castanopsis indica*	138.88	53.82	1.79	8.69
Nei Lieh	*Perilla frustescens*	629.90	83.11	1.13	34.56
Nei long	*Sesamum indicum*	201.53	40.79	0.37	17.78
MUSHROOMS					
Tit kjat syiar	*Clavaria flava*	26.13	260.00	3.51	250.39
Tit tyndong	*Gomphus floccosus*	18.14	3930.00	0.24	162.80
Tit Tap	*Pleutorus* sp.	28.31	31200.00	0.91	116.21
SPICES AND CONDIMENTS					
Rynsun Khasi	*Allium sativum*	33.67	BDL	0.10	1.2
Sying Smoh	*Kamferia galanga*	388	0.28	8.59	6.36
Sla Jaiur	*Zanthoxylum acanthopodium*	5534.82	1.35	0.53	541.53
Jaiur	*Zanthoxylum acanthopodium*	1241	0.12	1.22	2.36

Food intake of individuals

The mean daily intake of foods and mean/median consumption of nutrients of individuals by age/sex/physiological was analyzed. The average intake of cereals and millets among 1-9 years children was adequate and met more than one hundred percent of the recommended dietary allowances. Whereas, their average intake of pulses & legumes was three times lesser than the suggested level of 35 g/day. The mean intake of roots and tubers was very much higher (approximately 500% of the RDA) among all the age group. The average consumption of milk & milk products, fats & oils, GLV and sugar & jaggery was grossly inadequate, which shows <10% of the suggested RDA levels for children 1-7 years.

The mean intake of cereals and millets among adolescent boys age 10-12 years, 13-15 years and 16-17 years was 298.71 g, meeting about 70% of the suggested RDA level of 420 g/day. Whereas, among the adolescent girls of this age group the mean intake of cereals and millets was 416 g which was 9% more than the recommended intake and their average intake of pulses & legumes among boys and girls below the age of 18 years was three times less than the suggested level among the age of 13-15 years boy's and girls and 16-17 years boys and girls, the mean intake of cereals and millets was 418 g and 393g, and, 425g and 313g respectively. The intake of roots and tubers remained very high among both boys and girls in all the age group, whereas, the consumption of green leafy vegetables, fats and oils, and other vegetables was lower.

Among the adults engaged in sedentary work including non-pregnant non-lactating (NPNL) women had intakes of cereals and millets was 30% less than the RDA thereby meeting just about 70% of their requirements. Their average intake of pulses met only about 35% of the suggested daily intake in both men and women. Except for roots & tubers, the intake of all other foods was lower than the suggested levels. However, the extent of deficit compared to RDI was more with regard to milk & milk Products and fats & oils, followed by sugar and jaggery.

The mean intake of cereals and millets by pregnant women was 441.5 g, which was much higher than the figure observed among NPNL women (279 g). Barring Roots and tubers, other flesh foods and milk and milk products, the average intake of all other foods was found to be more or less similar to that of NPNL women. The average consumption of cereals and millets among lactating mothers was 385.9 g, which was higher than that observed among NPNL women (279g) but lower than that of pregnant women. The average consumption of pulses and legumes was very low (8 g). The intake of protective foods such as milk and milk products, Green Leafy Vegetables etc. was in general low.

Individual's nutrient intakes

Barring calcium, iron, vitamin C and riboflavin, the median intakes of all the nutrients among children 1-6 years, adequately met the RDA. The median intake of energy was 1015.40 Kcal and 1561 Kcal in 1-3 years and 4-6 years children respectively. The intake of protein intake was also 10% more than the suggested levels. The intake of vitamin A was more than the RDA, except among the age group 1-3 years which was15% below (342 mg/day) the RDA of 400 mg/day.

Among adolescent boys and girls (10-17 years), the median intakes of all the nutrients in general were less than RDA. With The median intake of energy ranging from 1721 Kcal against RDA of 2190 kcal in 10-12 years boys, 1807Kcal in girls, 2091 Kcal in 13 -15 years boys and 2073 Kcal in girls, 2092Kcal and 1940Kcal in boys and girls respectively of the age 16-17 years. The intake of protein was approximately less than 10% against the suggested RDAs. Vitamin A, thiamin and Niacin were in accordance with the recommended values for both adolescent boys and girls and it met the suggested recommended.

The median intakes of all the nutrients in adults doing sedentary work including non pregnant non lactating women, barring thiamine and riboflavin, in general with the exception of niacin, were less than the RDA. In adults the median intake of energy was 1685 Kcal against RDA of 2425kcal in men and 1875 Kcal in women and 1284Kcal for NPNL women. The intake of protein was about 47g as against RDA of 60g and 50g in men and women respectively, suggestive of a 10 to 20% deficit.

The median intakes of all the nutrients in general except for calcium, iron, folic acid and riboflavin, in pregnant women were adequate. The median intake of energy was 2470 Kcal against RDA of 2175 kcal. The intake of protein was about 68 g. as against RDA of 65 g. Whereas, in the case of lactating mothers, the nutrient intake was lesser than the suggested RDAs, with the mean intake of energy being 2127 Kcal against the RDA of 2275 Kcal. Their protein intake was 62g almost meeting the suggested RDA of 68g/ day.

CONCLUSIONS

1. Three hundred and seventy one plant and animal foods (both wild and cultivated/ domesticated) were identified by the study. These included insects and mushrooms.
2. Comprehensive nutrient composition database of 127 indigenous foods was generated.
3. The study for the first time had identified mushrooms consumed by people to be very rich in both proteins and vitamin D .
4. The oilseeds viz *Perilla frutescens* and *Sesamum indicum* was found to have high protein and fat content. The highest content of dietary fibre was found in the masticatory root *Potentilla polyphylla.* The rice land races also possessed high amount of dietary fibre than commonly consumed rice. Iron content was highest in condiment root *Kamferia galangal.* Sesamum indicum exibit the highest calcium content and in general all the green leafy vegetables had good amount of calcium content. Zinc content was highest in the stems of *Alocasia macrorrhiza.*
5. *Crassocephalum crepidiodes* leaves possess highest vitamin C content whereas folic acid content was highest in the leaves of *Sonchus asper.* The highest carotenoid content was observed in the leaves of *Passiflora edulis* and *Alocasia macrorrhiza.*
6. Stunting in <5 and >5 years children still exist at higher than reported by NNMB previously. But underweight and wasting were much lesser.
7. In adults Chronic Energy Deficiency (CED) still existed at 22 and 35 % in men and women respectively and overweight and obesity was lesser in woman (16 %) than men (32.2 %).
8. All individuals from children to adults of different physiological and activity status met at least 70% or more of the RDA for consumption of cereals. The intake of roots and tubers also remained very high across all age group, gender and physiological group.
9. The intake of other foods viz pulses and legumes; vegetables and fruits was very less than the RDA.
10. The median intakes of all the nutrients in adults doing sedentary work including non pregnant non lactating were less than the RDA.
11. The median intakes for calcium, iron, folic acid and riboflavin, in pregnant women were very less. In the case of lactating mothers, the nutrient intake was lesser than the suggested RDAs.
12. Among adolescent boys and girls (10-17 years), the median intakes of all the nutrients in general were less than RDA.
13. The intake of protein among children 1-6 years was 10% more than the suggested levels. The intake of vitamin A was also more than the RDA among 3-6 years.

VI. FOOD AND DRUG TOXICOLOGY RESEARCH CENTRE

1. DIFFERENTIAL PROTEIN EXPRESSION IN LIVER TISSUES IN FLUORIDE INTOXICATED RATS BY PROTEOMICS APPROACH

Due to scarcity of funds, we could not continue to study the differentially expressed serum and urinary proteome in control and fluoride intoxicated rats. Instead, we have planned to study the differential protein expression in the liver tissues in the control and fluoride intoxicated rats.

Endemic fluorosis is most severe and widespread in India and China and is endemic in at least 25 countries across the globe. In India, fluorosis is an endemic disease prevalent in 196 districts of nineteen states and is considered as a national health problem (Planning Commission, India, 2007).

Out of many preventive measures and approaches taken to mitigate the problem of fluorosis, a considerable research is needed on these multifactorial aspects of fluorosis, involving studies on epidemiological, nutritional, genetic aspects and at molecular level. There are very limited studies on understanding the processes of skeletal fluorosis and the underlying cause of the disease at the molecular level. Excessive consumption of fluorides in various forms leads to deleterious effects on different tissues of the body such as teeth, bone, kidney, brain, intestine and other soft tissues. Since there is no medicine is available for the treatment of the disease (dental and skeletal fluorosis), making medical intervention impossible. In such situation, early detection is more important. Biomarkers play major roles in early diagnosis, disease prevention, drug target identification, drug response etc. Till date, there are no specific studies on the potential biomarkers which can help us to assess the fluoride toxicity in earlier stages and to take earlier interventions to curtail the progression of fluoride toxicity on various vital tissues. The present study is aimed at exploring the differential expression of proteins in liver tissue that can be used to identify the biomarkers.

Hypothesis: Fluoride intoxication in rats results in the differential expression of proteins in liver.

OBJECTIVES

The present investigation is proposed with the following objectives:

- Identification of differential expression of proteins from the liver by 2D gel electrophoresis in fluoride intoxicated rats.
- Characterization of the differentially expressed proteins in liver by Mass spectroscopy in fluoride intoxicated rats.

METHODOLOGY

Materials*:* All other chemicals otherwise mentioned will be of analytical grade.

Animal Experiment

The animal experiment was initiated after the Institutional Animal Ethical Committee Approval. Wistar NIN rats were used for the study. The animal experiment was initiated in the month of March with 36rats randomly distributed into 6 groups with 6 rats in control group and 6 rats each in 5, 10, 15, 50 and 100 ppm F group. Animal care and experimental protocols are

followed in accordance with the Institutional Animal Ethical Committee. Rats are housed individually in stainless steel cages and are fed with standard laboratory diet. Control group rats received distilled water for drinking while the fluoride group received distilled water with 5, 10, 15, 50 and100 ppm F(sodium fluoride) in drinking water.

Collection of urine and blood: Urine and blood was collected at 1, 2 and at the end of the experiment (3 months) to study the fluoride levels in the control and fluoride intoxicated rats.

Body weight, diet and water intake: Daily diet intake [food given-(food left+wastage] was measured at intervals of 1 month for three consecutive days. Similarly, water intake was also measured [water given–water left]. Body weight was monitored weekly using a digital balance (Model BL 3, Sartorius, India).

***Euthanisation and collection of tissues*:** At the end of the experiment (3 months), all the animals were euthanized by CO_2 asphyxiation. At the end of 3 months, the animals were scarified by CO_2 euthanization and teeth, bones, liver tissues were collected. All the fluorosis related parameters were studied.

Urinary and serum F: Urinary F was analyzed by using an F ion selective electrode (Orion, USA) according to Tusl, 1970.

Teeth and femur bone F: Teeth and femur bone F were determined as previously described (Sly and Hu, 1995).Teeth and femur bone were weighed and dried at 50°C using the oven to make it moisture free. Both were defatted using chloroform and methanol (2:1) by keeping in soxhlet apparatus at 50°C for 48 h. Defatted teeth and bones were weighed and kept in a muffle furnace at 600°C for 24 h to make ash. Ashed teeth and bones were ground to fine powder and sieved using 125-micron pore size sieve. Twenty mg of sieved teeth and bone ash was taken in a clean plastic beaker in duplicate and dissolved completely in 0.5 M perchloric acid, pH was adjusted to 5.3 by adding 3 mL of 0.5 M sodium citrate buffer and F was analysed by adding 5 mL of TISAB I (Total Ionic Strength Adjustment Buffer I) using F ion selective electrode. The final value was represented as mg of F per g of dry teeth/bone.

Sample preparation for 2D gel electrophoresis: There were significant differences in the urinary and serum fluoride, teeth and bone fluoride in the 100ppm group compared to control group. Hence, the liver from the 100ppm group was taken for studying 2D gel electrophoresis followed by MALDI analysis. The livers from control and 100ppm fluoride group were homogenized in Tris-lysis buffer containing protease inhibitor cocktail (Roche) pH.7.4 using a homogenizer. Later, it was centrifuged at 10000rpm for 30 min at 4°C and the supernatant containing soluble proteins was recovered. The protein was estimated by Lowry method. After quantification, 500µg of liver proteins from each animal of the same group (n=6) were combined to constitute a pool that was submitted to proteomic analysis in triplicate.

***2D separation*:** Liver proteins 500µg were taken from each pooled sample and mixed in rehydration buffer to a volume of 130µl and were loaded on to 7cm IPG strips linear (pH 3-10) and kept for rehydration overnight. The first dimensional IEF was performed on an IEF system at 20°C (BIORAD). After IEF, second dimension of separation was performed on 12 % resolving gels. The resolved proteins spots were stained with coomassie brilliant blue R-250.The gels were scanned by Quantity one (BIORAD) software. The differential expression of liver proteins in the control and fluoride treated rats was analyzed by the PDQuest software (BIORAD). The 2D spots that exhibited a statistical significance were excised for future identification.

Mass spectroscopy: The differentially expressed proteins (2D spots) were given for MALDI analysis to CDFD, Hyderabad.

- The methodology in detail, the gel plug was washed with MS graded water at RT for 10 minutes at 600rpm using a thermo mixer. This procedure was done twice.
- The gel plug was then de-stained with Ammonium bicarbonate and Acetonitrile.

- The gel plug was furthered treated with DDT for reduction and Iodoacetamide for alkylation.
- Further, overnight Trypsin digestion was done at 37°C.
- After digestion, the peptides were extracted with equal proportion of Trifluroacetic acid (TFA) and Acetonitrile (ACN) followed by speedvac at 45°C
- The pellet was dissolved in TA buffer (TFA and ACN) and spotted along with HCCA Matrix onto the target plate. The spot was allowed to air dry.
- Mass spectrum was obtained using Bruker's MALDI-TOF/TOF UltraflexIII instrument.
- Instrument was first calibrated with peptide standards (Bradykinin, Angiotensin_II, Angiotensin_I, Bombesin, ACTH and Somatostatin)
- Mass spectrum (MS spectrum) of the sample was then obtained and saved along with respective Match ID.
- Peaks (M/z) with maximum relative abundance intensities (in MS spectrum) were taken for acquiring MSMS spectrum.
- MS and MSMS spectrum of each sample were combined in BrukerDaltonicsBiotools 3.2 software and the combined spectrum was further analyzed by Mascot search.
- Following parameters were used for online Mascot search.

Taxonomy	: Rattus	Enzyme	: Trypsin
Database	: NCBI	Missed cleavage	: 1
Mass tolerance	: 100ppm	MSMS tolerance	: 2Da
Fixed modifications	: Carbamidomethyl(C)	Variable modification	: Oxidation (M)

RESULTS

Bodyweight, Diet and water intake: The body weights of the rats at 1, 2 and 3 month were given in Table 1. There was a significant decrease in body weight in the 50 and 100 ppm F groups compared to control group. The diet and water intake of the rats at 1, 2 and 3 months was given in Table 2 & 3. There was a significantly decreased water intake in the 50, 100ppm F group compared to control group.

Urinary and serum F: There was a significantly higher excretion of fluoride in all the F groups than the control group (Table 4). There was a significantly increased serum fluoride levels in the 50 and 100 ppm F groups compared to control groups (Table 5).

Table 1. Bodyweight (g) of the rats in different groups at 1,2 and 3 months

Groups	I Month	II Month	III Month
Control	216.33 ± 23.269	267.8 ± 29.267	303.666 ± 33.206
5 ppm	219 ± 22.943	275.95 ± 33.073	305.833 ± 34.277
10 ppm	216.16 ± 14.08	261.7833 ± 18.218	288.333 ± 23.627
15 ppm	211 ± 29.705	254.3 ± 14.645	260.4 ± 12.354
50 ppm	193.166 ± 16.277a*b*c*	227.533 ± 20.222a*b*c*	237.5 ± 14.237a*b*c*
100 ppm	174.7143 ± 23.725a*b*c*	193.657 ± 20.710a*b*c*	220.285 ± 8.976a*b*c*

**Statistically significant (p<0.05). a=compared to control, b=compared to 5ppm fluoride, c=compared to 10ppm fluoride,*

Table 2. Diet intake (g) of rats in different groups at 1, 2 and 3 months

Groups	I Month	II Month	III Month
Control	14.82±0.89	16.82±1.03	18.92±1.02
5ppm	15.73±2.31	16.95±1.29	19.49±2.40
10 ppm	15.37±0.87	16.79±3.42	19.33±2.18
15 ppm	14.10±1.91	16.61±0.96	18.96±1.26
50 ppm	13.63±1.96	15.12±0.46	18.26±0.09
100 ppm	13.67±0.47	14.71±0.52	15.79±1.26 a*b*c*d*e*

**Statistically significant (p<0.05). a=compared to control, b=compared to 5ppm fluoride, c=compared to 10ppm fluoride, d=compared to 15 ppm fluoride and e=compared to 50 ppm fluoride.*

Table 3. Water intake (ml) of rats in different groups at 1, 2 and 3 months

Groups	I Month	II Month	III Month
Control	22.93±6.66	35.61±0.54	38.5±0.72
5ppm	25.90±7.62	33.61±2.27	37±1.83
10 ppm	25.37±2.60	32.05±3.66	34.06±2.59a*
15 ppm	24.53±2.11	29.39±3.26a*	31.56±2.15a*b*
50 ppm	22.83 ± 1.61	22.72 ± 1.36a*b*c*d*	24.06 ± 2.09a*b*c*d*
100 ppm	18.53 ± 1.86	21.33 ± 1.77a*b*c*d*	22.78 ± 1.78a*b*c*d*

**Statistically significant (p<0.05). a=compared to control, b=compared to 5ppm fluoride, c=compared to 10ppm fluoride, d=compared to 15 ppm fluoride and e=compared to 50 ppm fluoride.*

Table 4. Urinary F (mg F/24 hr) levels of rats in different groups at 1, 2 and 3 months

Groups	I Month	II Month	III Month
Control	0.084±0.004	0.094±0.0089	0.102 ± 0.0068
5ppm	0.089±0.003	0.148±0.007a*	0.172 ± 0.0087a*
10 ppm	0.099±0.017	0.154±0.0047a*	0.183 ± 0.0086a*
15 ppm	0.11±0.0160	0.19±0.0162a*b*c*	0.22 ± 0.0115a*b*c*
50 ppm	0.170±0.021a*b*c*d*	0.206±0.0166a*b*c*	0.296 ± 0.031a*b*c*
100 ppm	0.255±0.030a*b*c*d*e*	0.274±0.0160a*b*c*d*e*	0.355 ± 0.027a*b*c*

**Statistically significant (p<0.05). a=compared to control, b=compared to 5ppm fluoride, c=compared to 10ppm fluoride, d=compared to 15 ppm fluoride and e=compared to 50 ppm fluoride.*

Table 5. Serum F levels (µg/ml) of rats in different groups at 1, 2 and 3 months

Groups	I Month	II Month	III Month
Control	0.090 ± 0.005	0.089 ± 0.002	0.120 ± 0.004
5ppm	0.096 ± 0.003	0.101 ± 0.007	0.128 ± 0.020
10 ppm	0.100 ± 0.004	0.107 ± 0.006	0.131 ± 0.010
15 ppm	0.105 ± 0.003	0.115 ± 0.006	0.134 ± 0.003
50 ppm	0.115 ± 0.004a*b*	0.123 ± 0.003a*b*	0.143 ± 0.006a*b*
100 ppm	0.119 ± 0.002a*b*	0.132 ± 0.015a*b*	0.163 ± 0.008a*b*

**Statistically significant (p<0.05). a=compared to control, b=compared to 5ppm fluoride,*

***Teeth and bone fluoride*:** There was a significant increase in the teeth and bone fluoride levels in the F groups compared to control group (Table 6).

Table 6. Bone fluoride and teeth fluoride at 3 months

Groups	Bone fluoride (mg/g of dry bone)	Teeth fluoride (mg/g dry teeth)
Control	0.72 ± 0.06	0.16 ± 0.02
5ppm	0.91 ± 0.04	0.56 ± 0.06
10ppm	0.99 ± 0.04	0.62 ± 0.04
15ppm	1.09 ± 0.08a*	0.66 ± 0.02a*
50ppm	1.23 ± 0.12a*b*c*	0.82 ± 0.07a*b*c*
100ppm	1.58 ± 0.14a*b*c*	1.14 ± 0.12a*b*c*

**Statistically significant (p<0.05). a=compared to control, b=compared to 5ppm fluoride, c=compared to 10ppm fluoride*

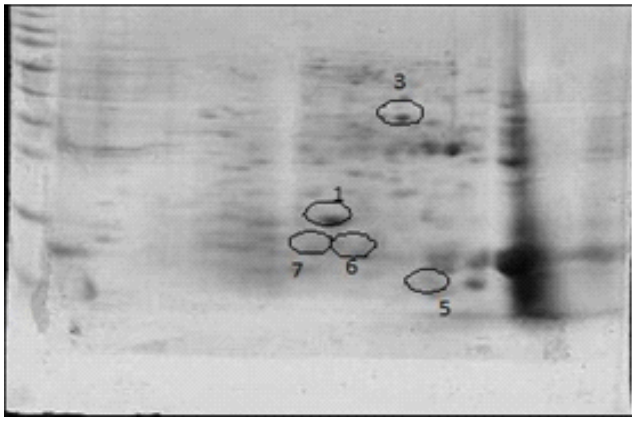
2D gel electrophoresis: As the significant differences in bodyweight, diet and water intake, urinary, serum and teeth and bone fluoride were observed in 100ppm fluoride group compared to control group, the livers from both the groups were studied for differential protein expression. The differential protein expression in the liver of control and 100 ppm fluoride group was observed.7 spots were identified as differentially expressed proteins by PD Quest software (BIORAD). The identified differentially expressed 7 proteins were labeled and shown in Fig 1A & 1B.

***MALDI analysis*:** The identified differentially expressed proteins were given for MALDI analysis to characterize the proteins. The proteins highly expressed in the control group compared to

Fig 1. 2D map of rat liver. Liver proteins were separated using pH gradient 3–10, followed by 12% SDS-PAGE. A. Control group B. 100ppm fluoride group

1A Control group

1B Fluoride group



fluoride were spots 2 and 4. The proteins highly expressed in the fluoride group compared to control group were spots 1,3,5,6,7. The proteins highly expressed in control group compared to fluoride group were glutathione-s-transferase and hemoglobin, alpha 2. The proteins highly expressed in the fluoride groups compared to control group were dismutase, arginase-1, T-cell receptor beta chain, D-dopachrome decarboxylase, D-dopachrome decarboxylase. The spectrum (lift spectra) or the individual spots are shown in Fig. 2 & 3.

Fig 2. MS MS spectra of liver upregulatedproteins in control compared to 100ppm fluoride group. A. Spot 2, B. Spot 4

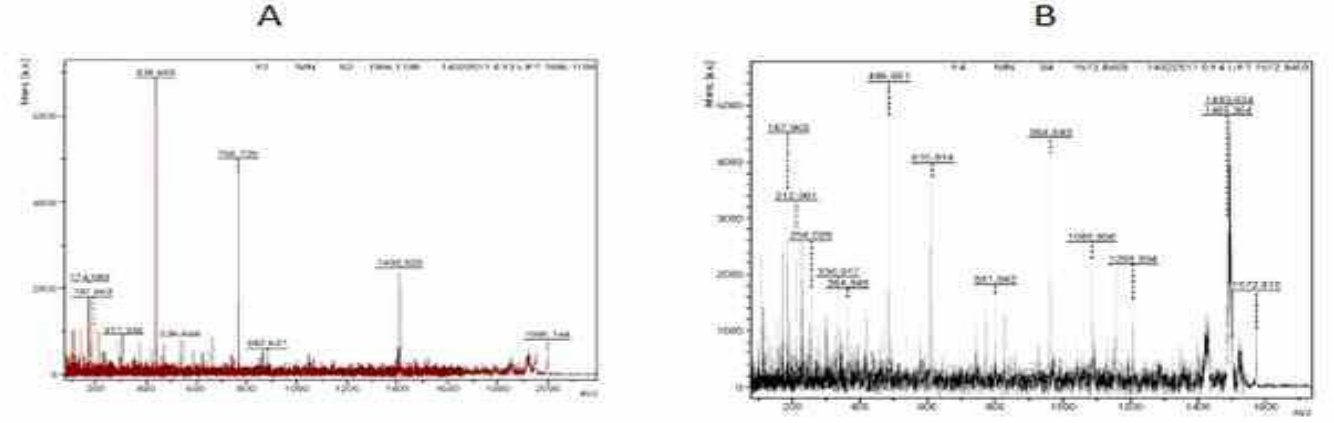
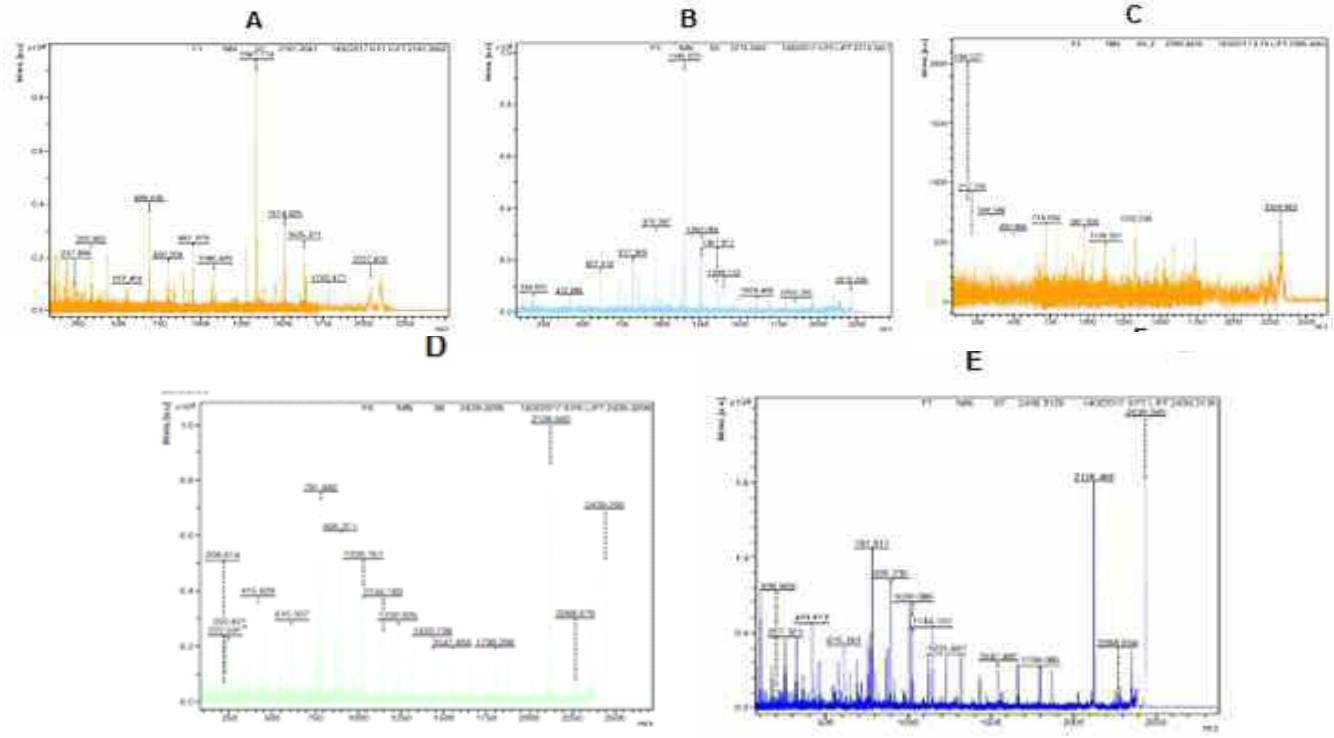


Fig 3. MS MS spectra of liver upregulated proteins in 100ppm fluoride group compared to control group. A. Spot 1, B. Spot 3, C. Spot 5, D. Spot 6, E. Spot 7.



CONCLUSION

Exposure to fluoride altered the expression of liver proteins belonging to antioxidant metabolism. Proteins that were not detected upon exposure to fluoride or that had their expression induced in the presence of fluoride were potential biomarkers of toxicity and should be better investigated. These findings indicate important pathways and cellular processes affected by exposure to fluoride and that should be addressed in more detail in future studies.

B. DRUG TOXICOLOGY

1. EVALUATION OF BIOAVAILABILITY OF ß-CAROTENE IN BIO-FORTIFIED FOOD CROPS

The research efforts to combat subclinical nutritional disorders due to micronutrient /vitamin deficiency are gaining much importance. Many efforts viz., bio-fortification, nutritional supplements etc., to reduce the risk are attempted. Among many such disorders, the clinical and sub-clinical ones due to vitamin A deficiency (VAD) across the globe was increasing. As a part of strategy, development of pro-vitamin A carotenoid-dense food crops is being prioritized.

In the last two decades, many foods (sweet potato, cassava, banana, etc) have been biofortified with pro-vitamin A carotenoids. The reports have also demonstrated substantial bioavailability during the *in vitro* experimentations. In India, maize hybrid lines biofortified with pro-vitamin A carotenoids are being developed by ICAR institutes (IARI, VPKAS, PJTSAU), as it is the third most important Indian cereal crop. However, bio-availability of vitamin A through these sources needs evaluation. In view of this, we had undertaken the investigations to assess the bioavailability of pro-vitamin A carotenoids. In the present study, the bioavailability of vitamin A carotenoids was assessed by the efficiency of micellarization of provitamin A carotenoids *in vitro* Caco-2 human intestinal cell line.

AIMS & OBJECTIVES

"Hybridization/ Genetic Modification in food crop for biofortification of β-Carotene, alters its bioavailability"

OBJECTIVES

- To estimate the carotenoid profile of selected biofortified food crops.
- To assess *in vitro* bioavailability of carotenoids in raw selected biofortified food crops.
- To determine *in vivo* bioavailability of carotenoids in selected biofortified food crops.
- To study the correlation between *in vitro* and *in vivo* bioavailability for model validation.

Work done during the year

Sample and Study Design: The institutes which had developed bio-fortified staple foods were identified, especially for maize crops. As a part of a collaborative program we received the maize hybrid seeds viz., normal maize cultivars (Madhuri Sweet Corn, DHM 121, 51014), quality protein maize (QPM) cultivars (104, 109), pro-vitamin A maize cultivars (118, 119, 120) and QPM + pro-vitamin A maize cultivars (114, 122) from Indian Agricultural Research Institute (IARI), New Delhi, Maize Research Centre and Professor Jayashankar Telangana State Agricultural University, Hyderabad. The pro-vitamin A maize and QPM + pro-vitamin A maize cultivars were selectively bred to enhance the β-carotene content in their kernels.

All the maize samples were subjected to proximate analysis including crude protein, crude lipids, moisture and carbohydrate by AOAC methods. The total carotenoids have been determined in powdered maize samples by modified method of Kurilich and Juvik, 1999 using HPLC to detect with a sensitivity of 1 ng/µL.

RESULTS

Proximate composition analysis: The proximates in normal and biofortified maize are given in Table 1, which were in line with the previously reported data. The data suggests a higher amount of moisture content in biofortified maize samples compared to the normal seed samples.

Caroteneoid content of maize geontypes: Carotenoid content of maize genotypes: Lutein (LUT), zeaxanthin (ZEA), β-cryptoxanthin (BCX), α-carotene (AC) and β-carotene (BC) were the major carotenoids in all the maize hybrids tested (i.e normal, QPM, proVA and QPM + proVA maize hybrids). The total carotenoid content in normal and biofortified hybrids of maize is given in Table 2. The highest concentration of BC (1036±13.91 µg/100g) was found in proVA biofortified Pusa-PV3 maize hybrid.

Table 1. Proximate compositional analysis of in various regular and biofortified hybrids of maize developed in India

S. No.	Genotype	Source	Moisture	Protein (DM Basis)	Total Fat
Normal					
1	BML7	IARI	5.33	1.98	8.77
2	Madhuri Sweet Corn	ANGRU	2.89	29.25	9.94
3	DHM 121		3.015	8.60	5.63
pro-vitamin A					
4	Pusa-PV2	IARI	10.43	14.67	6.73
5	Pusa-PV3		9.96	15.00	5.73
6	Pusa-PV4		7.89	15.19	7.48
QPM					
7	HM4Q	IARI	11.56	14.57	3.83
8	HQPM1		13.4	12.98	5.34
QPM+pro-vitamin A					
9	HM8Q-PV	IARI	9.57	13.69	5.30
10	HQPM4-PV		11.55	14.33	6.95

Table 2. Total carotenoid content and Retinol Equivalents (RE) of pro-vitamin A carotenoids in various regular and biofortified hybrids of maize developed in India, along with their individual percent contribution to Indian RDA of vitamin A

Hybrid	Trait	Total content of non-proVA carotenoids (µg/100g)		Total content of proVA carotenoids (µg/100g)			Ratio of non-proVA to proVA	Total RE from proVA/ 200g	% RDA of VA/ 200g
		Lutein	Zeaxan thin	ß-Crypto-xanthin	α-carotene	ß-carotene			
MS	Normal	1200.40± 19.91	766.94± 16.58	285.87± 24.06	61.99± 6.23	83.96± 7.37	4.56	119.00	20
DHM 121		329.74± 23.76	868.89± 14.44	667.54± 29.26	27.40± 12.65	89.53± 4.95	1.53	109.24	18
BML7		504.54± 10.23	2143.17± 47.00	1145.99± 21.35	41.91± 4.69	224.80± 6.46	1.87	204.68	34
HM4Q	QPM	459.19± 21.02	839.00± 23.02	232.00± 13.30	474.00± 10.61	50.50± 17.21	1.72	100.88	17
HQPM1		231.00± 27.52	136.00± 11.36	76.00± 19.21	307.00± 17.90	420.00± 18.87	0.39	152.88	25
Pusa-PV2	ProVA	209.39± 19.73	216.74± 32.18	61.00± 22.26	355.00± 15.72	759.00± 10.68	0.36	241.76	40
Pusa-PV3		349.00± 11.36	166.00± 29.52	60.00± 8.05	1084.00± 42.46	1036.00± 13.91	0.24	402.00	67
Pusa-PV4		152.00± 33.12	285.00± 21.95	112.00± 18.11	414.00± 16.92	602.00± 32.44	0.39	216.00	36
HQPM4-PV	QPM+ ProVA	133.63± 38.83	272.00± 13.77	83.00± 23.79	397.00± 34.75	766.00± 27.54	0.33	251.50	42
HM8Q-PV		814.00± 19.64	432.00± 11.57	752.00± 14.43	2046.00± 23.07	414.00± 19.71	0.39	453.26	76

The values are expressed as Mean±SD for six independent observations. The conversion ratio used for calculation of RE= 8:1 for β-carotene & 16:1 for α-carotene and β-Cryptoxanthin. RDA calculation was done using RDA for Indians, Indian Council of Medical Research, 2010, India

The concentration of BCX and AC, were highest in normal hybrid BML7 (1145.99±21.35 µg/100g) and double biofortified hybrid HM8Q-PV (2046±23.07µg/ 100g), respectively. The concentration of xanthophylls (LUT and ZEA) were maximum in Madhuri sweet corn (1200.40±19.91 µg/100g) and BML7 (2143.17±47.00 µg/100g), respectively. In general, the proVA carotenoids (BCX, AC and BC) content of biofortified maize were 2-10 fold higher compared to normal maize. The average ratio of non-proVA (LUT and ZEA) to proVA carotenoids was higher in normal maize (2.01) compared to biofortified maize (0.3).

The percent contribution of each maize hybrid to the retinol equivalents (RE) and percent contribution to the recommended dietary allowances (RDA) of vitamin A are given in Table 2. The consumption of 200 g per day of HM8Q-PV and Pusa-PV3 biofortified maize hybrids would alone contribute 76% and 67% of RDA of VA, respectively.

CONCLUSION

The results presented above indicates that biofortification of maize via marker assisted selection breeding via introgression of β-carotene hydroxylase enhances the provitamin A carotenoid content and reduces the xanthophylls. The biofortified PUSA-PV3 and HM8Q-PV maize hybrids containing high provitamin A carotenoid content provides nearly 70% and 80% of RDAs, respectively, at habitual intake levels 200 g/day.

VII. NATIONAL CENTRE FOR LABORATORY ANIMAL SCIENCES

A. SERVICES ACTIVITIES

1. Breeding and Supply of animals

During the period, a total of 22,928 animals were bred and out of which 18,093 animals were supplied to various other organizations and 2678 animals were supplied within the institute. An amount of ₹ 69,39,894/-(Rupees Sixty nine lakhs thirty nine thousand eight hundred and ninety four only) has been generated. Details of individual strains of animals bred and supplied are shown in Tables 1, 2 & 3.

2. Supply of Animal Feed

Stock Animal Feed

The stock feed of 57,200Kgs (Rat &Mouse feed 50,300 Kgs + Guinea pigs and Rabbit feed 6,900Kgs) was prepared during the period. Out of this, a total of 26,084Kgs feed (Rat & Mouse feed 24,893Kgs + Guinea Pigs & Rabbit feed 1191Kgs) was supplied to other institutions, generating an amount of ₹38,50,571/- (Rupees thirty eight lakhs fifty thousand five hundred and seventy one only). An additional 33,971Kgs of feed (Rat & Mouse feed 28,430 Kgs + Guinea Pigs & Rabbit feed 5541Kgs) was also supplied within the institute. The details of stock feed supplied are shown in Table 4 & 5.

a. Experimental Animal Feed

In addition, the centre also prepared 634Kgs of custom made experimental animal feed. Paddy husk 10kgs supplied to other institutions. An amount of ₹ 7,96,700/- (Rupees seven lakhs ninety six thousand and seven hundred only) was generated as shown in Table 6.

3. Blood and Blood Products

During the period, a total of 172ml of Blood and blood products have been supplied to 9 different institutions and also image scanning of samples from 4 different institutions had generated an amount of ₹ 1,25,450/- (Rupees one lakh twenty five thousand four hundred and fifty only) as shown in Table-7.

4. Human Resource Development

During this period, in the Junior level Laboratory Animal Technicians Training Course (LATTC), there were 15 participants who underwent training in Laboratory Animal Sciences. In the senior level Laboratory Animal Supervisors Training Course (LASTC), 11 candidates were trained. In the Ad-hoc training course, 12 candidates from different organizations were trained for a period of one week.

The Centre celebrated World Laboratory Animal Day on 24th April 2016 in association with ICMR and Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA). On this day, a one-day seminar on “Animal Welfare and Alternatives to Experimentation in Biomedical Research” was conducted. There were more than 180 delegates from private and Government Organizations who participated, including CPCSEA nominees of IAEC from various institutions. During these celebrations some of the retired staff of the NCLAS were also felicitated. Also members from local animal welfare organizations have been felicitated.

Details of Training Courses conducted by NCLAS (April- 2016 to March- 2017)

Sl. No	Type of Course and current number	Qualification & eligibility criteria	Duration	No. of Participants
1	48th Laboratory Animal Technician's Training Course (LATTC) 15th June - 29th July 2016	Undergraduate with knowledge of English read and write	6 weeks	15
2	36th Laboratory Animal Supervisor's Training Course (LASTC) 1st Sept -30th Nov 2016	Graduation in Life sciences , Medical & Veterinary sciences	3 months	11
3	Ad hoc or Modular Training Course – open throughout the year for National, International & WHO Sponsored candidates	Sponsorship from the Head of the institution	1 week	12

Table 1. Details of different species and strains of laboratory animals bred and supplied from NCLAS (April- 2016 to March - 2017)

Sl. No	Species	Strain or Breed	Stock as on	Total number of animals							Balance as on
				Bred during the period	Available	Supplied to NIN	Supplied to other institutions	Supplied Total	Died	Disp.	
1	Mouse	Balb/c An.N (in bred)	1343	5696	7039	775	4846	5621	63	-	1355
		C57BL/6J (in bred)	896	4896	5792	557	4362	4919	62	-	811
		NIH (S) Nude (in bred)	199	286	485	4	206	210	136	-	139
		NCr. Nude	162	425	587	55	75	130	136	-	321
		FVB/N (in bred)	71	32	103	-	30	30	39	-	34
		Swiss (in bred)	191	1474	1665	255	684	939	333	38	355
2	Gerbils	…	395	122	517	39	-	39	37	-	441
3	G.Pig	Dunkin Hartley (white)	518	1286	1804	-	1324	1324	104	25	351
		NIH (Colour)	58	207	265	2	40	42	53	12	158
4	Rabbit	New Zealand (white)	79	292	371	122	129	251	32	6	82
Total			3912	14716	18628	1809	11696	13505	995	81	4047

Table 2. Details of different species and strains of laboratory animals bred and supplied from NCLAS (April- 2016 to March - 2017)

Sl. No	Species	Strain or Breed	Stock as on	Total number of animals							Balance as on
				Bred during the period	Available	Supplied to NIN	Supplied to other institutions	Supplied Total	Died	Disp.	
1	Rat	CFY/NIN (inbred)	69	63	132	-	-	0	43	30	59
		Fischer 344 N (inbred)	353	78	431	14	32	46	14	70	301
		Holtzman (inbred)	80	85	165	-	16	16	26	42	81
		SD (Sprague Dawley)-Outbred	551	2303	2854	460	1666	2126	-	95	633
		Wkyoto (inbred)	92	91	183	30	-	30	21	44	88
		WNIN (inbred)	1631	3289	4920	170	3447	3617	-	-	1303
		WNIN / Gr-Ob	748	387	1135	42	-	42	51	281	761
		WNIN / Ob-Ob (inbred)	694	361	1055	93	-	93	61	203	698
		SD NIN Nude	212	133	345	-	-	0	45	90	210
2	Hamster	Golden (inbred)	230	1422	1652	60	1236	1296	82	-	274
3	Monkey	…	21	-	21	-	-	0	-	-	21
Total			4681	8212	12893	869	6397	7266	343	855	4429

Table-1 (Total)	**3912**	**14716**	**18628**	**1809**	**11696**	**13505**	**995**	**81**	**4047**
Table-2 (Total)	4681	8212	12893	869	6397	7266	343	855	4429
Grand Total	8593	22928	31521	2678	18093	20771	1338	936	8476

Table 3. Sale of animals from NCLAS
(April -2016 to March-2017)

Month	Animals																			Transport Boxes charges	Handling Charges	Transportation		Animal Sale Amount in Rs	Total Amount
	MICE						RAT									G. PIG						Road Transport	Air freight charges		
	BALB/c An.N	C57BL/6J	NIH Nude	NCr. Nude	FVB/N	Swiss	Fischer 344 N	Holtzman	Wkyoto	Sprague Dawley	WNIN	WNIN/Gr-Ob	WNIN / Ob-Ob	FVB	SD NIN Nude	D.Hartley (white)	NIH (Colour)	Hamster -Golden	RABBIT- New Zealand (white)						
April – 2016	220	101	--	8	--	15	--	--	--	--	50	--	--	--	--	--	--	--	--	300	4000	800	--	78550	83650
May-2016	556	423	--	8	--		--	--	--	120	312	--	--	--	--	110	--	216	10	26700	5850	20358	53522	442870	549300
June – 2016	547	422	16	8	--	112	--	--	--	198	580	--	--	--	--	254	--	220	20	42900	498500	32024	83204	962010	1618638
July– 2016	540	352	4	12	--	40	--	--	--	271	404	--	--	--	--	--	--	60	--	25000	10250	13320	37069	390750	476389
August– 2016	710	150	--	16	--	195	--	--	--	250	22	--	--	--	--	200	--	100	25	13300	8750	4082	3666	579200	608998
Sept– 2016	464	644	4	15	--	97	--	--	--	50	256	--	--	--	--	169	--	154	20	19602	7250	20880	49557	516800	614089
Oct– 2016	230	371	46	--	--	--	--	--	--	337	351	--	--	--	--	183	--	36	8	11300	9500	10566	22649	522650	576665
Nov – 2016	180	439	48	--	30	--	--	--	--	--	347	--	--	--	--	--	--	40	20	11700	12500	9234	23523	298100	355057
Dec– 2016	320	388	--	8	--	--	32	16	--	36	106	--	--	--	--	330	40	160	10	20800	7000	16855	41871	611100	697626
Jan – 2017	30	189	50	--	--	--	--	--	--	140	76	--	--	--	--	--	--	70	--	500	--	--	--	172150	172650
Feb – 2017	667	379	20	--	--	205	--	--	--	167	567	--	--	--	--	70	--	172	4	41700	4750	28324	74554	619500	768828
March– 2017	382	504	18	--	--	20	--	--	--	97	376	--	--	--	--	8	--	8	12	25500	3250	18990	49514	320750	418004
Grand Total	**4846**	**4362**	**206**	**75**	**30**	**684**	**32**	**16**	**--**	**1666**	**3447**	--	--	--	--	**1324**	**40**	**1236**	**129**	**239302**	**571600**	**175433**	**439129**	**5514430**	**6939894**

Table 4. Stock feed supplied from NCLAS (April-2016 to March–2017)

Month	(A) Diet formulated protein %		(B) Govt. Supply Protein %		(C) Private supply protein %		(D) Internal Supply Protein %	
	20 %	14 %	20 %	14 %	20 %	14 %	20 %	**14 %**
April-2016	3050	660	976	45	56		2248	**408**
May 2016	3950	600	1520	215	103	10	2552	**503**
June 2016	3400	420	505	--	303	--	2830	**408**
July 2016	4650	540	2750	4	50	--	2240	**399**
August 2016	4150	540	1670	--	176	3	2749	**612**
September 2016	4600	900	2604	165	300	2	2194	**606**
October 2016	3450	540	790	--	450	--	2334	**513**
November 2016	4250	600	1630	52	95	--	2207	**437**
December 2016	5200	600	2925	--	85	20	2415	**384**
January 2017	4300	540	1510	400	505	--	2537	**376**
February 2017	3000	480	1573	--	100	10	2152	**405**
March 2017	6300	480	2220	250	357	--	1972	**490**
Total in Kgs	50300	6900	20673	1131	2580	45	28430	**5541**
Grand Total	**57200**		**21804**		**2625**		**33971**	

Tables 5. Sale of feed from NCLAS (April - 2016 to March- 2017)

Sl. No	Month	Feed			Paddy husk Kgs	Transport charges Rs.	Handling Charges Rs.	Sale of feed Amount in Rs.	Total Amount in Rs.
		Rat, Mouse & Hamster feed in Kg (20% Protein)	Rabbit, G.Pig& Monkey feed in Kgs (14 % Protein)	High fat diet (Special) - Kgs					
1	April 2016	1032	45	101	--	17845	1250	245000	264095
2	May	1623	225	10	--	42012	1100	260750	303862
3	June	1108	15	90	--	15790	2050	262400	280240
4	July	2800	4	--	--	2659	2250	392400	397309
5	August	1846	3	125	10	40589	2450	457560	500599
6	September	3104	167	30	--	20159	2550	502250	524959
7	October	1240	100	7	--	8628	850	198730	208208
8	November	1725	52	245	--	55043	2700	507200	564943
9	December	3010	20	20	--	5007	2850	447170	455027
10	January 2017	2015	450	--	--	36926	2300	307650	346876
11	February	1673	10	6	--	20413	2500	241290	264203
12	March	3717	100	--	--		3000	533950	536950
Grand Total		24893	1191	634	10	265071	25850	4356350	4647271

Table 6. Special feeds / diets formulated and supplied from NCLAS (April 2016 - March 2017)

Si. No	Date	Bill No	PARTY	Type of Diet	Quantity Kgs	Amount Rs
1	07.04.2016	5470	Ms. NishaSinha, Panjab Univ. Chandigarh	HFD	1	2260
2	18.04.2016	5479	Dr.IshanPatro, Jiwaji University, Gwalior	8% Protein	50	22000
3	18.04.2016	5481	Dr. Banappa Unger, RMRC, Belagavi	HFD	50	73500
4	26.05.2016	5517	Dr. Jayasree, CCMB	High Salt Diets 2no.	10	9800
5	03.06.2016	5530	Dr. R.Ravindran,Univ. of Madras, Chennai	HFD	25	36750
6	17.06.2016	5553 5693	Director,MDRF, Chennai	HFD	40	64600
7	27.06.2016	5571	M/s Natreon Inc. Kolkata	HFD	25	45500
8	11.08.2016	5675	Director, NRIADD,Kolkata	HFD	50	101000
9	24.08.2016	5694	Director,MDRF, Chennai	HFD	50	80750
10	26.08.2016	5699	Dr.IshanPatro, Jiwaji University, Gwalior	8% Protein	25	13000
11	28.09.2016	5737	Director, MDRF, Chennai	HFD	30	48450
12	27.10.2016	5779	Prof.AllamAppaRao Univ. Of Hyderabad	HFD	7	11130
13	09.11.2016	5794	Dr.IshanPatro, Jiwaji University, Gwalior	8% Protein	150	78000
14	15.11.2016	5799	Director,MDRF, Chennai	HFD	30	60600
15	25.11.2016	5815	Dr.NHBalasinor,NIRRH Mumbai	6% Fat 22% Fat	35 30	65450 55500
16	01.12.2016	5822	Dr.Monita M.,St.Johns Res. Institute,Bengaluru	Iron Defi. Diet	10	7120
17	06.12.2016	5826	Dr.UmeshYadav,Central University, Gujarat	HFD	10	15900
18	22.02.2017	5916	Dr.DipayaChoudhuri, Tripura Univ. Tripura	Control Ca Deficient High Calcium	2 2 2	4890
	Total				634	7,96,200

Table 7. Sale of blood & blood products from NCLAS (April-2016 to March-2017)

Date	Bill No	Name of the Institute	Blood/ Plasma	Quantity in ml	Handling charges & Packing charges	Amount	Total
APRIL-2016							
15	5187	Mylan Laboratories Ltd., Hyderabad	Rat Plasma	20	1000	6000	7000
		Total		20	1000	6000	7000
MAY-2016							
4	5189	Dr.V.Sritharan, Global Medical Education & Res. Foundation Hyd.	Rabbit Plasma	4	300	1600	1900
5	5490	Director, Directorate of Poultry Res. Hyderabad	Rabbit Blood	10	1300	1000	2300
	Total			14	1600	2600	4200
JUNE-2016							
NIL							
JULY-2016							
1	5577	M/s GVK Bio Sciences Pvt Ltd, Hyd	Monkey Plasma	10	800	4000	4800
19	5596	M/s Aurigene Discovery Technologies, Hyderabad	Monkey Blood	80	1800	20000	21800
25	5198	Project Director, NIPER, Hyderabad	IVIS Scanning				4000
		Total		90	2600	24000	30600
AUGUST-2016							
2	5601	M/s GVK Bio Sciences Pvt Ltd, Hyd	Mice-Plasma	10	800	3000	3800
4	5665	M/s NatcoPharma Ltd., Hyderabad	IVIS imaging of samples (4000 x 8 No's)				32000
10	5671	Mrs.AneesFathima, G. Pulla Reddy College of Pharmacy, Hyderabad	Evaluation of Tissue Samples, Photos and Posures (250x7Nos, 50x9nos)				2200
24	5692	Dr. DR. K Gouthamrajan, JSSCP, Ooty, HCU, Hyderabd	C57Bl/6J Plasma	5	--	--	750
25	5696	M/s Tergene Biotech Pvt Ltd	Serum	10	2500	--	6500
	Total			25	3300	3000	45250
SEPTEMBER- 2016							
8	5607	Ms.Pamu Sandhya, M/s Shadan Womens College of Pharma, Hyderabad	Plasma	3	750	1200	1950
	Total			3	750	1200	1950
OCTOBER- 2016							
1	5783	Directorate of Poultry Research, ICAR, Hyderabad	Blood	10	1300	1000	2300

Table 7. Sale of blood & blood products from NCLAS (April-2016 to March-2017) (Contd..)

Date	Bill No	Name of the Institute	Blood/ Plasma	Quantity in ml	Handling charges & Packing charges	Amount	Total
2	5784	Dr Hrudayesh Prakash, University of Hyderabad	Histo pathology Scanning	27 samples	--	12150	12150
Total				37	1300	13150	14450
NOVEMBER- 2016							
NIL							
DECEMBER- 2016							
16	5844	M/s Tergene Biotech Pvt Ltd., Hyderabad	Serum	10	2500	4000	6500
Total				10	2500	4000	6500
JANUARY-2017							
NIL							
FEBRUARY - 2017							
NIL							
MARCH- 2017							
30	5975	The Director, CSIR-CCMB, Hyderabad	IVIS imaging of samples (2000 x 3 No's)				6000
31	5978	Dr. B. Bhima, Dept of Microbiology, OU, Hyderabad	Histopathology Scanning	52	--	9500	9500
Total				52	--	9500	15500
Grand Total				172	12300	62250	125450

RESEARCH ACTIVITIES

1. EFFECT OF FICUS RACEMOSA BARK METHANOLIC EXTRACTION ON FERTILITY RESTORATION IN WNIN OBESE FEMALE RATS

Body weight reduction and physical exercise have been shown to help obese women with infertility. We studied the effect of Hesperidin and Chlorogenic phenolic compounds present in *Ficusracemosa* bark methanolicextraction (FRBME) in WNIN/GR-Ob obese infertile rats.

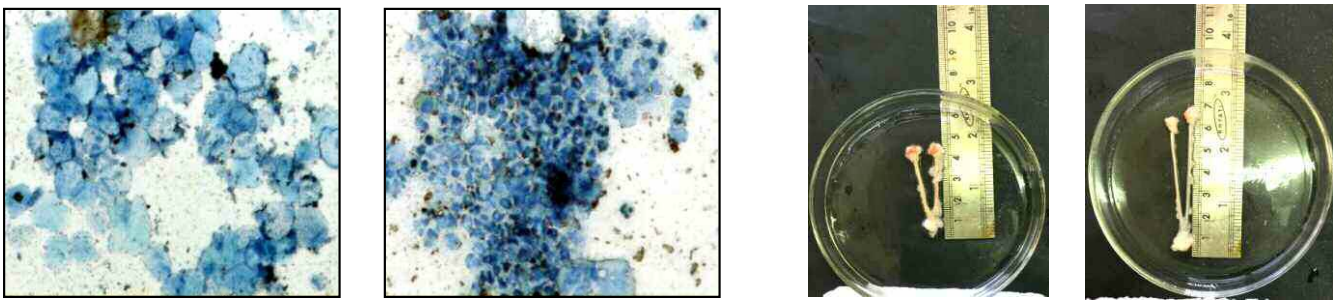
METHODS

24 obese female obese rats of 35 days (12) and 60 days (12) of age were taken for the study and divided into four groups. Group I, III were controlled and group II, IV rats received 200 mg of FRBME for a period of 30 days and 60 days under meal feeding training program. Body composition was determined by the total body electrical conductivity (TOBEC). Ex vivo biochemistry in blood was carried for lipids and reproductive hormones and histology by haematoxylin-eosin stain. Ovaries and uterine horn morphology was studied by scanning electron microscope (SEM).

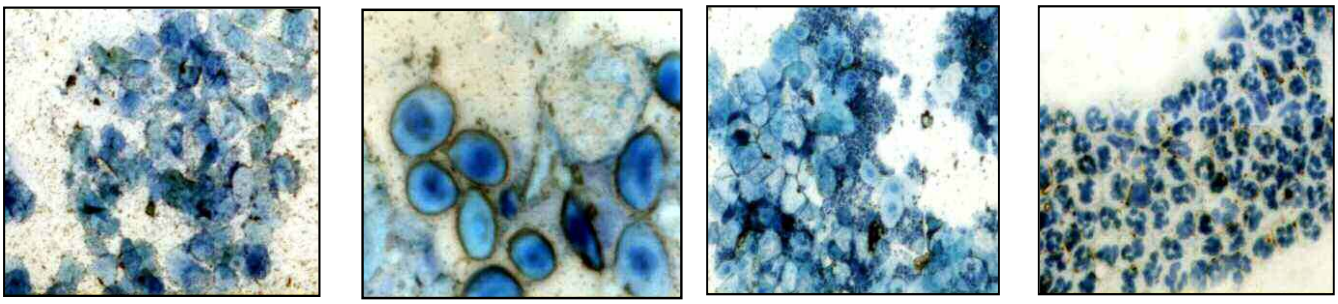
RESULTS

- Administration of FRBME has been shown to advance the age of the puberty, and it restored regular estrus cycles in obese rats (Fig. 1).
- TOBEC analysis showed a significant increase in lean body mass and reduced body fat in the treated rats.
- Glucose, insulin and lipid levels were significantly reduced and the characteristic peak of estrogen and progesterone was restored.
- Uterine horn length increased and SEM analysis showed the presence of corpora lutea in the ovaries (Fig. 2).

Fig 1. Estrus cycle restoration and increase in length uterine hornes in *F. racemosa* treated obese rats



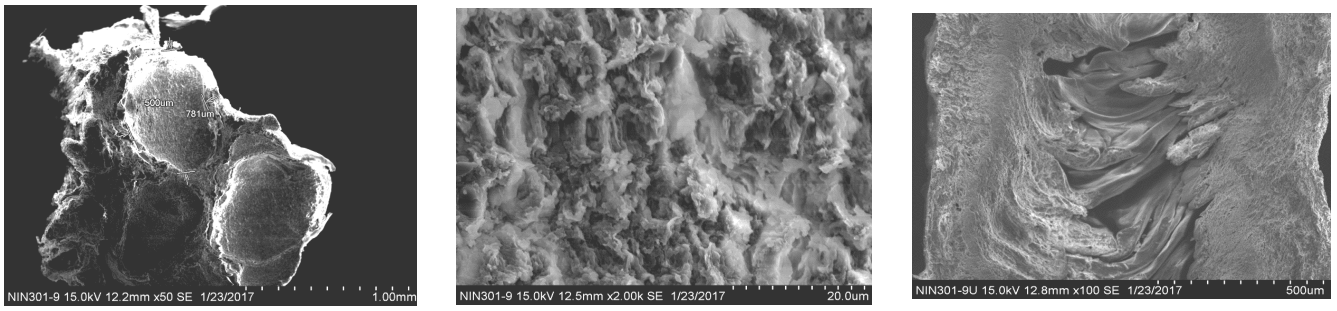
Control (only two stages) Uterine horn length



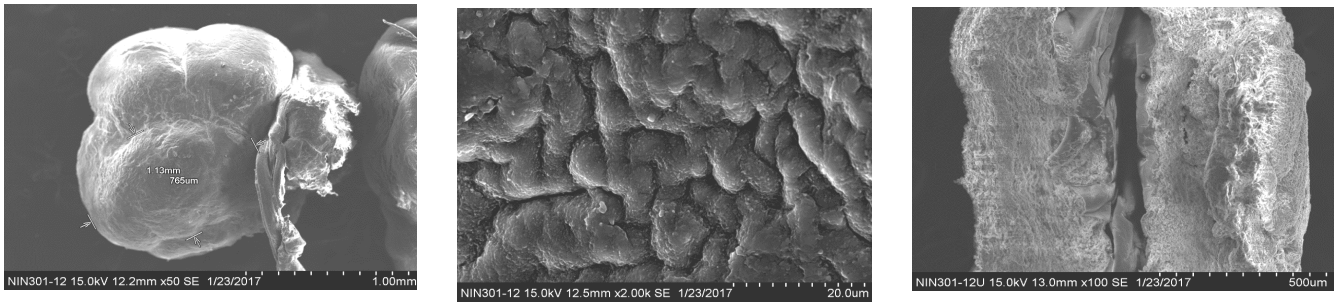
Treated with *F. racemosa* (All four stages were observed)

Fig 2. SEM analysis of ovaries of control and *F.racemosa* treated WNIN obese rats

Control D



F.racemosa treated



CONCLUSION

In this study, FRBME improved the insulin resistance, reproductive indices and also prevented the cystic carporaluteae and congestion of ovaries, in WNIN obese rats. The phenolic compounds like Hesperidin and Chlorogenic present in *F.racemosa*, can be used as a new therapeutic molecules for the reproductive health of obese women.

2. STUDY OF TONGUE MUSCLE FAT IN FILTRATION WNIN OBESE, CALORIE RESTRICTED OBESE AND LEAN RATS AND ITS RELATIONSHIP WITH OBSTRUCTIVE SLEEP APNEA

Obstructive sleep apnea has been linked to obesity, which results from abnormal upper airway structures. The main objective was to see the fat infiltration into the tongue of calorie restricted rats and its consequences on upper airway obstruction in comparison with lean and *ad lib* fed obese rats.

METHODOLOGY

A total of 18 adult Lean (223.33 g ±20.42), Obese (584.22 g ± 5.65) and calorie restricted (303.00 g ± 16.97) obese rats were taken for the study. Body fat and LBM was determined by TOBEC, radiographs of upper airway structure were analyzed by digital X-ray. Ex-vivo biochemistry was carried for leptin, and lipids in blood/tissues, and fat infiltration by ORO. The tongue, masseter muscle fat, fibre and taste buds morphology was assessed by SEM.

RESULTS

- TOBEC analysis showed higher body fat and low LBM, X-ray displayed excess fat in the neck region (Fig. 1).
- TG levels were high and leptin levels were reduced in CR rats compared to lean and ad lib fed obese rats.
- ORO staining of tongue showed fat accumulation of varying degree in different groups (Fig 2).
- SEM analysis demonstrated an increase in fat infiltration with an increase in fat droplets (Fig. 3).
- Tongue muscle fibres showed rugged abnormal folding, with an increase in width in obese rats. The distance and width between the taste buds of CR reduced to levels of lean rats (Fig. 3)

Fig 1. X-ray photographs of the whole body of lean, obese and calorie restricted WNIN rats.

(a) Dorsal view, (b) Lateral view and (c) View up to thoracic

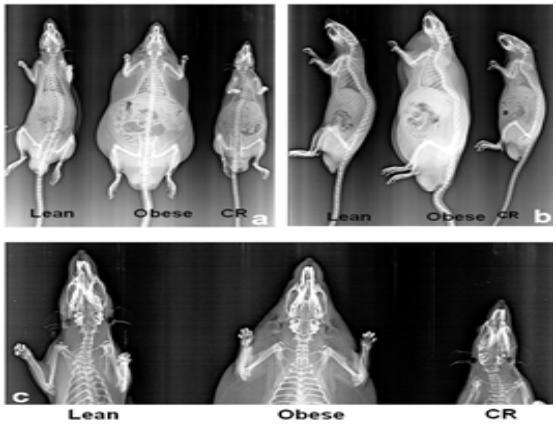


Fig. 2 Photo micrographs of H&E stained tongue and masseter muscle of lean *(a. Tongue and b. masseter at 10X magnification), obese (c. Tongue and d. masseter at 10Xmagnification) and calorie restricted rats (e. Tongue and f. masseter at 10Xmagnification).2-II. Fat infiltration photo micrographs (LS) of ORO stained tongue lean (a. at 20X and b. at 40X magnifications), obese (c. at 20X and d. at 40Xmagnifications) and calorie restricted obese rats (e. at 20X and f. at 40X magnifications). 2-III. Fat infiltration photo micrographs (LS) of ORO stained masseter muscle of lean (a. at 20X and b. at 40 X magnifications), obese (c. at 20X and d. at 40Xmagnifications) and calorie restricted obese rats (e. at 20X and f. at 40Xmagnifications).*

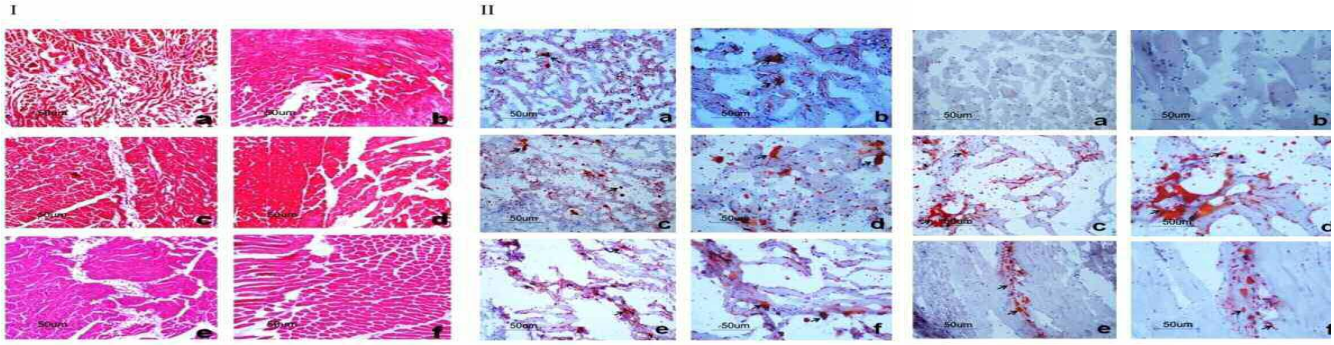
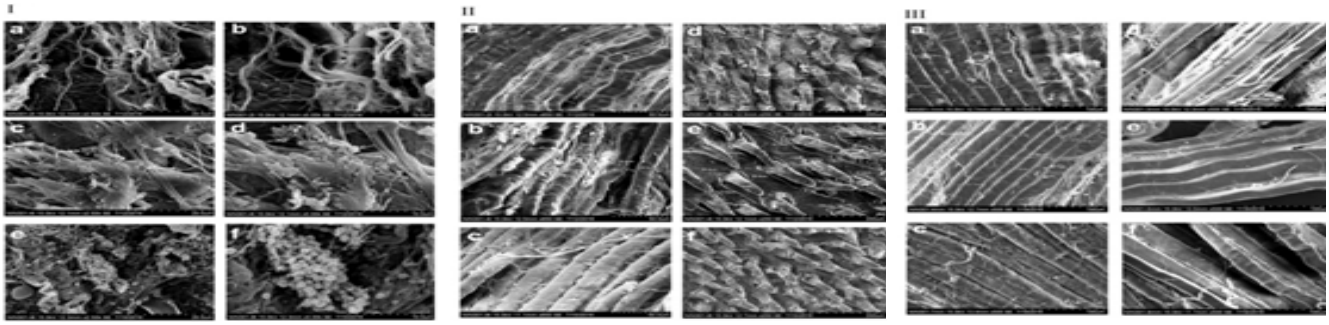


Fig.3 Fat infiltration micrographs of tongue in lean *(a. at 2.50K and b. at 5.0 K magnifications), obese (c. at 2.50K and d. at 5.0 K magnifications) and calorie restricted obese rats (e. at 2.50K and f. at 5.0 K magnifications) by scanning electron microscopy (Longitudinal section). 3-II.Photo micrographs of tongue fibres and taste buds of lean, obese and calorie restricted obsess rats by scanning electron microscopy (Longitudinal section). (a, b, c tongue fibers of lean, obese and calorie restricted rats at 650SE magnifications and d, e, f taste bud morphology of lean, obese and calorie restricted rats at 250SE magnifications) respectively.3-III. Fat infiltration (a, b, c) and fibre width (d, e, f) micrographs of masseter muscle in lean, obese and calorie restricted obese rats at 500SE magnification respectively.*



CONCLUSION

The calorie restriction in WNIN/Ob obese rats improved the upper airway structure morphology, increased stiffness of the tongue and lung volume. Calorie restriction can be used as non-pharmacological intervention therapy for obesity associated OSA.

3. SEQUENCING, CONFIRMATION AND FUNCTIONAL ANALYSIS OF OBESITY MUTANT GENE IN WNIN / OBESE RAT

The National centre for Laboratory Animal Sciences (NCLAS) situated at National Institute of Nutrition has been maintaining one of the oldest Wistar rat strains, in the world (over 90-100 generations) dating back to 1918. From this rat stock of WNIN (inbred Wistar strain maintained at NIN), we isolated and established an obese mutant rat designated as WNIN/Ob, which showed all the traits of obesity in terms of physical, biochemical and behavioural factors.

These rats had unique characteristics such as a 'kinky' tail, incomplete dominant inheritance and other physical and biochemical traits. The known fa/fa and cp/cp mutations contributing to Zucker and Koletsky mutant obese rats had a defect at leptin receptor loci. However in WNIN/Ob rat strain, as per the present evidence available, the mutation appears to be at a new locus. Much of the last decade's progress in obesity research is attributable to the cloning of obesity genes from the experimental models available and it is expected that the present new mutant gene may add to our understanding of the pathogenesis of obesity.

A genetic dissection work to find out the gene mutation responsible for the development of obesity in this mutant was undertaken. Crosses were made between WNIN/Ob and Fisher-344 (un related strain) and F2 population was generated. The F2 population (N=320) along with parental lines were genotyped using SSLP markers (according to Rat Genetic Database), and the mutation was found to be on chromosome number 5. The preliminary data on genome analysis resulted in the identification of a highly polymorphic region on chromosome number 5 which spanned about 54 Mb. Further fine mapping identified the body weight QTL spanning 4.4cM (manuscript communicated for publication). The gene expression, sequencing of differentially expression genes along with their promoters DNA sequences of this body weight locus would lead to the identification of actual genetic mutation causing obesity in WNIN/Ob rats.

OBJECTIVES

- Differential gene expression of genes located in body weight QTL (completed)
- Candidate gene (s) and their promoter DNA Sequencing
- Identification and validation of mutation causing obesity

METHODOLOGY

Animal Experiment: Obese trait in the WNIN/Ob animals is an incompletely dominant allele and obese progeny can be generated by mating heterozygous carriers as obese male and female animals are infertile. Heterozygous carriers were obtained from WNIN/Ob colony established in the Animal facility of the Institute. These animals were selectively bred to raise Obese, carriers and lean animals in each generation. After 2 generation of selective breeding, 5 male Obese animals along with their lean counter parts were chosen for expression studies. Their body

weights were monitored.These animals were sacrificed at 3months of age and tissues were collected and stored at -80^{0}C for further studies.

Gene Expression Studies: These 51 known genes in the identified QTL region which include Leptin Receptor, Leptin Receptor Overlapping Transcript, Janus Kinase1, have been reported to be a candidate for obesity. Of the 61 genes, 10 are hypothetical genes whose function is yet to be known. The genes which are closely linked to D5WOX37 were identified and considered for the expression studies. Primers were designed as per the number of exons of each gene.

The tissues collected from the above animal groups were used for expression studies. Hypothallamic region was separated from the brains and RNA was isolated from different tissues using Trizol method. Obese animals (N=6) and lean animals (N=6) were used for the study. RNA was converted to cDNA using Oligo dT as priming source. Such cDNA is used for Expression analysis. Real Time PCR (BioRad CFX 1000) was performed to compare the expression of the genes in the two experimental groups. The results are presented in relative fold change of expression.

Sequencing: The differentially expressed genes will be identified and sequenced along with their promoter region.

Identification and validation of mutation: The identified SNP will be validated in mapping population and randomly selected WNIN/Ob and lean colony animals.

RESULTS

It was identified that the Obese mutants tend to show increased body weight at an age as early as 21days when compared to their Lean litter mates. The increased body weight gain in Obese mutant rats continued till 90 days of experimentation (Fig-1).

Gene expression studies: The body weight QTL gene expression studies in hypothalamus showed that of all the genes, only two genes showed a 2+ fold change up regulation (Table 1). A majority of genes showed a down regulation trend. Interestingly, lowest trend was seen in JAK1, which is closely linked to leptin receptor and it also is a part of JAK-STAT pathway in brain. It was noticed that POMC, an anorexic gene, not in the QTL region shows a very high up regulation (Fig 2).

Gene expression studies in adipose and liver: Gene expression studies were carried out in liver and adipose tisuue based on the leads from the hypothalamus studies. But none of the genes showed significant changes in gene expression (Table 2 & 3). Most of the genes showed the down regulation trend in liver, whereas, in adipose it was not observed.

Fig 1. Weekly Body Weight Gain Pattern

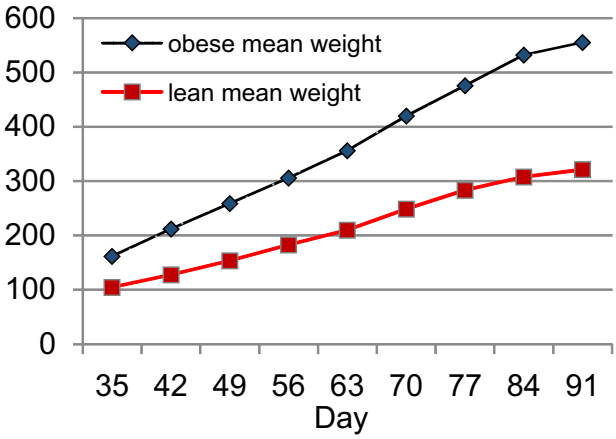


Table 1. Gene expression in hypothalamus

S. No	Gene	Expression in Obese	Fold Change
1	LepR_3-4	↓	0.23
2	LepR_12	↓	0.05
3	LepR_B	↑	2.58
4	LepRot	↑	0.58
5	JAK1	↓	0.9
6	AK4	↓	0.5
7	EFCAB7	↓	0.1
8	ATG4C	↓	0.5
9	USP1	↓	0.5
10	DNAJC6	↑	0.62
11	RAVER	↑	0.9
12	LOC100359934	↑	3.44
13	ROR1	↑	0.74
14	Inadl	↑	0.8
15	FGGY	↑	0.81
16	HOOK1	↑	2.14
17	NPY	↑	0.57
18	POMC	↑	151.41

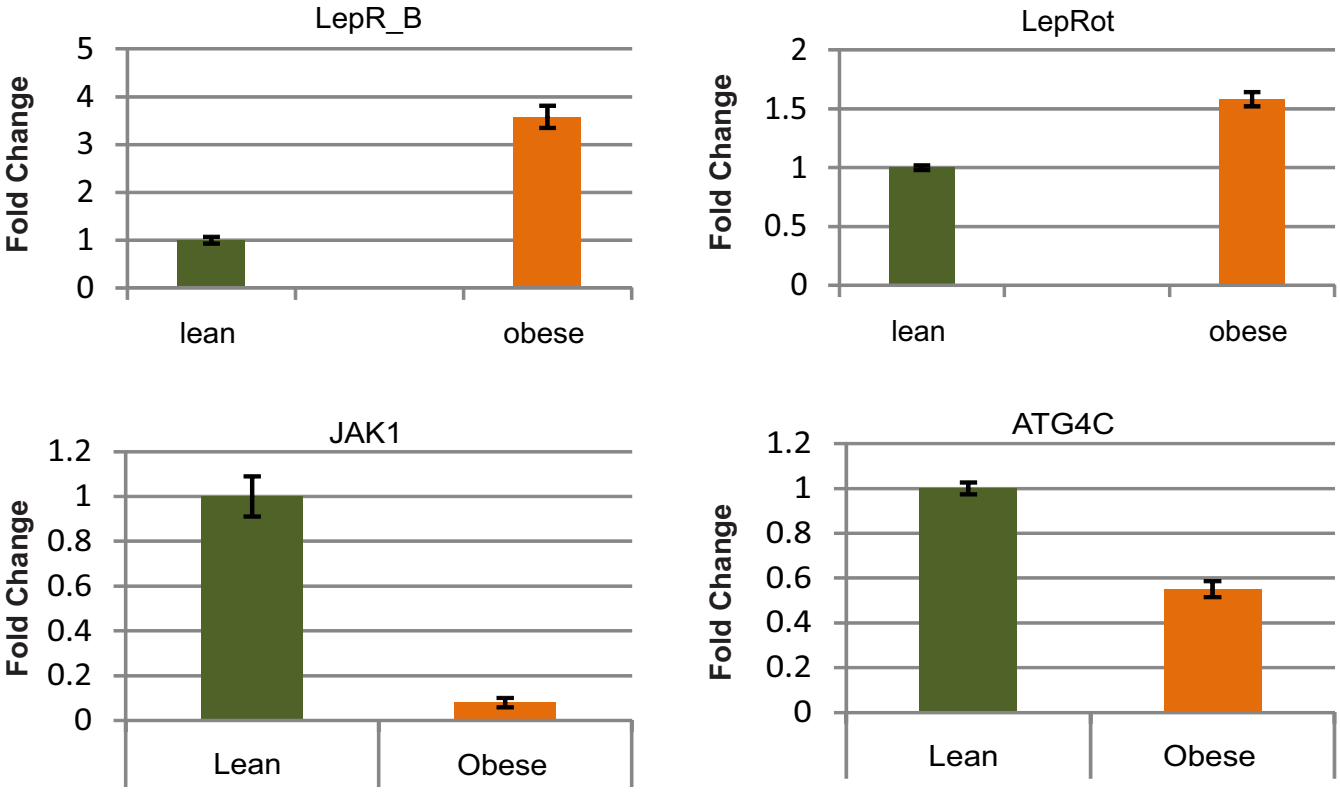
Table 2. Gene expression in adipose tissue

S. No	Gene	Expression in Obese	Fold Change
1	LepR_B	↓	0.65
2	JAK1	↓	0.36
3	ATG4C	↓	0.38
4	LOC100359934	↓	0.20
5	USP1	↑	2.86

Table 3. Gene expression in liver tissue

S. No	Gene	Expression in Obese	Fold Change
1	LepR_B	↑	0.12
2	JAK1	↓	0.05
3	AK4	↑	1.18
4	ATG4C	↑	0.02
5	LOC100359934	↑	0.74
6	FGGY	↑	0.52
7	HOOK1	↓	0.18

Fig 2. Gene expression studies of hypothalamus



CONCLUSIONS

- In the initial screening of QTL genes, 25 genes were studied in hypothalamus known to influence the food intake of the animal. The genes which showed differential gene expression were screened in Liver and Adipose tissue.
- Gene expression in Liver and adipose did not show significant difference in expression of genes in the QTL region.
- In hypothalamus, the genes closely linked to D5WOX37 showed differential expression.

The differentially expressed genes found to span in a genomic region of 200 kb. These genes have to be sequenced along with their promoter region.

4. BIOCHEMICAL, IMMUNOLOGICAL CHARACTERIZATION OF SPRAGUE DAWLEY/ NIN HAIRLESS MUTANT RAT DERIVED FROM SD/NIN STRAIN

The hairless rats were observed in Sprague Dawley/ NIN out bred rat colony maintained at NCLAS, National Institute of Nutrition. The parents were identified, isolated and further propagation was carried out by selective breeding methods. The morphological features, breeding details in each generation were recorded. The distinct feature of the nude mutant rat is absence of thymus. To know the presence or absence of thymus gland nude rats were dissected and it was noticed that these rats had thymus gland. The hairless character is known to be hereditary in laboratory animals. These models offer several advantages for experiments in dermatology and pharmacology. Hence, there is a need to analyze various parameters including morphological genetical, biochemical and immunological characters of this mutant rat.

The hypotrichosis is a heritable character in this mutant. The factors and mechanism involved in preventing normal hair development needs to be identified. Therefore it was proposed to characterize the new SD/ NIN hr (hairless) rat in terms of their physical, physiological biochemical, immunological, molecular characterization to establish these parameters in new rat model.

MATERIALS AND METHODS

24 rats of both sex 60 - 70days old were taken from SD/ NIN hr. colony after taking approval from IAEC. Each group consist of six animals.
Group 1 : hairless females (homozygous)
Group 2 : had females with hair (heterozygous)
Group 3 : males without hair (homozygous)
Group 4 : males with hair (heterozygous).

The rats were housed in individual cages 30gms of standard stock diet was given/day and water was provided ad libitum. A temperature of 22+2^{0}C and relative humidity of 45-55 percentage,12 hrs light and dark cycle was maintained in the in the room. The study was carried out for a period of one month to determine the growth pattern of these rats. Weekly body weights and daily food intake of each animal was recorded. Blood samples were collected from retro orbital sinus puncture under mild anesthesia for clinical, biochemical and immunological parameters. Physical parameters like body composition and bone mineral contents were measured by TOBEC and DEXA. At the end of the study, rats were euthanized and gross necropsy was studied and major tissues were given for histopathological examination.

Biochemical Estimation

Total cholesterol, triglycerides, bilirubin, urea, albumin, alkaline phosphatase, SGOT, SGPT, creatinine and total protein determined in plasma by autoanalyser method (AC ALERA).

Body Composition

Body composition of experimental animals were determined by using total body electrical conductivity TOBEC specific for small animal body composition analysis (Model SA 3000 multi detector, EMSCAN, spring field-II).

Body composition and bone density was estimated by dual energy x-ray absorptionmetry (DXA) which is a non-invasive technique for the determination of body composition like lean tissue, fat mass and bone mineral density.

RESULTS

The body weights were not significantly different in hairless and normal animals. However the food intake was higher in hairless rats, but showed lower body weights compared to normal heterozygous rats. (Table 1). Plasma albumin, creatinine, bilirubin, total protein. SGPT and SGOT did not show much variation in all the four groups studied.

The glucose values in female hairless were less when compared with other groups. The levels in hairless female were significantly lower compared to its normal counterpart. The Hairless male showed higher glucose levels when compared to other groups. This group of rats showed significantly higher values compared with normal male rats. The triglyceride values are similar in both sex of hairless rats but are significantly lower when compared to normal rats. The female hairless rat showed higher levels of urea than other groups the hairless stain of rats showed higher values of urea when compared to their normal counterparts. The Alkaline phosphatese and total cholesterol levels were significantly different than other groups.

The vital organs brain, heart, lungs, liver, spleen, kidneys, sex organs and thymus were evaluated for histopathology. No abnormal lesions were observed and a few lesions were seen in lungs and liver that are common to colony bred rats.

Table 1. Food intake and body weight of SD/NIN hr mutant rat (n=6; mean ±SE)

Group	1st week		2nd Week		3rd week		4th week	
	Food intake (gm)	**Body Weight** (gm)	**Food intake** (gm)	**Body Weight** (gm)	**Food intake** (gm)	**Body Weight** (gm)	**Food intake** (gm)	**Body Weight** (gm)
Hairless Female	22.75 ± 0.63*	248.00 ± 1.00	22.06 ± 0.39*	255.41 ± 3.34*	22.18 ± 0.57*	262.29 ± 7.80*	23.14 ± 0.41*	264.78 ± 7.04*
Normal Female	15.76 ± 0.80	271.00 ± 3.46*	17.54 ± 0.17	281.08 ± 2.95	15.48 ± 0.43	289.45 ± 7.06	17.26 ± 0.12	294.80 ± 7.00
Hairless Male	27.06 ± 0.94*	376.00 ± 5.30*	26.99 ± 0.37*	381.69 ± 4.17*	27.27 ± 0.36*	389.37 ± 9.40*	27.29 ± 0.51*	395.27 ± 9.70*
Normal Male	19.36 ± 0.67	384.00 ± 5.70	19.51 ± 0.47	420.65 ± 1.89	19.28 ± 0.68	425.57 ± 3.37	22.31 ± 0.56	431.26 ± 3.51

Table 2. The Haematological profile of SD/NIN hr mutant rat (n=6; mean ±SE)

Group	WBC	RBC	HCB	HCT	MCV	MCH	MCHC	PLT	Neutro	Lymphocytes	Monocytes	Eosmpohils	Basopl us
Hairless Female	6.35 ± 0.33	9.66 ± 0.13	16.78 ±0.21	43.85* ±0.62	48.41* ±0.52	18.56* ±0.12	38.38* ±0.27	989.50 ±55.42	24.67 ±1.2	70.67 ±1.14	2.67 ±0.51	1.83 ±0.67	0
Normal Female	6.58 ±0.32	9.06 ±0.27	16.51 ±0.37	42.9* ±0.70	47.86* ±0.98	18.83* ±0.22	39.58* ±0.40	964.83 ±19.85	24.00 ±2.2	69.33 ±4.55	2.50 ±0.54	2.0 ±0.44	0
Hairless Male	7.00 ±0.20	9.13 ±0.08	23.30 ±7.5	42.53* ±0.24	45.86* ±0.62	18.16* ±0.13	39.45* ±0.12	1010.1 ±31.43	23.00 ±0.89	71.00 ±1.2	28.3 ±0.75	1.67 ±0.21	0
Normal Male	6.47 ±0.25	9.17 ±0.08	16.13 ±0.27	41.78* ±0.28	46.33* ±0.33	18.30* ±0.18	39.23* ±0.07	1052.0 ±48.95	24.17 ±1.7	68.6 ±2.3	2.50 ±0.54	2.50 ±0.22	0

**Values were significantly different from experimental group animals. ($p<0.05$)*

Table 3. The SD/NIN hr mutant rat Biochemical profile
Clinical Chemistry parameters (n=6;mean ±SE)

S.No	Parameters	Hairless Female	Normal Female	Hairless Male	Normal Male
1	Glucose (mg/dl)	93.00 ± 2.25*	112.33 ± 9.92	120.83 ± 5.09*	105.20 ± 1.24
2	Billirubin (mg/dl)	0.235 ± 0.016	0.206 ± 0.012	0.223 ± 0.019	0.235 ± 0.021
3	Total Protein (g/dl)	6.44 ± 0.058	6.31 ± 0.109	6.45 ± 0.124	6.80 ± 0.201
4	Triglycerides (mg/dl)	27.33 ± 0.715*	35.33 ± 1.14	27.33 ± 2.71*	32.60 ± 1.74
5	Urea (mg/dl)	47.33 ± 3.40*	38.17 ± 3.07	38.17 ± 2.27*	34.60 ± 1.47
6	Albumin (g/dl)	4.013 ± 0.033	3.78 ± 0.098	3.25 ± 0.546	3.82 ± 0.156
7	ALP (µu/dl)	115.17 ±10.41*	91.83 ± 10.59	117.17 ± 8.7	119.20 ± 9.23
8	SGPT (µu/dl)	28.00 ± 2.29	24.50 ± 1.76	31.17 ± 3.28	30.00 ± 2.14
9	SGOT (µu/dl)	40.50 ± 2.17	40.17 ± 3.19	39.83 ± 4.14	40.35 ± 1.50
10	Total Cholesterol (mg/dl)	73.83 ± 1.24	71.17 ± 2.33	56.83 ± 1.04	55.60 ± 1.74
11	Creatinine (mg/dl)	0.515 ± 0.032	0.588 ± 0.015	0.576 ± 0.049	0.566 ± 0.018

**Values were significantly different from experimental group animals. ($p<0.05$)*

Table 4. Bone mineral density (DEXA) of SD/NIN hr rats (n=6 mean±SE)

Parameter	Hairless Female	Normal Female	Hairless Male	Normal Male
B.Wt	274.2 ± 40.36	295.31 ± 16.52	420.2 ± 38.36	437.86 ± 21.41
LBM	215.5 ± 16.57	228 ± 13.23	314.67 ± 23.31	331.6 ± 10.43
Fat	59.13 ± 14.31	59.3 ± 8.46	95.48 ± 16.59	95.56 ± 11.34
Fat%	22.33 ± 3.55	20.5 ± 2.42	23.17 ± 2.3	22 ± 1.73
BMC	8.19 ± 0.81	8.51 ± 0.45	10.36 ± 0.55	11.14 ± 0.72
BMD	0.158 ± 0.004	0.16 ± 0.004	0.17 ± 0.012	0.174 ± 0.007

Table 5. Body Composition of SD/NIN hr rat (n=6 mean±SE)

Parameter	Hairless Female	Normal Female	Hairless Male	Normal Male
B.Wt	281.8 ± 7.08	307.83 ± 3.53	429.0 ± 37.3	437.53 ± 11.3
LBM	266.97 ± 4.01	282.59 ± 4.78	407.5 ± 21.5	404.0 ± 6.3
Fat	14.82 ± 4.0	25.23 ± 3.97	21.46 ± 5.5	33.79 ± 6.4
Fat %	5.14 ± 1.29	8.18 ± 1.28	5.29 ± 1.6	7.69 ± 1.4
TBN	579.09 ± 7.05	602.54 ± 12.7	868.31 ± 55.9	849.17 ± 17.08
TBNA	961.26 ± 11.75	1000.3 ± 21.32	1443.3 ± 93.3	1411.4 ± 28.4
TbK	1970.47 ± 52.03	2047.85 ± 42.21	2924.89±184.7	2861.7 ± 56.3

CONCLUSIONS

- The mutant rats had thymus gland which is absent in most of the nude strains of rats
- Body weights were similar in hairless and normal rats.
- Significantly higher food intake was observed in hairless rats.

- Clinical parameters like serum albumin alkaline phosphates, creatinine, bilirubin, total protein and triglycerides did not show variation in all four groups.
- Glucose showed variation among both male and female hairless rats.
- Increase in serum urea levels was observed in both sexes of hairless rats.

ALP and total cholesterol levels was significantly high in hairless females compared to other groups.

5. THE STUDY OF DERMAL ARCHITECTURE OF SD/ NIN HAIRLESS MUTANT RAT

Spontaneous mutation provides potential models for human disorders. The development of inherited model or any mutant discovered must be described in enough detail to determine their scientific value in research.

Hairlessness (hypotrichosis) is known to be a heritable character. Laboratory animals that lack normal hair growth provide valuable models to study hair and skin abnormalities. Several mutations are known to lead to hypotrichosis in rats, including fuzzy(fZ), hairless(hr), Shorn (shn), Rowett (rnu) Charles River (CR), hairless Wistar (hW) which have been reported to influence hair follicle development. The hairless SD/NIN mutant has been identified, isolated from the SD rat colony at our NCLAS. Biochemical, immunological characterization of this mutant rat were studied.

OBJECTIVE

To study the comparative histopathological changes in skin of both hairless and heterozygous phenotypes of SD/NIN hr (hairless) mutant rat models.

Hypothesis

Laboratory rat models that lack normal hair coat provide distinct advantages in studies of percutaneous drug absorption, skin pharmacology and photobiology. The study of histological changes in hairless mutant rat would provide an attractive model in experimental dermatology and skin pharmacological studies.

Study Design

Rat pups of normal SD, SD Homozygous, SD Heterozygous (both phenotypes) 3, 7, 14, 21, 35, 49 & 70 days (with and without hair) were taken and sacrificed by carbondioxide (CO_2) asphyxiation. Skin specimens from cranium and dorsal regions were fixed in buffered 4% paraformaldehyde, sectioned processed for H & E staining.

RESULTS

Histopathological examination of the skin revealed that irrespective of the age of the rats hair of all homozygous hairless rats was under developed with prominent signs of atrophy like atrophic dermal papillae, hair matrix, internal and external root sheaths. The hair canals were also empty and dilated and cystic in most of the rats. The hair shafts also appeared to be thinner and improperly keratinized especially in the upper portion. In heterozygous counterpart animals, hair follicles were normal with normal dermal papillae, hair matrix and shaft and with normal cortex and medulla.

VIII. PRE-CLINICAL TOXICOLOGY RESEARCH CENTRE

1. PRE-CLINICAL TOXICITY EVALUATION OF HERTUZAB BIO-SIMILAR TRASTUZUMAB

The HER receptors are proteins that are embedded in the cell membrane and communicate molecular signals from outside the cell to inside the cell, and turn genes on and off. The HER proteins regulate cell growth, survival, adhesion, migration, and differentiation functions that are amplified or weakened in cancer cells. In some cancers, notably some breast cancers, HER2 is stuck in the "on" position, and causes breast cells to reproduce uncontrollably, causing breast cancer.

Antibodies are molecules from the immune system that bind selectively to different proteins. When it binds to defective HER2 proteins, the HER2 protein no longer causes cells in the breast to reproduce uncontrollably.

Clonz Biotech Private Limited has recently developed HERTUZAb using recombinant DNA technology with an intention to promote it for treatment of breast cancer. As per regulatory requirements, it is mandatory to undertake Pre-clinical toxicity evaluation of the product as per DBT guidelines.

METHODOLOGY

Preclinical Evaluation

Acute Toxicity Study in SA Mice: Swiss albino mice (5M+5F), aged 4 - 6 weeks old, weighing 20 - 25gm were received from the National Center for Laboratory Animal Science (NCLAS) with approval of IAEC (P5F/IAEC/2014/II/BDK). All the mice were caged individually for seven days for acclimatization. This was followed by randomization, the total volume of administration for males was 0.32ml / animal and for females it was 0.3ml/ animal given in two divided doses (first dose at 0^{th} hour and second dose at 4^{th} hour). Each time, 0.16ml for male and 0.15ml for female was given based on the average body weight. The animals were observed daily for mortality and general behavior. Routine physical and physiological examinations were recorded bi-weekly in all animals till the end of the experiment. All animals were euthanized, subjected for gross necropsy and vital organs were collected (pharynx, larynx, esophagus, abdominal aorta, brain, pituitary glands, thymus, spleen, bone marrow, kidney, pancreas, skin, heart, lung, trachea, thyroid, adrenals, sternum, liver, sciatic nerve, gastrointesinal tract, epididymis, uterus, mammary glands, testes/ovaries & tail).

Acute Toxicity Study in SD Rats: Sprague Dawley rats (5M+5F), aged 4 - 6 weeks old, weighing 160 - 180gm were received from the National Center for Laboratory Animal Science (NCLAS) with approval of IAEC (P5F/IAEC/2014/II/BDK). All the rats were caged individually for seven days for acclimatization. This was followed by randomization, the concentration of the test compound was maintained constant for male's 45.8mg/1.8ml per animal and for females 43mg/1.72ml depending on the average body weight and administered in two divided doses (First dose at 0^{th} hour and second dose at 4^{th} hour). Each time, 0.9ml for males and 0.86ml for

females was administred. The animals were observed daily for mortality and general behavior. Routine physical and physiological examinations were recorded bi-weekly in all animals till the end of the experiment. All animals were euthanized, subjected for gross necropsy & vital organs were collected (pharynx, larynx, esophagus, abdominal aorta, brain, pituitary glands, thymus, spleen, bone marrow, kidney, pancreas, skin, heart, lung, trachea, thyroid, adrenals, sternum, liver, sciatic nerve, gastrointestinal tract, epididymis, uterus, mammary glands, testes/ovaries & tail).

Sub Chronic Toxicity Study : The study has been conducted in Sprague Dawley rats (24M+24F), aged 4 – 6 weeks old, weighing 150 – 180 gm, obtained from NCLAS, NIN, Hyderabad with approval of IAEC (P5F/IAEC/2014/II/BDK). The rats with normal health report have been conditioned for 6 days in the experimental room. This was followed by randomization and divided into five groups viz., Vehicle control (VC), Therapeutic dose (TD), Average dose (AD, five times of TD), High Dose (HD, ten times of TD) & Innovator Drug (Reference drug) to receive various concentrations of test compound by intravenous route. The loading dose was given on 0th day as single exposure with total volume of 2ml per animal in two divided doses each time 1ml in 24 hrs. The test compound has been administered in Therapeutic Dose (5.04mg/0.2ml), Average Dose (25.2mg/1ml), High Dose (50.4mg/2ml) and Innovator Dose (5.06mg/0.23ml). The maintaining dose was given weekly once for three weeks (7th, 14th & 21st day) with total volume of 1ml per animal. The test compound was administered in Therapeutic Dose (2.52mg/ 0.1ml), Average Dose (12.6mg/ 0.5ml), High Dose (25.2mg/ 1ml) and Innovator Dose (2.52mg/ 0.11ml). The innovator was administered equivalent to therapeutic dose.

S. No	Groups	Test compound
1	Vehicle Control	Formulation Buffer
2	Therapeutic Dose (TD)	Hertuzab, a Biosimilar Trastuzumab
3	Average Dose (5X TD)	
4	High Dose (10XTD)	
5	Innovator Dose (ID)	Trastuzumab (Herclon ™)

Study parameters

The animals were observed daily for mortality, live phase, and cage side, physical, physiological and neurological activities till end of the experiment. Food intake and body weights were recorded bi-weekly. Urine analysis (qualitatively) was monitored pre- and post exposure in all groups of animals. The biochemistry and hematology parameters were undertaken in all groups after 48hrs of last exposure followed by euthanization of 50% of animals from groups VC, TD & HD and all animals from groups AD & ID for gross necropsy and histopathology of all vital organs (pharynx, larynx, esophagus, abdominal aorta, brain, pituitary glands, thymus, spleen, bone marrow, kidney, pancreas, skin, heart, lung, trachea, thyroid, adrenals, sternum, liver, sciatic nerve, gastrointestinal tract, epididymis, uterus, mammary glands, testes/ovaries & tail). On 16th day of post exposure the remaining 50% of animals from groups VC, TD & HD were subjected to study biochemistry and hematology related parameters, followed by euthanization, to collect all major organs for gross necropsy and histopathology.

RESULTS

Acute – Mice

- No pre-terminal deaths were recorded in mice exposed to test compound.
- No significant effect on body weight gain were recorded
- Clinical signs, behavioral activity were normal.
- No gross necropsy changes were recorded.

Acute – Rat

- No pre-terminal deaths were recorded in rats exposed to test compound.
- No significant effect on body weight gain were recorded
- Clinical signs, behavioral activity were normal.
- No gross necropsy changes were recorded.

Sub-chronic

- No pre-terminal deaths were recorded in rats exposed to test compound and innovator group.
- No abnormal clinical signs, behavioral activity etc., were observed in animals which received test compound as compared to vehicle control.
- No significant effect on food intake and body weight gain was observed in animals which received test compound as compared to vehicle control.
- The clinical chemistry parameters viz., blood glucose levels, kidney and liver function tests were found to be in normal range in all groups of animals when compared to vehicle control.
- There were no significant changes in hematology parameters except RBC levels of the TD group in final term showed high levels when compared to vehicle control but were within normal range and hence not considered significant.

Histopathology

Midterm: Changes of histological significance were observed only in liver, lungs and trachea. However, the histological changes observed in all these organs of the experimental groups were also observed in the control group and hence cannot be considered significant.

Final term: Changes of histological significance were observed only in the liver, lungs and testis. In both liver and lungs, histological changes observed in the experimental group were also observed in the control group and hence not considered significant. However, one animal in the HD group showed degeneration in one testis.

CONCLUSIONS

Acute – Mice

No pre-terminal deaths were recorded in mice which received test compound. There were no abnormalities in live phase, physical activity and neurological activity throughout the study period. There was no significant difference in body weight. All the animals were active throughout the study period. No gross necropsy changes were observed.

Acute – Rat

No pre-terminal deaths were recorded in rats which received test compound. There were no abnormalities in live phase, physical activity and neurological activity throughout the study period. There was no significant difference in body weight. All the animals were active throughout the study period. No gross necropsy changes were observed.

Sub-chronic

There was no mortality in animals exposed to test compound and vehicle Control. There were no abnormal changes in physical, physiological, clinical and neurological activities. All clinical parameters including clinical chemistry and hematology were recorded in normal range. Histopathology did not reveal changes of significance in all the organs except in a single organ (testis) in the HD group.

2. PRE CLINICAL TOXICITY EVALUATION OF BIVALENT VACCINE (TD) FOR ADULTS

Immunization is one of the most well-known and effective methods of preventing childhood diseases. With the implementation of the Universal Immunization Programme (UIP) by the Government of India, significant achievements have been made in preventing and controlling vaccine-preventable diseases (VPDs).

Indian Immunologicals Limited has recently developed Bivalent vaccine for adults is a combination vaccine consisting of concentrated and purified Diphtheria toxoid and purified tetanus toxoid adsorbed on a mineral carrier (Aluminium Phosphate). The vaccine is used for active immunization of children over 7 years of age and adults against Diphtheria & Tetanus. As per the regulatory requirements, it is mandatory to undertake Preclinical toxicity evaluation of Bivalent vaccine (Td) as per DBT[1] guidelines.

METHODOLOGY

Preclinical Evaluation

Acute Toxicity Study in SA Mice: The study has been conducted in Swiss Albino Mice (6M+6F), aged 4-6 weeks old, weighing 18-20gm, obtained from NCLAS, NIN, Hyderabad with approval of IAEC (P7F/IAEC/I/2015/BDK).The mice with normal health report have been conditioned for 7 days in the experimental room. This was followed by randomization; the test compound was administered in High Dose (5X Human Dose – Diphtheria toxoid – 20Lf, Tetanus toxoid – 37.5Lf). The total volume of administration was 0.5ml / animal and given in two divided doses first dose at 0^{th} hour 0.3ml (0.15ml at right thigh + 0.15ml at left thigh) and second dose at 6^{th} hour 0.2ml (0.1ml at right thigh + 0.1ml at left thigh). The animals were observed daily for mortality, live phase, cage side, physical, physiological, neurological activity till the end of the experiment. The feed intake, body weights were recorded bi-weekly. All animals were euthanized, subjected for gross necropsy and vital organs were collected (pharynx, larynx, esophagus, abdominal aorta, brain, pituitary glands, thymus, spleen, bone marrow, kidney, pancreas, skin, heart, lung, trachea, thyroid, adrenals, sternum, liver, sciatic nerve, gastrointestinal tract, epididymis, uterus & testes/ovaries) including site of injection.

Acute Toxicity Study in Duccan Hartley Guinea pigs: The study has been conducted in Duccan Hartley Guinea pigs (6M+6F), aged 3 months old, weighing 250 – 300gm, obtained from NCLAS, NIN, Hyderabad with approval of IAEC (P7F/IAEC/I/2015/BDK). The Guinea pigs with normal health report have been conditioned for 18 days in the experimental room to get appropriate body weight. This was followed by randomization and the test compound was administered in High Dose (5XHuman Dose – Diphtheria Toxoid – 20Lf, Tetanus Toxoid – 37.5Lf) and total volume of administration is 0.5ml / animal and given at right (0.25ml) and left (0.25ml) dorsal area of thigh muscle keeping in view the maximum volume to be administered. The animals were observed daily for mortality, live phase, cage side, physical, physiological, neurological activity till end of the experiment. The feed intake, body weights were recorded bi-weekly. All animals were euthanized, subjected for gross necropsy and vital organs were collected (pharynx, larynx, esophagus, abdominal aorta, brain, pituitary glands, thymus, spleen, bone marrow, kidney, pancreas, skin, heart, lung, trachea, thyroid, adrenals, sternum, liver, sciatic nerve, gastrointestinal tract, epididymis, uterus & testes/ovaries) including site of injection.

RESULTS

Acute – Mice

- No pre-terminal deaths or morbidity were recorded in mice exposed to test compound.

- No significant effect on body weight gain were recorded
- Clinical signs, behavioral activity were normal.
- No gross pathological changes in the vital organs were recorded on necropsy.

Acute-Guinea pigs

- Mortality was recorded in one male animal on 13th day of post exposure.
- No significant effect on body weight gain were recorded
- Clinical signs, behavioral activity were normal.
- No gross pathological changes in the vital organs were recorded on necropsy.
- The histopathological results of organs collected during autopsy showed changes in lungs, liver, bladder and testes. Lungs showed changes of Chronic interstitial pneumonitis a congested while liver showed focal area of necrosis. Urinary bladder showed acute chronic inflammation changes and testes showed maturation arrest.

CONCLUSIONS

Acute-Mice: No pre-terminal deaths were recorded in mice which received test compound. There were no abnormalities in live phase, physical activity and neurological activity throughout the study period. There was no significant difference in body weight. All the animals were active throughout the study period. No gross necropsy changes were observed.

Acute-Guinea pigs: Mortality was recorded in one male animal on 13th day of post exposure. There were no abnormalities in live phase, physical activity and neurological activity throughout the study period. There was no significant difference in body weight. All the animals were active throughout the study period. No gross necropsy changes were recorded and no significant changes in organ weights were observed. Based on the results of autopsy of one male animal which died on the 13th day of post exposure, it was found that it was due to pneumonitis and congestion in lungs with convergent asphysia and death.

LIBRARY AND DOCUMENTATION SERVICES

Library continued to cater to the documentation and information needs of the Institute and other Research Organizations, Home Science and Medical Colleges. The library had played a key role in reference activities by offering information dissemination services like MEDLINE Searches, Proquest Medical Library Full Text Database of journals and other online retrieval activities using the LAN Network of the Institute. Library continued to participate in exchange of data, journals and information using the URL<http://Groups.yahoo.com/group/ICMR Librarians>.

Resource Sharing and User Education Programmes etc. are continuously being undertaken by the Library. Institute's Scientific papers going in for publication in Scientific Journals etc., are being routed through the Library and a data-base of the published papers is also made accessible through on-line services using NIN Website (www.ninindia.org).

The Library services are being further strengthened by continuously receiving support from Indian Council of Medical Research for accessing E-journals from JCCC@ICMR and J-Gate database. The Library is also a member of ERMED Consortia of National Medical Library, New Delhi provided by ICMR for accessing E-journals Online Subscription of 4 Core Journals such as LANCET, NATURE, NEJM, SCIENCE has been renewed by ICMR.

The Library has continued to provide an excellent Photostat support to the scientists, technical as well as to the administrative staff.

The following library services were expanded as detailed below:

1. NEW ADDITIONS

Books	. . . .	5
Reports	. . . .	56
Thesis / Dissertations	. . . .	5
CDROMS	. . . .	13
PC Quest CD's	 3	
General CD's	. . . 10	

NEW JOURNALS ADDED

Foreign Journals

1 American Journal of Clinical Pathology
2 Biochemica Biophysica Acta: Molecular Basis of Disease
3 Bone
4 Cell
5 Endocrinology
6 Food and Chemical Toxicology
7 Human Pathology
8 I L A R Journal
9 International Journal for Vitamin and Nutrition Research
10 International Journal of Sport Nutrition and Exercise Metabolism
11 Journal of Biological Chemistry
12 Journal of Clinical Investigation

13 Journal of Health Communication
14 Journal of Nutrition Education & Behaviour
15 Journal of Nutritional Biochemistry.
16 Journal of The Royal Statistical Society: Series C (Applied Statistics)
17 Lab Animal
18 Lipids
19 Medicine and Science in Sports and Exercise
20 Pharmacogenomics journal
21 Public Health Nutrition
22 Stem Cells

2. JOURNALS DELETED

Foreign Journals

1. Acta Paediatrica
2. American Journal of Epidemiology + Epidemiologic Reviews (Combind)
3. American Journal of Medicine
4. Analytical Chemistry
5. Annals of Clinical Biochemistry
6. Annals of Nutrition & Metabolism
7. Asia Pacific Journal of Clinical Nutrition
8. Biochemical Journal
9. Biochemical Pharmacology
10. Biochemistry
11. Clinical Nutrition
12. Comprehensive Reviews in Food Science and Food Safety
13. CRC: Critical Reviews in Food Science & Nutrition
14. Diabetes Care
15. Diabetologia
16. Epidemiologic Reviews
17. European Journal of Nutrition
18. International Journal of Food Safety, Nutrition and Public Health
19. International Journal of Food Science and Nutrition
20. International Journal of Obesity
21. Journal of Clinical Biochemistry and Nutrition
22. Journal of Experimental Medicine
23. Journal of Food Online
24. Journal of Food Safety
25. Journal of Food Science
26. Journal of the American Dietetic Association-2012
    (Journal of the Academy of Nutrition and Dietetics)
27. Nutrition & Cancer
28. Nutrition & Dietetics
29. Nutrition Research Reviews
30. Nutrition, Metabolism & Cardiovascular Diseases
31. Progress in Lipid Research
32. Science (by Airmail)

3. OTHER ACTIVITIES

Journals Bound	. . . .	219
Visitors using the Library	. . . .	3996
Circulation of Books/Journals etc	. . . .	1401
No. of E-mails sent outside	. . . .	1504
No. of E-mails received	. . . .	5,764
Photocopying (No. of pages)	. . . .	1,39,421
No. of INTERNET Searches provided	. . . .	134
No. of Reprints sent	. . . .	140

4. TOTAL LIBRARY COLLECTIONS

Books	. . . .	18,111
E – Books	. . . .	36
Journals (Bound Volumes)	. . . .	40,202
Journals subscribed for 2016	. . . .	112
E – Journals subscribed for 2016	. . . .	17
Journals received (Gratis/Exchange) for 2016	. . . .	108
Microforms (Microfiche)	. . . .	1,080
Slides	. . . .	280
Reports	. . . .	13,839
Theses & Dissertations	. . . .	424
MEDLINE CDROMS Discs	. . . .	383
Current Contents on Diskettes with abstracts	. . . .	664
Proquest (Full Text E-Journals) on CD ROMS	. . . .	495
General CD's	. . . .	322

Ph.D PROGRAMMES

Research scholars registered for PhD

S. No	Name & Year of joining	Title of the thesis	Supervisor	University
1	A. Kiran Kumar (2012)	Metabolic response of zinc depletion and excess in contrasting cells: Studies in osteoblasts, myocytes and enterocytes.	Dr. K. Madhavan Nair	Osmania
2	N. Himaja (2012)	Effects of Fos coated probiotics on fetal immune-programming and other health benefits	Dr. R. Hemalatha	Dr.NTRUHS
3	S Vishwaraj (2012)	Role of molecular chaperones in chronic tissue remodeling diseases	Dr. G.Bhanuprakash Reddy	Osmania
4	Sneha Jakhotia (2012)	Role of small heat shock proteins in diabetic nephropathy	Dr. G Bhanuprakash Reddy	Osmania
5	K Shruthi (2012)	Role of Ubiquitin proteosome system In diabetic complications	Dr. G Bhanuprakash Reddy	Osmania
6	T Shalini (2012)	Assessment of nutritional status of geriatric population	Dr. G Bhanuprakash Reddy	Osmania
7	J Sugeetha (2012)	Impact of dietary saturated fatty acids on the progression of Nonalcoholic fatty liver disease in fructose induced model of Steatosis-Role of adipose tissue insulin sensitivity and secretory function.	S. Ahmed Ibrahim	Osmania
8	Daniella Chyne (2012)	Studies on the biodiversity of food resources in Meghalaya	Dr. R. Ananthan	Osmania
9	K. Mangthya Naik (2012)	Studies on gastro protective effects of Naga King chili	Dr. R. Ananthan	Osmania
10	S.Alekhya (2012)	Identifying microbiological and hygienic factors affecting safety of stress foods and addressing them through vendor education.	Dr. V.Sudershan Rao	Osmania
11	Prashanthi PS (2012)	Studies on Xanthophylls: dietary sources , processing , bioavailability and biological effects	Dr.K. Bhaskarachary	Osmania
12	J.Sreenivas Rao (2012)	Effect of Cooking / Processing on the Bioavailability of Provitamin A carotenoids in Indian foods	Dr.K. Bhaskarachary	Osmania
13	M. Srujana (2012)	Effect of pesticide exposure among the farm children and their mothers on the various biochemical parameters associated with reproduction, neurotoxic enzymes, oxidative stress and impact on the micronutrient status.	Dr. J Padmaja Rambabu	Osmania

S. No	Name & Year of joining	Title of the thesis	Supervisor	University
14	Venkat Reddy.B (2012)	Monitoring of organophosphate pesticide metabolites in commonly used fruits, juices, vegetables and urine samples of urban children and its toxic effect	Dr.S.N.Sinha	Osmania
15	Archana Konapur (2013)	Targeted nutrition communication for promoting consumption of variety of foods for improving micronutrient status of rural families	Dr. K. Madhavan Nair	Osmania
16	Dripta Roy Choudhury (2013)	Functional benefits of inclusion of fruits in supplementary nutrition programme (SNP): A randomized community trial among ICDS preschool beneficiaries on micronutrient status ,gut health, growth and development	Dr. K. Madhavan Nair	Osmania
17	M Siva Prasad (2013)	Status of Micronutrients and its influence on Molecular Mechanisms in Diabetic Nephropathy	Dr. G Bhanuprakash Reddy	Osmania
18	Raja Gopal Reddy (2013)	Role of vitamin A metabolic pathway on the development of non-alcoholic fatty liver disease: A study on nutrient-nutrient interactions	Dr.SM.Jeya Kumar	Osmania
19	MVS Prasad (2013)	Biochemical and Molecular studies on role of diet in the induction of obesity: Rat as a model system	Dr. K. Rajender Rao	Osmania
20	D M Dinesh Yadav (2013)	Studies on identification of candidate gene(s) associated with obesity in WNIN/Ob rat	Dr. K. Rajender Rao	Osmania
21	Keren Susan Cherian (2013)	A study on body composition and energy balance in selected groups of junior athletes	Dr.Y. Venkata Ramana	Osmania
22	Vilasagaram Srinivas (2013)	Role of maternal long chain fatty acids on angiogenic factors in first trimester placenta and their invasive properties:implication to feto placental growth	Dr.Sanjay Basak	Osmania
23	Rishika Jada (2013)	Effect of Cowpea isoflavones as a natural source for treatment of os teoporosis in MG-63 human osteosarcoma cells and to assess its synergetic role with Vitamin D in bone formation.	Dr.C.Suresh	Osmania
24	G. Srividya (2014)	Anticancer and proteasome inhibitory potential of cinnamon in prostate cancer: In vitro and In vivo studies	Dr. Ayesha Ismail	Osmania
25	P.Kondaiah (2014)	Effect of zinc supplementation prior to iron on iron absorption, and iron status in deficient rats: in vitro and in vivo studies	Dr.P.Raghu	

S. No	Name & Year of joining	Title of the thesis	Supervisor	University
26	K.Narendra Babu (2014)	Probiotic potential and other beneficial effect of ocimum, ginger and piper nigum on immune-inflammatory disease conditions	Dr. B.Dinesh Kumar	Dr.NTR UHS
27	D.Vasundhara (2014)	Effect of probiotic supplementation on bacterial vaginosis (BV) in pregnant women	Dr. R. Hemalatha	Dr.NTR UHS
28	Nivedita Dubey (2014)	Nutritional composition bioavailability and allergenicity profile of nutritionally enriched GM food crops.	Dr.B. Dinesh Kumar	Osmania
29	Anita Singh (2014)	Development of herbals(*Asparagus racemosus, BacopaMonnieri, WithaniaSomnifera, Convolvulus pluricaulis, Tribulusterrestris, Phyllanthusamarus)* and their combinationsas potential immunomodulators and anti-inflammatory products.	Dr. B.Dinesh Kumar	
30	K.Rajeshkumar (2014)	Role of advanced glycation end products in chronic tissue remodeling diseases	Dr. G Bhanuprakash Reddy	Osmania
31	A. Kiranmayee (2014)	Impact of Statins in vitamin D deficiency and Genetic polymorphism in Indian population	Dr.B. Dinesh Kumar	
32	Bidyalakshmi Loukrakpam (2014)	Studies on the food system of the Meitei community of Manipur and its nutritional implications	Dr. R. Ananthan	Osmania
33	Kondeti Suresh (2014)	Studies on regulation of FGF21 in obese and prediabetic rat models	Dr. K. Rajender Rao	
34	S G D N Lakshmi Reddy (2014)	Development and validation of an index for assessing food safety at household level	Dr. V.Sudershan Rao	
35	U.V. Rama Krishna (2014)	Isolation, characterization and anti cancerous activity of bio active molecules from camellia sinensis	Dr.S.N.Sinha	
36	Neelima AS (2014)	Intracellular mechanism of naturally available neuroprotective compounds in mitigating the combined toxicity generated by Lead and beta amyloid peptides in human brain cells .	Dr.C.Suresh	Osmania
37	Talari Aruna (2014)	Nutritional quality, prebiotic potential and other health benefits of Raffinose family oligosaccharides of Pigeon Pea (Cajanus Cajan, L)	Dr.S.Devindra	Osmania
38	S.Kiruthika (2014)	Agricultural interventions for improving nutritional status among<5 year old rural Indian children.	Dr.Bharati Kulkarni	
39	G.Sumalatha (2014)	Isolation and identification of Vit-B12 producing probiotic strains from dairy products	Dr.M.Shiva Prakash	

S. No	Name & Year of joining	Title of the thesis	Supervisor	University
40	Mohd. Shujauddin	Dynamics of intrauterine inflammation in relation to malnutrition during pregnancy – foetal outcome and metabolic changes in adulthood	Dr. R. Hemalatha	
41	Mr.V.Sudershan Reddy	Iron homeostasis in adolescent girls with iron deficiency anemia –Role of genetic varients and gut microbiome	Dr. R. Hemalatha	
42	K. Divya Shoshanni	Assesment of nutritional status, morbitity status and utilisation of health care facilty in the elderly population age 60yrs and above	Dr.P. Suryanarayana	
43	M.Thirupathi Reddy	Big data and data mining techniques in nutrition	Dr.M.Vishnu Vardhana Rao	
44	Ankita Mondal	Seasonal Variation in MaInutrition among Rural Women and Children	Dr.BharatiKulkarni	
45	Richa Pande	Study the influence of media on food choices and development and assessment of a SBCC program in rural Telangana	Dr.Bharati Kulkarni	
46	KB.Chathyushya	Development of Multiplex PCR for rapid detection of probiotic microflora in human breast milk	Dr. M. Shiva Prakash	
47	G.Ramesh (2016)	Moleculed mechanism involved in vitamin D deficiency induced muscle atrophy	Dr. Ayesha Ismail	
48	Swetha Boddula	Development and validation of a tool to assess diet quality and associated factors among adolescents in India	Dr M.S.Radhika	

AWARDS / HONOURS CONFERRED ON SCIENTISTS

Name of the Scientist	Awards/ Honour
Mr.J.Sreenivas	Best presentation award at the 2nd International Conference on "Current Trends in Mass Spectroscopy", held in Chicago (July 20-22)
Dr.Raja Sriswan	Selected for the ICMR-IIT Kharagpur MedTech Internship Program for eight weeks from May 9 to July 3, 2016
Dr.R.Hemalatha	Honoured with 7th Dr.Rajammal P.Devadas Memorial award – 2016 by Nutrition Society of India. Elected as Fellow of National Academy of Medical Sciences – 2016
Dr.G.M.Subba Rao	Awarded Dr.V.N.Patwardhan Prize 2014 from Indian Council of Medical Research
Dr.B.Naveen Kumar	Awarded second prize in Evaluation of 16th Foundation course for scientists and technologists, IIPA, New Delhi on Feb 10, 2018

Participation of Scientists in International Meetings/ Workshops/ Conferences/ Training Programmes

S.No	Name of the Scientist	Meeting/ Conference attended	Date
1	Mr.J.Sreenivas	2nd International Conference on "Current Trends in Mass Spectroscopy", in Chicago. Presented research work titled "Determination of heavy metal contents in Indian foods using ICP-MS after closed vessel microwave digestion".	July 20-22
2	Dr. G. M. SubbaRao	Conference of the International Association of Media and Communication Research (IAMCR), University of Leicester, United Kingdom. Presented a paper on "What's cooking? Exploring the connect and the disconnect in media portrayal and public perceptions on food safety associated health risks in India" in the Health Communication and Change Working Group.	July 27-31
3	Mr.T.Longvah	Workshop on "Traditional, indigenous and cultural food & nutrition", organized by National Nutrition & Food Technology Research Institute, Tehran, Iran. Participated as a Taskforce member at the Workshop.	Sept. 8-9
4	Dr.Bharathi Kulkarni	Participated in the "Health birth, growth and development knowledge integration, convening" as a part of the "Grand Challenges Annual Meeting", organized by Bill and Melinda Gates Foundation, in London, UK.	Oct. 24-28
5	Mr.Naveen Kumar, Ms.Alekhya (UGC-SRF), Ms.SGD.Nagalakshmi (ICMR-SRF)	4th Asia-Pacific International Food Safety Conference" & 7th Asian Conference on Food and Nutrition Safety, in Penang, Malaysia	Oct. 11-13
6	Dr.P.Raghu & Dr.R.Ananthan	Visited Medical Research Institute, Colombo, Sri Lanka to train scientists and technical staff on serum vitamin A analysis by HPLC and food mineral analysis by atomic absorption spectrometry.	Oct. 24-28
7	Dr.B.Dinesh Kumar	24th Meeting of the working group on the "Safety of Novel Foods and Feeds" in Paris, France organized by OECD	Mar. 30-31, 2017
8	Dr.GM.Subba Rao	International Union of Nutrition Sciences (IUNS) Workshop on "Capacity and Leadership in Nutrition Sciences", held at National Institute of Nutrition and Health, Tokyo, Japan.	Mar.7-9, 2017

Workshops/ Conferences/ Seminars/ Training Programmes held at NIN

1. Workshop on “Big Data and Analytics in Epidemiology” was jointly organized by ICMR and DST, wherein a mobile app on “Dietary Guidelines for Indians” was launched by Dr.Sowmya Swaminathan, DG, ICMR & Secretary, DHR (April 29-30).
2. A one-day seminar on “Animal Welfare and Alternatives to Experimentation in Biomedical Research” was conducted on the occasion of World Laboratory Animal Day by National Centre for Laboratory Animal Sciences (NCLAS), ICMR & Committee for the Purpose of Control & Supervision of Experiments on Animals (CPCSEA) (Apr.24).
3. Twentieth meeting of the Subject Expert Committee (SEC) – Life Sciences, was conducted by Department of Science and Technology-Women Scientist Scheme (WOS-A) (May 18-20).
4. Department of Food and Drug Toxicology Research Center (NIN) conducted the training program for laboratory technicians who were appointed under “National programme for prevention and control of fluorosis” (July 25-29).
5. XXXIII Conference of the International Society of Fluoride Research on the theme “Debilitating fluorosis: Current status, Health challenges & Mitigation Measures”, was organized by the International Society of Fluoride Research in association with NIN (Nov. 9-11).
6. International symposium on “Food Composition in Nutrition and Health” was organized in New Delhi. The “Indian Food Composition Data Tables – 2017” was released by Shri.J.P.Nadda, Hon'ble Minister of Health and Family Welfare, Govt. of India (Jan. 18).
7. A Regional Review Meeting cum Capacity Building Programme under National Programme for Prevention and Control of Fluorosis was held during March 2-3, 2017.
8. ICMR-NIN Workshop on Research Methodology, in association with Directorate of Medical Education, Govt. of Telangana (March 21-23).

Services rendered towards income generation

1. Pathology Services

During the year, a total income of ₹ 2,47,150/- was generated from various projects of Institute's Histopathology and Electron Microscopy.

2. Training Programmes

I An amount of ₹ 6,20,000/- was generated from the tuition fee collected from the first and second year participants of 2 year MSc (Applied Nutrition) course (1st year-16 & 2nd year-15 candidates).

ii. An amount of ₹ 2,10,000/- was generated from private candidates admitted to the regular training programme viz., Post Graduate Certificate Course in Nutrition and Training Course on Endocrinological Techniques and their Applications.

SCIENTIFIC PUBLICATIONS - 2016

A. PAPERS PUBLISHED IN SCIENTIFIC JOURNALS

1. Abdeen ZA, Arlappa N, Balakrishna N, Hari Kumar R, Laxmaiah A, Mallikharjuna Rao K, Meshram II : Worldwide trends in diabetes since 1980 : a pooled analysis of 751 population-based studies with 4.4 million participants. Lancet. 387: 1513-1530, 2016
2. Amruth Rao P, Brahmaiah U, Hemalatha R: Phyrnoderma: Associated nutritional co-morbodities among children attending at Nutrition clinic, Osmania General Hospital, Hyderabad, India. Indian J Paediat Dermatol. 17: 108-111, 2016.
3. Amruth Rao P, Ramulu P, Paul Marx K, Tirumala Rao B, Venkata Ramana Devi: Association of post prandial hyper triglyceridemia and carotid intima media thickness in patients with type-2 diabetes mellitus. Int J Diab Res. 5:87-91, 2016.
4. Anderson I, Harikumar R, Mallikharjuna Rao K, Laxmaiah A, Meshram I, Balkrishna N, Arlappa N: Indigenous and tribal peoples' health (The Lancet- Lowitja Institute Global Collaboration) : a population study. Lancet. 388:131-157, 2016.
5. Anil Kumar P, Swathi Chitra P, Bhanuprakash Reddy G: Advanced glycation end products mediated cellular and molecular events in the pathology of diabetic nephropathy. BioMol Concepts. 7:293-309, 2016.
6. Anil Kumar P, Welsh GI, Raghu G, Menon RK, Saleem MA, Bhanuprakash Reddy G: Carboxymethyl lysine induces EMT in podocytes through transcription factor ZEB2: Implications for podocyte depletion and proteinuria in diabetes mellitus. Arch Biochem Biophys. 590: 10-19, 2016.
7. Anil Sakamuri, Sujatha P, Uday Kumar P, Sugeedha Jayapal, Sailaja P, Sai Santosh V, Nagabhushan Reddy K, Siva Sankara Vara Prasad S, Ahamed Ibrahim: Transient decrease in circulatory testosterone and homocysteine precedes the development of metabolic syndrome features in fructose-fed sprague dawley rats. J Nutr Metab. Article ID 7510849, 11 pages, 2016.
8. Arlappa N, Balakrishna N, Laxmaiah A, Brahmam GNV. Vitamin A deficiency disorders among the rural pre-school children of South India. Int J Nutr. 2: 8-11, 2016.
9. Arlappa N, Qureshi IA, Ravikumar BP, Balakrishna N, Qureshi MA: Arm span as an alternative to standing height for calculation of body mass index (BMI) amongst older adults. Int J Nutr. 2: 12-24, 2016.
10. Aruna P, Uma A, Raghunath M: Telomere length as a biomarker of ageing and associated metabolic disorders: a review. HFSP J. 10:64-95, 2016.
11. Aruna T, Devindra S: Nutritional and anti-nutritional characteristics of two varieties of red gram (Cajanus Cajan, L) seeds. Int J Sci Res Pub. 6: 567-572, 2016.
12. Ayesha Ismail, Bindu Noolu, Ramesh G, Shyam P, Rajanna A, Babu SK: Cytotoxicity ad proteasome inhibition by alkaloid extract from Murraya koenigii leaves in breast cancer cells-molecular docking studies. J Med Food. 19: 1155-1165, 2016.
13. Bhagyasri A, Naveen Kumar R, Balakrishna N, Sudershan Rao V: Exposure assessment of

artificial sweeteners among type 2 diabetic, overweight and obese individuals. Indian J Nutr Diet. 53: 268-276, 2016.

I14. Bhaskarachary K, Sudershan Rao V, SubbaRao GM, Joshi AK: Traditional foods, functional foods and nutraceuticals. Proc Indian Natl Sci Acad. 82:1565-1577, 2016.

15. Black MM, Sylvia Fernandez-Rao, Hurley KM, Tilton, N, Balakrishna N, Harding KB, Reinhart G, Radhakrishna KV, Madhavan Nair K: Growth and development among infants and preschoolers in rural India: Economic inequities and caregiver protective/promotive factors. Int J Behav Dev. 40: 526-535, 2016

16. Bindu Noolu, Ramesh G, Mehrajuddin Bhat, Qadri SSYH, Sudhakar Reddy V, Bhanuprakash Reddy G, Ayesha Ismail: *In Vivo* Inhibition of proteasome activity and tumour growth by *Murraya koenigii* leaf extract in breast cancer xenografts and by its active flavonoids in breast cancer cells. Anticancer Agents Med Chem. 16: 1605-1614, 2016.

17. Biswas P, Venkata Ramana Murthy C, Agnello G, Stone E, Chakarova C, Duncan JL, Kannabiran C, Homsher M, Bhattacharya SS, Naeem MA, Kimchi A, Sharon D, Iwata T, Riazuddin S, Bhanuprakash Reddy G, Hejtmancik JF, Gerogiou G, Amer Riazuddin S, Radha A: A missense mutation in the ASRGL1 is involved in causing autosomal recessive retinal degeneration. *Hum Mol Genet*. 25: 2483-2497, 2016.

18. Devindra S, Aruna T: Analysis of available carbohydrate fractions from Indian foods by using a modified AOAC total dietary fiber method. Indian J Sci. Res. 7:1-9, 2016.

19. Deethu Sara Varghese, Sreedhar S, Balakrishna N, Venkata Ramana Y: Evaluation of the relative accuracy of anthropometric indicators to assess body fatness as measured by air displacement plethysmography in Indian women. Am J Hum Biol. 28: 743-745, 2016.

20. Himadri Singh, Parthasarathy V, Farouk M, Vijayalakshmi V: Progenitor cells may aid successful islet compensation in metabolically healthy obese individuals. Med Hypotheses. 86: 97-99, 2016.

21. Himadri Singh, Vijayalakshmi Venkatesan: Beta-cell managaement in Type 2 diabetes: Beneficial role of nutraceuticals. Endocr Metab Immune Disord Drug Targets. 16: 89-98, 2016.

22. Kesavan R, Chandel S, Upadhyay S, Bendre R, Gangula R, Potunuru UR, Giri H, Sahu G, Uday Kumar P, Bhanupradesh Reddy G, Joksic G Bera AK, Dixit M: Gentiana lutea exerts anti-atherosclerotic effects by preventing endothelial inflammation and smooth muscle cell migration. Nutr Metab Cardiovasc Dis. 26:293-301, 2016.

23. Lakshmi Ramakrishnan, Shreena Ramanathan, Santhakumar Aridoss, Diwakar Yadaw, Boopathi K, Goswami P, Subramanian Th, Adhikary R, Venkaiah K, Bitra George, Paranjape RS: Declining trends in risk behaviors and HIV/STIs among male clients of FSWs in India: Findings from large scale bio-behavioral surveys from three high prevalent Southern States. Int J Health Sci Res. 6:1-11, 2016.

24. Lakshmi Reddi SGDN, Naveen Kumar R, Subba Rao GM, Vishnu Vardhana Rao M, Sudershan RV: Bacteriological quality of drinking water at point of use and hand hygiene of primary food preparers: Implications for household food safety. J Water Sanit Hyg Dev. 6:224-230,2016.

25. Little Flower Augustine, Madhavan Nair K, Sylvia Fernandez Rao, Vishnu Vardhana Rao M, Ravinder P, Laxmaiah A: Exploring the bio-behavioural link between stress, allostatic load & micronutrient status: A cross-sectional study among adolescent boys. Indian J Med Res. 144: 378-384, 2016.

26. Madhavan Nair K, Little Flower Augustine: Food synergies for improving bioavailability of micronutrients from plant foods. Food Chem. 238: 180-185, 2016.

27. Madhavan Nair K, Little Flower Augustine, Archana Konapur: Food-based interventions to modify diet quality and diversity to address multiple micronutrient deficiency. Front Public Health. Article 277, 3: 1-14, 2016.

28. Manoj Kumar, Yadav AK, Verma V, Birbal Singh, Gorakh Mal, Ravinder N, Hemalatha R: Bioengineered probiotics as a new hope for health and diseases: an overview of potential and prospects. Future Microbiol. 11: 585-600, 2016.

29. Mansoori T, Vijay Kiran S, Balakrishna N: Plateau Iris in primary angle closure glaucoma: An ultrasound biomicroscopy study. J Glaucoma. 25: e82-e86, 2016.

30. Mariachiara Di Cesare, Laxmaiah A: Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19.2 million participants. Lancet. 387: 1377-1396, 2016.

31. Matsuzaki M, Sullivan R, Ekelund U, Radhakrishna KV, Bharati Kulkarni, Collier T, Ben-Shlomo Y, Sanjay K, Kuper H: Development and evaluation of the Andhra Pradesh children and parent study physical activity questionnaire (APCAPS-PAQ): a cross-sectional study. BMC Public Health. 16:48, DoI: 10.1186/s 12889-016-2706-9, 2016.

32. Meshram II, Balakrishna N, Sree Ramakrishna K, Mallikharjun Rao K, Hari Kumar R, Arlappa N, Manohar G, Gal Reddy Ch, Ravindranath M, Sharad Kumar S, Brahmam GNV, Laxmaiah A: Trends in nutritional status and nutrient intakes and correlates of overweight/obesity among rural adult women (≥ 18-60 years) in India: National Nutrition Monitoring Bureau (NNMB) national surveys. Public Health Nutr. 19: 767–776, 2016.

33. Meshram II, Mallikharjun Rao K. Gal Reddy Ch, Ravindranath M, Sharad Kumar, Sree Ramakrishna K, Hari Kumar R, Venkaiah K, Laxmaiah A: Prevalence of under nutrition and its predictors among under 5 year children in Surat region, Gujarat, India. Clin Nutr Diet. 2: 1-12, 2016.

34. Meshram II, Vishnu Vardhan Rao M, Sudarshna Rao V, Laxmaiah A, Polasa K: Regional variation in the prevalence of overweight/obesity, hypertension and diabetes and their correlates among the adult rural population in India. Br J Nutr. 115: 1265-1272, 2016.

35. Nagpal R, Kumar M, Yadav AK, Hemalatha R, Yadav H, Marotta F, Yamashiro Y: Gut microbiota in health and disease: an overview focused on metabolic inflammation. Benef Microbes. 7: 181-194, 2016.

36. Naveen Kumar B, Meshram II, Vijayabhaskara Rao D, Radhika MS, Venkaiah K: Ranking of the districts using a composite index based on the nutritional status of under 5 years children in Telangana State. Int J Nutr Diet. 4:71-83, 2016.

37. Naveen Kumar B, Venkaiah K: A New algorithm for generating Gaussian random numbers. Int J Agric Stat Sci. 12:307-310, 2016.

38. Naveena N, Vishnuvardhan Rao M, Bhaskarachary K: Effect of boiling and juicing on the content of polyphenols, Invitro bioaccessibility and antioxidant activity of commonly consumed vegetables and fruits. Indian J Nutr Diet. 53: 365-379, 2016.

39. Noor Ahmed M, Sinha SN, Sudershan Rao V, Sivaperumal P, Vasudev K, Shaik Ashu, Vishnu Vardhana Rao M, Bhatnagar V: Accumulation of polychlorinated biphenyls in fish and assessment of dietary exposure: a study in Hyderabad City, India. Environ Monit Assess.188: 94, DOI 10.1007/s, 2016.

40. Purna Chandra M, Kondaiah P, Ravindranadh P, Ghosh S, Mahavan Nair K, Raghu P: Eicosapentaenoic acid inhibits intestinal β-carotene absorption by downregulation of lipid transporter expression via PPAR-α dependent mechanism. Arch Biochem Biophys. 590: 118-124, 2016.

41. Radha Krishna KV, Hemalatha R, Raja Sriswan M, Babu Geddam JJ, Balakrishna N: Do South Indian newborn babies have higher fat percentage for a given birth weight? Early Hum Dev. 96: 39-43, 2016.
42. Raghu G, Sneha Jakhotia ,Yadagiri Reddy P, Anil Kumar P, Bhanuprakash Reddy G: Ellagic acid inhibits non-enzymatic glycation and prevents proteinuria in diabetic rats. Food Func. 7: 1574-1583, 2016.
43. Ramakrishna D, Nagasuryaprasad K, Venu Gopal G, Suresh P, Vijayalakshmi Venkatesan: Carcinosin 30 C protects islets against STZ induced beta cell dysfunctions. World J Pharm Res 5: 753-768, 2016.
44. Ramakrishna UV, Shyam Perugu, Sinha SN, Vakdevi V: Drug-repositioning of the gallate group from EGCG for the repurposing. Eur J Pharm Med Res. 3: 253-262, 2016.
45. Ramulu P, Amruth Rao P, Dhanunjaya D: Prevalence of non-alcoholic fatty liver disease (NAFLD) in type 2 diabetic patients in correlation with coronary artery disease. IAIM. 3: 118-128, 2016.
46. Ramulu P, Amruth Rao P, Kanthi Swaroop K, Paul Marx K, Venkata Ramana Devi Ch: A Prospective study on clinical profile and incidence of acute kidney injury due to hair dye poisoning. Int J Res Med Sci. 4: 5277-5282, 2016.
47. Ramya Y, Naveen Kumar R, Sudershan Rao V, Balakrishna N, SubbaRao GM: Food safety in domestic refrigerators - a mixed methods study to identify key messages to promote safe storage practices among households. Indian J Nutr Diet. 53: 1-14, 2016.
48. Shalini T, Sivaprasad M, Ayesha Ismail, Bhanuprakash Reddy G: Emerging problem of vitamin D deficiency in India. Proc Indian Natl Sci Acad. 82: 1381-1394, 2016.
49. Shruthi K, Sreenivasa Reddy S, Yadagiri Reddy P, Shivalingam P, Harishankar N, Bhanu Prakash Reddy G: Amelioration of neuronal cell death in a spontaneous obese rat model by dietary restriction through modulation of ubiquitin proteasome system. J Nutr Biochem. 33: 73-81, 2016.
50. Sinha N, Bhattacharya A, Deshmukh PR, Tanmay Kanti P, Yasmin S, Arlappa N: Metabolic syndrome among elderly care-home residents in southern India: a cross- sectional study. WHO South East Asia J Public Health. 5: 62-69, 2016.
51. Sinha SN, Paritosh Kar K, Shyam P, Ramakrishna UV, Thakur CP: Adefovir dipivoxil-A possible regimen for the treatment of dengue virus (DENV) infection. Chemometr Intell Lab Syst. 155: 120-127, 2016.
52. Sivaprasad M, Shalini T, Balakrishna N, Sudarshan M, Lopamudra P, Suryanarayana P, Arlappa N, Ravikumar BP, Radhika MS, Bhanuprakash Reddy G: Status of vitamin B12 and folate among the urban adult population in South India. Ann Nutr Metab. 68: 94-102, 2016.
53. Sivaprasad M, Shalini T, Das S, Vijayalakshmi V, Bhanuprakash Reddy G: Vitamins B6, folic acid, and B12: Molecular functions and prevalence of deficiency in India. Proc. Indian Natl Sci Acad. 82: 1395-1412, 2016.
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55. Sudhakar Reddy V, Bhanuprakash Reddy G: Role of crystallins diabetic complications. Biochim Biophys Acta. 1860: 269-277, 2016.
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