वार्षिक प्रतिवेदन

Annual Report

2018-19

icmr INDIAN COUNCIL OF MEDICAL RESEARCH

NIN NATIONAL INSTITUTE OF NUTRITION

आई सी एम आर – राष्ट्रीय पोषण संस्थान

ICMR - NATIONAL INSTITUTE OF NUTRITION

भारतीय आयुर्विज्ञान अनुसंधान परिषद

Indian Council of Medical Research

हैदराबाद, तेलंगाना, भारत

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RELEASE OF ICMR-NIN 'MY PLATE FOR THE DAY' AND ‘FIVE KEYS FOR FOOD SAFETY’

Shri M. Venkaiah Naidu, Hon'ble Vice-President of India during his visit to the Institute in December, 2018 has released the 'My Plate for the Day. This model plate visually depicts how a daily plate has to look if the foods one has to consume in a day are kept in a raw form in a plate to meet 2000 Kcal or energy requirements. My Plate is designed in such a way that cereals to meet not more than 45% of the total calories, while pulses, legumes including meat or egg should provide about 15% calories, while visible fats and oils should be limited to not more than 15% of energy. And, at least 10% each from food groups like nuts and oil seeds, fruits and vegetables; milk and curd. The diet with the suggested amounts of all the food groups will provide the necessary essential amino acids (proteins) and the essential fatty acids (vitamins & minerals). This plate is designed based on the habitual dietary intakes of Indians and if followed will not only help maintain health and nutritional status, but will also ensure that micronutrient requirements are met and NCDs are kept at bay.

Based on the cultural and contextual factors that determine the safety of foods at household level, ICMR-NIN has come up with the unique 5-keys for food safety. This was based on a study conducted to develop and validate the Household Food Safety Index based on the associations between food safety knowledge, practices and enabling assets and the microbial load in food samples. After applying factor analysis technique out of 87 such variables, 11 variables have explained the microbiological contamination of foods in home kitchens. Five -key messages were developed for household food safety based on these 11 variables to ensure safety of home cooked foods. The 5-keys were developed into an innovative 'palm-shaped' pamphlet in three languages – Hindi, English and Telugu.

These were also released by Shri M. Venkaiah Naidu, Hon'ble Vice President of India during his visit.

1. Public Health Nutrition

- In spite of India's economic surge, the malnutrition (double burden of disease) is a major public health problem in India. In pursuance of goals of National Nutrition policy, sustainable developmental goals, WHO Nutrition Frame work and India's five year plans, emphasized to establish 'Nutrition Surveillance System (NSS) in India to provide early warning signs of malnutrition among vulnerable population groups to enable us to take immediate actions to mitigate the problem of malnutrition. In order to comply with the policy recommendations, the ICMR-National Institute of Nutrition Hyderabad has established NSS in 6 select states (Kerala, Maharashtra, Madhya Pradesh, Meghalaya, Odisha and Telangana) in India on a pilot basis. The main objective of the NSS is to track the Nutrition status of vulnerable population on regular basis and also presenting the real time data by using Tabs by AWWs to enable administrators to take immediate remedial actions for prevention and control of malnutrition.
- The burden of anemia is widely prevalent among all age groups, gender, physiological groups, which is a persistent public health problem in India. In spite of initiation of several nutrition supplementation programs like iron and folic acid in India, the prevalence anemia was not declined since several decades. Thus, it is time to look beyond iron and folic acid supplementation, and may consider for B12 deficiency. Therefore NIN has taken up B12 deficiency mapping in India and we would like to estimate exclusive contribution of B12 deficiency in total burden of anaemia. The findings of the study may enable us to recommend B12 vitamin supplementation.
- The high prevalence of stunting and low birth weight is also major public health problem in India. In order to prevent and control, NIN has developed a district level multi-component health and nutrition education intervention model to prevent and control malnutrition among vulnerable population groups. The intervention model has been implemented in 16 high burden districts in 5 states viz., Andhra Pradesh, Gujarat, Jharkhand, Telangana and Uttar Pradesh to prevent and control stunting and LBW in these districts. The study was implemented for last three years and a significant reduction in undernutrition among children and reproductive women was observed.
- As we know that the nutritional status of women living with HIV/AIDS is sub-optimal and improvement of their nutrition is very important in order to see good prognosis among them. For this purpose, NIN has developed special full meal supplementation program and implemented the same for 1 year. A significant improvement has been observed in their body weight, BMI and CD4 count.

2. Impact evaluation of Karnataka Multi Sectoral Nutrition Pilot Project

The Government of Karnataka initiated the Comprehensive Nutrition Mission to address the underlying prevalence of undernutrition and to clip the gaps in the existing/on-going nutrition programs. The mission has been implementing Karnataka Multi-sectoral Nutrition Pilot (KMNP) project with the objective to reduce malnutrition by increasing utilization of services related to nutrition services for children <3 years, adolescent girls, pregnant women and lactating mothers in the selected two blocks on a pilot basis. An impact evaluation was carried out by collecting quantitative and qualitative data using mixed methods approach with the objective to assess the impact of the KMNP interventions on the nutritional status of under three-year children and adolescent girls. The findings show that there was a significant difference in the intervention blocks compared to the control blocks in the nutritional status as indicated by lower stunting of children and lower anemia in adolescent girls in the Intervention group compared to the control group. There was a significant difference in the intervention blocks compared to the control blocks on awareness of nutrition, health and sanitation related issues and utilization of various government programs, which were better off in the Intervention blocks compared to the control block.

3. Development of easy to use R programming package "ICMR_NIN_STAT" for commonly used statistical tests in medical research

Commonly used software/programs in medical research include SAS, SPSS, Stata, graph pad, Epi-info and Excel, which are cost prohibitive to purchase for researchers from developing countries. R programming language or Environment has become the lingua franca of statistical software over the last two decades. The R Programming Environment is a widely used open source system for statistical analysis and statistical programming. The objective of this study was to develop easy to use R programming package for commonly used statistical, medical and nutrition diagnostic functions in medical and nutrition research. An easy to use package was developed and it includes several functions for data importing and exporting, screening of data, data cleaning, data preparation, descriptive statistics, inferential statistics and reporting and common diagnostic functions in used in medical and nutrition research.

4. Technology development and efficacy testing of extruded rice fortified with iron, folic acid and vitamin B12

Fortification of rice with micronutrients using extrusion technology is considered a sustainable strategy to prevent nutritional deficiencies in general population. The objective of the present study is to assess the retention, stability and iron bioavailability from indigenously developed triple fortified rice (iron, folic acid, vitamin B_{12}) during rinsing and different cooking methods. Further, we also assessed the acceptability of fortified rice in adult human volunteers. The retention of iron during rinsing with excess water was >95%, while folic acid and vitamin B_{12} levels were reduced by ~25%. Watertight cooking (in electric cooker or on flame) of rice had no additional effect on the nutrient levels as compared to rinsed rice, implying their stability during cooking. However, cooking with excess water followed by decanting led to 50% loss of iron and ≥75% loss of folic acid and vitamin B_{12}. The dialyzable iron and ferritin synthesis in Caco-2 cells was higher from fortified rice compared to unfortified rice. Further, triangle tests in adult human subjects revealed that there are no significant sensory differences among fortified and unfortified rice. These results suggest that the iron, folic acid and vitamin B_{12} fortified rice has high retention and stability of fortified nutrients and is acceptable for consumption in adult human volunteers.

5. Effect of zinc supplementation prior to iron on iron absorption, and iron status in deficient rats: report of *in vitro* studies

The absorption of dietary iron is influenced by numerous dietary and physiological factors. We have previously demonstrated that zinc treatment of intestinal cells increases iron absorption via induction of the apical membrane iron transporter divalent metal iron transporter-1 (DMT1). Now we have investigated the effect of zinc on iron uptake, iron transporter and iron regulatory protein (IRP 1 and 2) expression and the impact of the PI3K pathway in differentiated Caco-2 cells, an intestinal cell culture model. It is found that zinc induces DMT1 protein and mRNA expression. Zinc-induced DMT1 expression and iron absorption were inhibited by siRNA silencing of DMT1. Further, zinc treatment led to increased abundance of IRP2 protein in cell lysates and in polysomal fractions, implying its binding to target mRNAs. Zinc treatment induced Akt phosphorylation, indicating the activation of thePI3K pathway. LY294002, a specific inhibitor of PI3K inhibited zinc induced Akt phosphorylation, iron uptake, DMT1 and IRP2 expression. Further, LY294002 also decreased the basal level of DMT1 mRNA but not protein expression. siRNA silencing of IRP2 led to down regulation of both basal and zinc induced DMT1 protein expression, implying possible involvement of post-transcriptional regulatory mechanisms. In agreement with these findings zinc treatment stabilized DMT1 mRNA levels in actinomycin-D treated cells. Based on these findings, it can be concluded that zinc-induced iron absorption involves elevation of DMT1 expression via stabilization of its mRNA, viaaPI3K/IRP2-dependent mechanism.

6. Status of micronutrients and its influence on molecular mechanisms in diabetic nephropathy

Diabetic nephropathy (DN) is the most frequent cause of end-stage renal insufficiency. The results of study demonstrate altered vitamin status in chronic kidney disease (CKD) patients (with and without diabetes). Retention of vitamins in the circulation of CKD patients was associated with molecular mechanisms of kidney dysfunction. The plasma levels of minerals were found to be low in the CKD patients. These results indicate blood/plasma levels of vitamins might not necessarily represent their adequacy/inadequacy status in DN patients because cellular nutrition depends on the normal uptake of nutrients at the cellular level followed by effective utilization at the tissue level.

7. Developed a non-invasive nanoparticle mediated delivery of triamcinolone acetonide for diabetic retinopathy

Diabetic retinopathy (DR) is the leading cause of visual impairment and blindness worldwide. Current day treatment of DR relies heavily on invasive techniques such as intravitreal injections of therapeutic agents. To date, there has been no non-invasive drug delivery system reported for DR treatment. We developed a core-shell nanoparticle-based delivery system loaded with triamcinolone acetonide and evaluated its efficacy in a DR rat model. The drug loaded nanoparticles significantly improved structural and functional aspects of retina as compared. This study demonstrates the potential of a nanoparticulate delivery system for use as a topical formulation for treating DR.

8. 4-PBA prevents diabetic muscle atrophy by modulating ER stress response and ubiquitin-proteasome system

In this study the role of ubiquitin-proteasome system (UPS) and ER stress in the brain of diabetic rats and examined the therapeutic effect of a chemical chaperone, 4-phenylbutyric acid (4-PBA) was investigated. 4-PBA intervention attenuated ER stress, alterations in ubiquitin-proteasome system (UPS), and ER-associated protein degradation (ERAD) components in diabetic rats. Importantly, neuronal apoptosis was lowered in 4-PBA-treated diabetic rats. These studies suggest that altered UPS could be one of the underlying mechanisms of neuronal apoptosis in diabetes and chemical chaperones such as 4-PBA could be potential candidates for preventing these alterations under hyperglycemic conditions.

9. Proteasome inhibitory potential of cinnamon extract in prostate cancer: *In vitro* and *in vivo* studies

Cinnamon extract and its components (*Procyanidin B2, Cinnamaldehyde, Cinnamic acid and Eugenol*) inhibited the catalytic enzymes of the 26S proteasome, decreased cell viability and led to apoptotic cell death of human prostate cancer cells. Treatment with cinnamon and its compounds also resulted in suppression of angiogenic and anti-apoptotic gene markers. Interestingly, cinnamon extract and its bioactive compounds had a minimal effect in inhibiting the proteasome or decreasing the viability in normal cells. In conclusion, the results from this project demonstrate that cinnamon and its active components act as proteasome inhibitors and anti-cancer agents.

10. Impact of vitamin D deficiency on the cardiovascular function in a rat model

Herein, whether vitamin D deficiency induces oxidative stress and fibrotic changes in the rat heart and whether the changes observed can be reversed upon rehabilitation of the vitamin D deficient rats with control diet was assessed. Vitamin D deficiency led to increase in protein carbonyls, altered activity of antioxidant enzymes and increase in nitrate levels in the heart. These results suggest that vitamin D deficiency leads to increase in oxidative and nitrosative stress in the rat heart. Further, vitamin D deficiency appeared to lead to fibrotic changes in the heart. Supplementation of the deficient rats with vitamin D corrected all the changes.

11. Studies on Xanthophylls: Dietary sources, processing, bioavailability and biological effects

A study was carried out to screen common plant foods for xanthophylls content and studied stability during storage, processing, bio-accessibility and bioavailability in rats and humans. The study generated a database on the composition of bioactive xanthophylls in commonly consumed foods using validated HPLC techniques. The study demonstrated that domestic cooking methods decreased the xanthophyll contents as compared to the fresh samples. The retention was higher in most samples when cooked by microwave or steaming methods. The storage stability of xanthophylls in fruits studied at room temperature showed higher levels as compared to those stored in refrigerator. The invitro bioaccessibility of xanthophylls lutein and zeaxanthin in vegetables ranged from 30-59%. The invivo bioavailability studies of lutein in gerbils from cooked green leafy vegetables amaranthus and spinach was 59 and 55%, bioavailability of zeaxanthin from cooked maize was 24% and bioavailability of β-cryptoxanthin from ripe papaya was 36%.

12. Prebiotic effect of legume raffinose family oligosaccharides

Effect of commonly consumed legume prebiotic oligosaccharide on high fat induced obese mice model was studied. The gain in body weight was observed to be more in high fat fed group whereas green gram fed animal group exhibited lower body weights when compared to other legume fed groups. After 18 weeks of the study, oral glucose tolerance test (OGTT) was carried to see the effect of legume prebiotics on the amelioration of insulin resistance. The results of oral glucose tolerance test revealed that high fat fed group exhibited higher glucose levels for all the time-points i.e 0,30,60,90,120 minutes respectively. Green gram fed group showed lower glucose levels when compared to other legume fed groups.

The oligosaccharide fermentation in the caeco-colon by the bacteria can give many positive health benefits as prebiotics. The prebiotic potential of legume oligosaccharides on the control of obesity high fat induced obesity was carried out in animal model. The results of the prebiotic potential of legume oligosaccharides were also promising since there was a decrease in blood glucose level, improved lipid profile, and improved body mass composition.

The ceacum sample analysis showed that increase in the shot chain fatty acid. The ceacum content analysis for gut bacteria by conventional method showed that increase in the colonies of beneficial bacterial counts.

13. Gluten intolerance in India: Prevalence, food gluten level and intake rates and whether fermented products are remedy for celiac disease?

Determination of levels of gluten in non-gluten foods such as oat and rice as well as gluten free products to measure gluten cross contamination. Estimation of gluten contamination in flour samples collected directly from mills in different rural areas to analyze cross contamination in non gluten products.

About 90-92% of food samples labeled as gluten-free are strict gluten-free, while other samples contained gluten levels marginally above levels of codex standards. Nearly 70% of local brands, 30% of mill samples and 13% of branded samples were contaminated with gluten levels above codex standards in naturally gluten-free food samples. Natural gluten-free flour samples collected directly from local mills and unbranded products contained a considerably high amount of gluten. Over 36% and 9.8% of unclaimed and claimed gluten-free products contained gluten levels above codex standards (20mg/kg).

In unclaimed products, the category in grain flours (35.9%) and oat (85%, 11.67-1830mg/kg) as the main ingredients are highly contaminated with gluten. The consumption of oats and flours from local brands and flours obtained from common flouring mill could be a high concern in celiac patients although it is of non-gluten origin, as even trace levels of gluten are risky for celiac patients.

14. A Hospital based survey on the prevalence of food allergy was conducted with the objective to assess the prevalence of reported food allergy at hospitals, in and around Hyderabad and to list the food items causing allergy, as reported by subjects which was confirmed by using skin prick test (SPT), serum IgE, serum histamine and food specific IgE estimates. The results of the study showed that almost 17% of the patients were atopic and were most sensitive to orange, papaya, guava, drumstick, prawns etc,

15. *Staphylococci* contamination and the risk associated with production of toxin in milk products

The study was planned with the objective to isolate and identify Staphylococcus from milk products and to evaluate the percentage of enterotoxin producing coagulase negative and positive strains of Staphylococcus in milk products. The results of the study showed that among 400 milk products analyzed S. aureus contamination was found to be highest in Khoa (66%) when compared to other milk products.

16. Studies on the food system of the Meitei community of Manipur and its nutritional implications

The study assessed the availability, accessibility and dietary utilization of foods consumed by the Meitei community of the lowland valley from ten different villages. Total of 313 foods were recorded to be consumed by the Meitei community through focus group discussion and indepth interview. Among those, 95 foods found to be indigenous thus analyzed for the nutrient composition and phytonutrient profile. Many of the indigenous leafy vegetables were found to be rich in micronutrients like B1, B5, B9, vitamin C, β-carotene, and minerals such as Fe, Mn, K, and Mg. High antioxidant activity was found in the leafy vegetables such as *Cissus adnata, Nelumbo nucefera* and fruit like *Euphoria longana.* Nutritional status of the Meitei community was studied in 1920 households by measuring basic anthropometry and dietary intakes were studied in 240 households. Low intake of milk and milk products was observed in all the age groups of the community. The community also had very low intake of vitamin A. However, both over-nutrition and under-nutrition was found in the adults of this community.

17. Dietary intake of aflatoxins from spices risk assessment

The study was undertaken to assess the extent of aflatoxin and ochratoxin contamination in various spices and spice blends and to perform risk assessment of aflatoxin exposure from spices from the available data on aflatoxin levels and dietary spice intake in the Indian context. Aflatoxins were detected in a total of 61/80 samples at levels ranging from 2.0-37µg/kg. The number of contaminated samples was more in chilli powder, nutmeg, spiced tea mixes, and RTE spice mix samples. All the chilli powder, nutmeg and spiced tea mixes analysed showed presence of aflatoxins. Levels exceeded the FSSAI specified maximum limits of 30 µg/kg in one chilli powder and 2 spiced tea mix samples. The highest mean and maximum aflatoxin level detected was in spiced tea mix samples followed by chilli powder samples. Co-occurrence of aflatoxins with ochratoxin A was detected in 56% of the samples. The level of aflatoxin intake calculated from mean and maximum aflatoxin levels was maximum from chilies followed by cumin powder, black pepper, nutmeg and mace.

18. Assessment of mycotoxin contamination in processed foods containing maize and groundnut

The study was undertaken to assess occurrence of aflatoxins, fumonisins, and ochratoxins in selected processed foods consumed as snacks and dietary accompaniments and based on maize and groundnut and other cereal products. The aflatoxin exposure from ready to eat (RTE) groundnut snacks was calculated from the aflatoxin levels present in different RTE groundnut products and amount of the product consumed. The study indicated that aflatoxin levels were highest in fried and roasted groundnut snacks particularly when they contained discoloured kernels. Around 60% of these samples contained discoloured kernels which were found to

contain aflatoxin levels ranging from 0.13-357µg of aflatoxin that was translated to 0.01-2.8 µg/g sample that exceeded the FSSAI limits of 10 µg/kg. Majority (78-100%) of the aflatoxin levels in these samples were contributed by discoloured kernels. From a total of 103 groundnut snack products, aflatoxins were detected in 51% of the samples with levels ranging from 1.0-660.0 µg/kg. Around 14% of the samples in fried groundnut, chikki, groundnut chocolate bars, groundnut masala powders and peanut butter had aflatoxin levels that exceeded the FSSAI limit of 10 µg/kg in RTE groundnut products. Highest aflatoxin level was observed in chikki and groundnut masala powders.

19. Investigation of mycotoxin contamination in herbal and medicinal plants and products to formulate prevention and control strategies

The present study was undertaken to investigate fungal and mycotoxin contamination in selected herbal and medicinal plants that are being utilized for health or therapeutic benefits. A total of 35 samples comprising 15 types of botanicals that are routinely used for health or medicinal benefits and included in the list provided by FSSAI and 51 powdered herbal mixes were analysed for aflatoxins, ochratoxin A and fumonisin B1. The results of analysis showed that majority contained aflatoxins (85%), 18.8% contained ochratoxin A and none showed presence of fumonisins. High aflatoxin levels that exceeded 20 µg/kg were detected in Daru haldi, Brahmi, Bhoj patra, Amla seeds, and dry ginger. The study carried out indicated the potential for many botanicals that are being consumed for therapeutic or health benefits to get contaminated with mycotoxins through the use of contaminated raw materials in their preparation.

20. Assessment of chemical contaminants in fresh/ packaged/ bottled tender coconut water

Samples of fresh tender coconut water (FTW) collected from Andhra Pradesh and Kerala (n=127) did not show pesticide residues. While, samples (FTW) collected from Tamilnadu (n=34) were detected with Monocrotophos (n=4) (TxD, Malayan Dwarf, COD) and Malathion (n=5) (COD and TxD) residues. Similarly, packaged tender coconut water (PTW) samples (n=126) also contained Monocrotophos (n=1) (Real active) and Malathion (n=4) (Cocojal, Madhura Coco Fresh, Cocoma and Coconad) residues. Both FTW and PTW samples contained heavy metals except Mercury and Arsenic. Only one sample of FTW collected from Kerala contained Arsenic.

21. Assessment of children who are helping their parents in agricultural farms of their own and monitoring their health and the health of their mothers with respect to exposure to pesticides

The community based cross-sectional study among the farm women (24-45 years, exposed=129, control = 134) (belonging to Ranga Reddy district of Hyderabad, Telangana), were detected with residues of OPs (Chlorpyrifos, Diazinon, Malathion and Monocrotophos) in their blood samples. This might have led to alterations in the Acetylcholinesterase (AChE) levels, oxidative stress parameters, CD cell markers, hormone levels and low levels of micronutrients viz., vitamins A, D, E, Calcium, Copper, Magnesium, Manganese and Zinc. Similar observations were made among the farm children (9-12 years: exposed = 66, control = 69 and 13-15 years: exposed = 63, control=65). However, supplementation with micronutrients (9-12 years: n = 54 and 13-15 years: n = 56) has improved the levels of vitamin E, copper, magnesium, zinc (both age groups), manganese (13-15 years) and enzymatic activities like AChE, lipid peroxidation (both age groups), catalase levels (13 - 15 years) among the post-supplemented children. This could be due to enhanced metabolism/excretion of residues in the body.

22. Development and validation of a comprehensive index for assessing food safety at household level

A cross-sectional study conducted among primary home food preparers (N=400) in rural and urban (@200 each) areas of Telangana helped develop and validate a household food safety index (HFSI). An 87-item comprehensive index questionnaire covering variables like knowledge, practices and enabling-environment was developed and associated with presence of high risk food borne pathogens in samples. Of them, 11 index variables were found to be significantly associated with food contamination. These 11 key variables were used to develop a household food safety index (HFSI) that can rapidly ascertain food safety status at household level. These 11 parameters were collapsed into five context-specific 5 key messages. A communication campaign was carried out among households (N= 120) using the 5-keys to food safety and after the campaign, significant improvement was observed in HFSI scores.

I. PUBLIC HEALTH NUTRITION

1. Improving health and nutritional status of vulnerable segments of population by implementing multi-component health and nutrition education intervention as a sustainable model of intervention

Despite the implementation of a number of nutritional programs for more than four decades, impact evaluations at different points of time showed limited effects. Under nutrition remains a resistant problem with 45% of children under the age of five being underweight, 22% of newborns being low birth weight and 55% of women and 70% of children being anaemic *(NFHS 3)*.

This paved way for a set of recommendations to identify a few implementable strategies to combat the problems of undernutrition, which could be taken up in few severely affected districts in India. However, one of the major recommendations was to develop a model that could promote multi-disciplinary convergence to identify the existing lacunae in implementation of the current programs, promote effective implementation of the same by instituting an independent supervisory and quality control mechanism, which would be in a self-sustaining nature by the government. The aim of the project is to develop a district specific intervention model in high burden districts in India to reduce the persistent problem of undernutrition (stunting and anaemia) among mothers and children in the select states. For the purpose, each district specific multi-component health and nutrition education intervention strategies were developed and implemented in 3 phases in 7 Districts of Gujarat, 5 districts of Jharkhand, one district each in Andhra Pradesh and Telangana by the ICMR-National Institute of Nutrition, Hyderabad during 2016-2020.

Objectives

- The first, second and third phases were formative research, development of intervention strategies & implementation, and evaluation of the effect of the interventions respectively.
- Based on the formative research in phase-I, in phase-II nutrition education intervention strategies were developed, pretested, piloted, finalized and implemented for at least one and half years and evaluated for their impact. In the final phase, impact evaluation was carried out after one and half years of regular implementation of an intervention programme.

Methodology

Sampling Design: It was a community based cross sectional study carried out by using systematic sampling procedure. 7 districts and 2 blocks from each district were selected. From each block, 5 villages and 50 HH from each village were covered. From each selected HH, one under 5 child was covered for various investigation such as ANC care practices of mothers of <12 month old children, Infant and young child feeding practices (IYCF), feeding practices, Vitamin A supplementation, Iron and folic acid (IFA) supplementation, hand washing practices of mothers and anthropometric measurements of mother and children were carried out. Haemoglobin analysis was done using the indirect cyanmethmoglobin method.

Intervention materials

These included posters, banners and flow charts that were explained to all the beneficiaries in the house to house visits and also group meetings were held with pregnant and lactating

women, adolescent girls and health and nutrition education were imparted to them through posters and banners. Every household (HH) having pregnant women, lactating mothers and mothers having at least one under 5-year child was visited at least 3 times by the project staff to impart health and nutrition education.

Types of interventions: Based on the findings of formative research, district specific intervention strategies were developed and implemented in 14 districts in 4 states and the important education material such as posters, flip charts, table calendars, banners, which were developed, finalized after pre-testing and used for imparting education to the adolescent girls, pregnant women, lactating mothers. The mode of education was person to person, group meetings, and it was ensured that at least 7-9 times the project staff has interacted with each of the beneficiaries in the target areas for counselling. The results are very encouraging observed in the state of Jharkhand in the service delivery, practices of mothers (Fig.1) and reduction in the undernutrition (Fig 2).

Fig 1. Percentage changes in the infant and young child feeding (IYCF) practices of mothers of young children in the state of Jharkand after 18 months of interventions

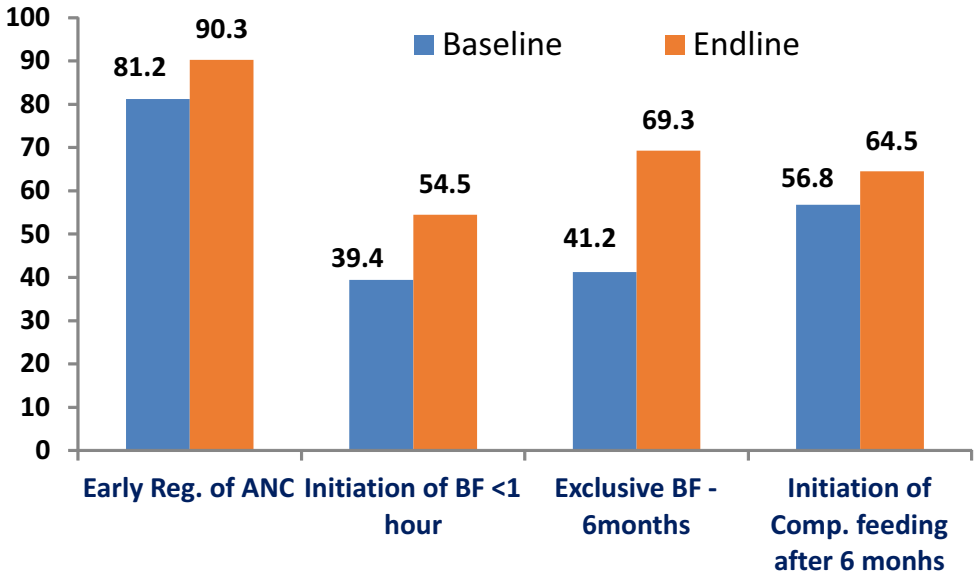
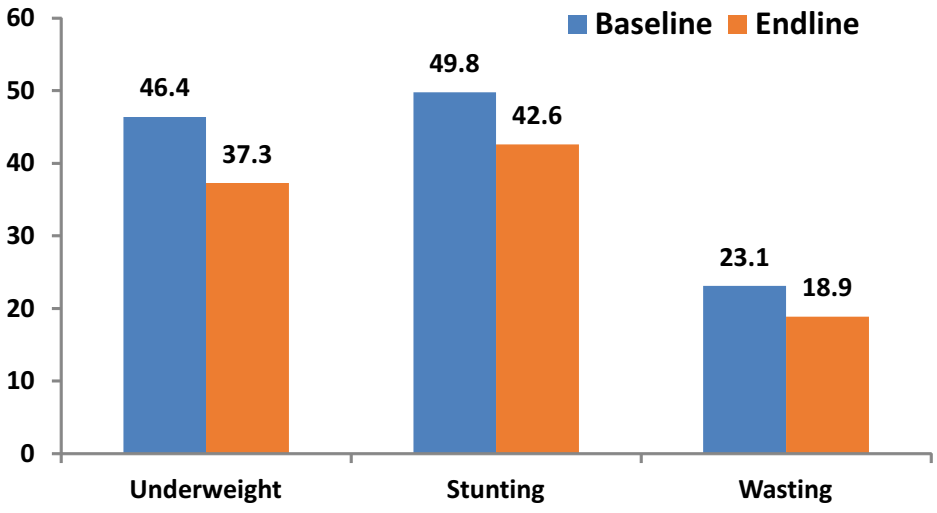


Fig 2. Prevalence (%) of undernutrition (underweight. Stunting and wasting) among under five-year children in Girdi districts of Jharkhand



Similarly, very encouraging results were observed in the state of Gujarat, where the intervention model was implemented in 7 districts (Fig 3 & Fig 4).

Fig 3. Percentage changes in the infant and young child feeding (IYCF) practices of mothers of young children in the state of Gujarat after 18 months of interventions

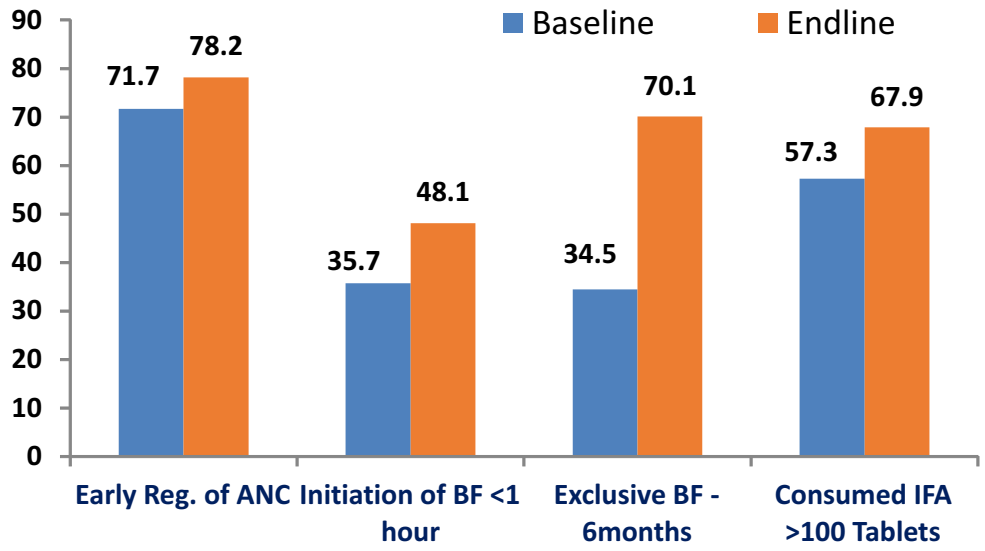
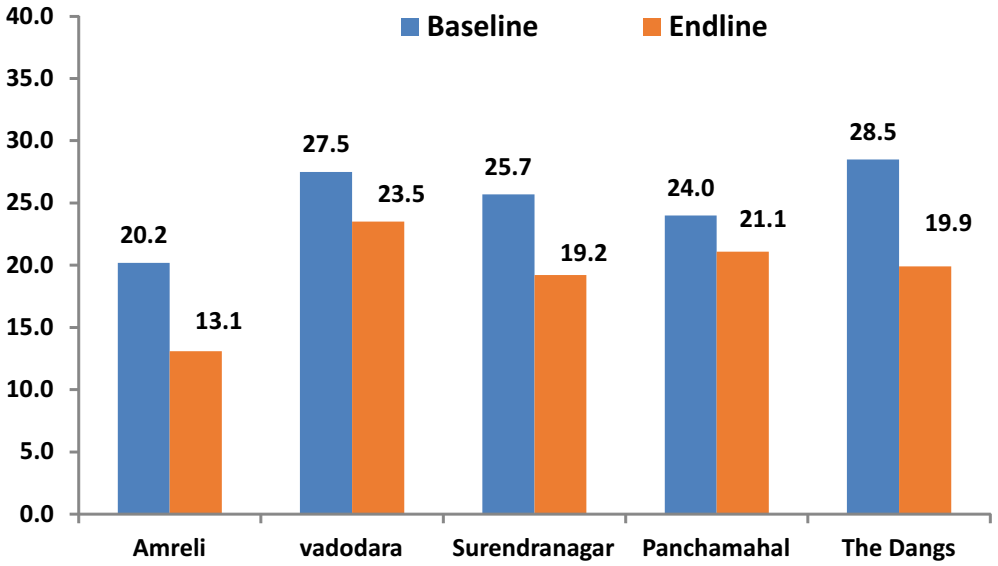


Fig 4. Prevalence (%) of wasting among under five-year children in 5 districts of Gujarat



2. Nutritional status of women living with HIV/AIDS and impact of food based approach on disease progression

Deficiency of micronutrients such as Vit A, E, B, selenium, zinc has been associated with accelerated disease progression and increased mortality and it is also known that micronutrient supplementation can delay HIV disease progression and reduce mortality in HIV-positive people. Therefore, a study was carried out to understand the impact of food and micronutrient

support on disease progression. The study was conducted with the objective to assess the nutritional status of adult female people living with HIV (PLHAs) and to assess the impact of food-based approach with micronutrient supplementation on disease progression.

Objectives

To assess the nutritional status of adult female people living with HIV (PLHAs) attending ART centers and impact of food-based approach with micronutrient supplementation on disease progression.

Methodology

The subjects were selected by simple random sampling procedure from the Pre-ART registers and were purposively allocated to two groups based on their CD4 cell count at baseline. The purpose of categorizing the study subjects into two groups was to examine the impact of the intervention of food on the subjects who were receiving ART and compare with the Pre ART subjects who were not receiving ART treatment.

All the adult females attending the ART centres were considered as prospective subjects. The study population consisted of female PLHAs who were registered members of the ART centre. They were initially identified from the pre-ART registers and all those willing to participate in the study were selected and recruited into the study. Eligible participants were consecutively assigned to either of the two groups based on the CD4 cell count till the total sample size of 100 is achieved. All the recruited subjects were provided with the designed food basket (Table 1 & 2).

Table 1. Protein and Energy Composition of the Supplementation per subject/day

Ingredients	Parts	Quantity (gms)	Energy (kcal)	Protein (gms)
Rice	3	100	345	6.8
Pulses (red gram)	2	60	201	13.4
Egg	1	50	87	6
Milk powder	2	50	230	10
Total			863	36.2

Table 2. Composition - Micronutrient Premix

Components	Quantity	RDA	Components	Quantity	RDA
ß-Carotene	5000 mcg	2400 mcg	Piridoxin Vit-B6	5 mg	2.0 mg
Vit – D3	200 IU	600 IU	Folic acid Vit-B9	0.20 mg	100 mg
Vit – C	50 mg	40 IU	Calcium	1000 mg	400 mg
Thiamine Vit- B1	2 mg	0.9 mg	Zinc	5 mg	8 mg
Riboflavin Vit-B2	2 mg	1.1mg	Selenium	30.0 mcg	55 mcg
Niacin Vit -B3	25mg	12mg			

Results

A total of 199 (ART -100, Pre ART- 99) female PLHAs were recruited into the study and baseline investigations were carried out on them. Twenty-four-hour recall diet survey was done on 190 subjects (ART-94, Pre ART- 86). The intervention food basket consisting of food as well as micronutrient premix was given to 178 subjects. However, only 153 subjects could be retained at the end of 12 months follow up and for end line investigations.

- About 40% subjects in the pre-ART group and 51% of subjects in ART group were divorced.
- About half of the subjects, among both the groups were having a monthly income of Rs.5000-10000/-.
- The consumption of different foods by the subjects in both the groups was less than the RDI, but current consumption levels in the pre-ART group were marginally better as compared at baseline.
- Similarly, the intake of different nutrients was less than RDA among both groups, however, its consumption was better among the ART group as compared to the pre-ART group, at baseline.
- With the supplementation of foods and vitamins and mineral premix, there was a marked increase in the intake of foods and nutrients in both the groups at the end line.
- The prevalence of CED (BMI <18.5) was high among the subjects of ART group (35%) as compared to those in the Pre ART group (10.1%), at baseline.
- There was a marked reduction in the prevalence of CED among both the groups after 12 months of food, nutrient and mineral supplementation (Pre-ART: 10.1% to 5.6%; ART: 35% to 16.7%).
- The mean CD4 cell count was 598 cell/cumm in pre-ART group and 276 cell/cumm in ART group.
- The mean serum albumin levels ranged between 4.13g/dl to 4.64g/dl, among the both the group of subjects at baseline and endline, which was well within the normal range limits (3.8g/dl-5.5g/dl).
- The proportion of subjects with serum pre-albumin levels <15mg/dl declined among subjects in both the groups, from 21% to 9% in pre-ART group and from 39% - 20% in the ART group.

3. Monitoring of the national non-communicable diseases (NCDS) targets 2016-17: A multi-centre study

The WHO estimates of 2010 for India indicated an estimated 60% of all deaths attributed to NCDs in India. The Global Burden of Disease 2010 estimates for India show an increase in DALYS for ischemic heart disease, stroke, diabetes, major depressive disorders, chronic obstructive disorders, cirrhosis of liver, migraine and low back pain. The leading burden of disease attributable to 15 leading risk factors in 2010, expressed as a percentage of India DALYs are dietary risks, household air pollution, smoking, high blood pressure, childhood underweight, occupational risks, ambient particulate matter pollution, high fasting blood glucose, iron deficiency, alcohol use, physical inactivity, suboptimal breastfeeding, high body mass index, high cholesterol and sanitation. Several surveys and studies have shown the high prevalence of risk factors like tobacco consumption, alcohol use, low consumption of fruits and vegetables, physical inactivity, high blood pressure, elevated blood glucose levels, overweight and obesity in the population. The key determinants driving these risk factors include low levels of education, poverty, poor housing conditions, inadequate spaces for physical activity etc.

Recognizing the limitations, challenges and the need for a robust system for monitoring and evaluation of NCDs and their risk factors to produce evidence for policy and strategies for

prevention and control of NCDs, the Indian Council of Medical Research (ICMR) was identified as the nodal agency for monitoring, evaluation and surveillance of the national NCD monitoring framework. At recommendations of the ICMR's Apex Committee on NCD Surveillance and the National Technical Working Group (TWG), assessing the status of NCD status in the country in order to achieve the national NCD targets in 2017, 2020 and 2025. The same protocol would serve as a prototype for future such surveys with some modifications as felt appropriate by the TWG at the time of the future surveys (2020, 2025). States could adopt the protocol to conduct similar surveys to arrive at State based estimates in the corresponding time periods. ICMR will provide all technical and operational support in conducting these surveys. These activities will help in strengthening national and sub-national capacities to monitor NCDs and their risk factors and setting up of their surveillance mechanisms.

The national NCD monitoring framework has 21 indicators, of which 3 are related to adolescents (defined as 10-19 yr age group), and the remaining to adults (18-69 years age group) and health system coverage. In order to maximize ethical and operational logistics, the present survey covered 15-69 years, in which adolescent age categories from 15-19 years were covered.

Objectives

Primary objective

To generate country/national level estimates of key NCD related indicators (risk factors and health system response) identified in the national NCD monitoring framework for the year 2017.

Secondary objectives

1. To create a central and regional pool of resources (protocols, standard tools, training manuals etc.) to support the conduct of similar surveys at state level.
2. To strengthen capacities for monitoring of NCDs at the national and sub-national level

Methodology

The detailed methodology of the present study was given in the last year Annual Report (Plan of work 2017-18). It is a cross Sectional community based study carried out by adopting a stratified sampling procedure. It was a multi-centre study carried out at 11 sites in India by various Institutions and research organizations. The target age group for the present study was 15 – 69 year age group. The overall sample size of 7,200 for adults (20-69 years) is based on 20% relative precision and the sample size of 4,800 for adolescent (15-19 years), (to estimate the prevalence of obesity and tobacco use), is based on relative precision of 30%. The design effect is 1.5 and the response rate is 85% for adults and adolescents.

Investigations include an assessment of Socio-economic status, Anthropometric measurements, blood pressure measurement, risk behaviours and estimation of biomarkers like fasting blood glucose and urinary sodium excretion (on sub-sample) in spot urinary sample was carried out. In sampled health centres, health facility assessment was also carried out.

Results

A total of 10,659 households were covered for this survey in India. Of them, 45.1% were pucca houses and 38.2% of rural households had no access to a toilet facility. The mean number of servings of fruit and/or vegetables per day was 1.7 and the consumption of fruits and/or vegetables was inadequate with 98.4% of adult reported consuming less than five servings per day (men 98.0% and women 98.8%). The estimated mean salt intake per day was 8.0g (8.9g in men and 7.1g in women per day); and Mustard oil was the most frequently used oil for cooking (48.8%).

The prevalence of current use in any form of tobacco was 32.8% and 28.0% used tobacco daily. A total of 15.9% (men 28.3% and women 2.4%) of respondents reported drinking alcohol

in the past 12 months, and only one in every 20 respondents (5.9%) was an episodic heavy drinker. With respect to adolscents, 3.5% were ever users of alcohol, and 1.3% reported consuming alcohol in the last 12 months.

Almost half of the adult respondents (41.3%) did not meet WHO recommendations on physical activity of 600 Metabolic equivalents (METS) per week and the corresponding figure reported for adolescent respondents was 25.2%.) One in every five (19.9%) and more than one in twenty (6.2%) were overweight and obese, respectively. The overall prevalence of overweight with BMI ≥25.0 Kg/m2 was 42.5% and 18.0%, in urban and rural areas, respectively. The prevalence of overweight and obesity among adolescents 6.2% and 1.8%, respectively. The prevalence of hypertension among the adult population in India was 28.5%.Of them 20.6% of cases were newly diagnosed, while 7.9% were reported cases. Similarly, the prevalence of diabetes mellitus in India was 9.3%.

Conclusions

The National Non-communicable Disease Monitoring Survey has provided a comprehensive data set for the selected indicators of the National NCD monitoring framework for India. The presence of NCD risk factors at high proportions among adults and adolescents residing in urban and rural areas calls for stepping up of response to provide affordable healthcare in an accessible manner universally to all. Efforts to strengthen surveillance of NCDs need to be put in place and strong multisectoral actions will help in mitigating several NCD risk factors.

4. Effectiveness of diet and life style intervention through information education communication (IEC) tools with anganwadi workers (AWCS) as the centre of knowledge dissemination for hypertension (including hypercholesteremia and diabetes) risk reduction - A cluster randomized controlled trial

Hypertension is one of the important risk factors for cardiovascular diseases (CVD) and it was estimated (2000) that there were 972 million hypertensives, of which 333 million are in developing countries and 639 million in economically developing countries and it has been projected that by the year 2025, this will reach to 1.56 billion i.e. approximately 1 in 3 adults aged over 20 years will have hypertension, according to an analysis published in the January 15 issue of *The Lancet*. The overall prevalence of hypertension was 26.4% in 2000, and is projected to increase to 29.2% by 2025.

Very few studies have been carried out in tribal population to address the problem of hypertension. A recent study carried out by NNMB/NIN (ICMR) during 2008-09 in the tribal population in nine States (Kerala, Karnataka, Tamil Nadu, Andhra Pradesh, Maharashtra, Madhya Pradesh, Gujarat, Orissa and West Bengal) reported that the prevalence of hypertension was about 25% in men and 23% in women.

Hypertension is mostly due to a sedentary lifestyle, lack of physical activity, consumption of more fatty food, overweight/obesity and alcohol intake, apart from other non-modifiable factors such as age, sex, occupation, noise pollution and family H/o hypertension. It was

observed that the knowledge about hypertension was very low among tribals. Hence it was proposed to carry out an intervention study among the tribal population to know the effects of nutrition and health education on blood pressure level and other cardiovascular risk factors among the adult tribal population in Adilabad district of Telangana State.

Objectives

General objective

To assess the effectiveness of diet and life style intervention through information education communication (IEC) tools with anganwadi workers (AWCs) as the centre of knowledge dissemination for hypertension (including hypercholesteremia and diabetes) risk reduction.

Secondary objectives

- To assess the operational feasibility of integrating NCD risk reduction in community health programs through existing community level healthcare volunteers such as ASHA or equivalent
- To assess the usefulness of trained healthcare workers to affect changes in dietary fat, fibre and salt, tobacco and alcohol consumption and increasing physical activity and
- To assess the efficacy of these interventions to evaluate changes in lipid levels and glycemic control.

Methodology

The details of the methodology were given in the Annual Report of 2014-15. It was a community-based, cluster randomized trial, and was carried out in the selected villages of integrated tribal development agency areas (ITDA), Adilabad district, Telangana. Twelve anganwadi villages were covered randomly for the study, and 6 AWCs were experimental and another 6 were control villages (cluster randomization). Anganwadi villages formed the sampling frame. All the HHs in the selected AWC village were enumerated first and then 100-130 HHs were selected randomly to get a sample of 300 subjects' $\geq$18 years of age for the survey. So, a total of 1800 subjects from each group was covered. The investigations carried out under the project was an assessment of SES, anthropometric measurements, BIA, blood pressure measurement, estimation of fasting blood sugar, haemoglobin and lipid profile at baseline and end line after 18 months of intervention.

Results

The total number of tribal adults (>18 years and above) who participated in the study were 3630 (1804 intervention group) at baseline and 2168 (1444 intervention group) were at end line evaluation. Almost all the sample characteristics of the control and intervention group of adults at baseline were no statistically different. After 18 months of multi-component health and nutrition education intervention through the anganwadi workers, a significant reduction in the mean systolic ($p<0.007$) and diastolic ($p<0.023$) blood pressure was observed among the intervention group, but it was not observed in the control group. A significant reduction in the prevalence of overweight and obesity and abdominal obesity was observed in both delete “the” groups.

Conclusion

The study has been carried out as one of the multi-centric studies among the tribal adult population in Adilabad district of Telangana State. The study has shown a significant reduction in the mean systolic and diastolic blood pressure after 18 months of nutrition and health education intervention. A similar study may be scaled up in other districts of the state where hypertension and NCDs prevalence was high.

II. CLINICAL EPIDEMIOLOGY

1. Impact evaluation of Karnataka multi-sectoral nutrition - Pilot project

Under nutrition continues to be a major public health problem in the developing countries, including India, the most vulnerable groups being women and young children. Proper nutrition is necessary for the adequate growth and development of children. Under nutrition has a multi-factorial etiology, which include both nutrition and non-nutrition factors. Keeping in view the magnitude of under nutrition as well as micronutrient malnutrition, the Government of Karnataka has initiated the Comprehensive Nutrition Mission to address the underlying prevalence of under nutrition and to clip the gaps in the existing/on-going nutrition programs. The mission has been implementing the Karnataka Multi-sectoral Nutrition Pilot (KMNP) project with the objective to reduce malnutrition by increasing utilization of services related to nutrition services for children <3 years, adolescent girls, pregnant women and lactating mothers in the selected two blocks on a pilot basis. The KMNP was implemented in Chincholi (Gulbarga district) and Devadurga (Raichur district) blocks of Karnataka since 2015. KMNP envisaged a lifecycle nutrition supplementation intervention that seeks to provide support at the critical phase of growth for pregnant women, young children and adolescent girls. Effectively, there are three important components of KMNP of which Components 1 and 2 were the focus of this evaluation; the third and final component focuses on administration, capacity building and internal activities for KMNP.The intervention continued till the end of September 26, 2018 and ICMR-NIN under the ambit of MoU with KCNM has carried out the impact evaluation of the KMNP project by assessing the inputs against outcome indicators with neighboring non-intervention blocks in the same district as control.

Methodology and Results

This impact evaluation was carried out by collecting quantitative and qualitative data using a mixed methods approach. It was a community-based case control study that adopted a cluster sampling procedure. Similar blocks in the respective districts in the human development index (HDI) were taken as intervention and control blocks. Chincholi and Jewargi blocks of Gulbarga and Devdurga and Lingasugar blocks of Raichur were the blocks chosen for the survey. Chincholi and Devdurga blocks were the Intervention blocks while the other two were taken as control blocks (no intervention). Study subjects were mothers of under 3 children and adolescent girls who were current beneficiaries. For the purpose of the survey, in each arm, a total of 30 villages representing the entire intervention blocks were selected by adopting a systematic random sampling procedure. In each of the selected village, a total of 20 households having at least one index child delete "of" under 3 years of age who was a current beneficiary was covered by adopting the cluster sampling method. Socio-economic and demographic particulars were collected along with details on Ante-natal Care (ANC) particulars, immunization history and morbidity, investigations like anthropometry (height, weight and Mid Upper Arm Circumference (MUAC)), haemoglobin, nutrition history, child care practices, hygiene, dietary intakes from mothers and adolescent girls. For qualitative data, Information on knowledge and practices (K&P) of adolescent girls, mothers on infant and child nutrition as well as socio-cultural aspects of food consumption were collected employing standard methodology till theoretical saturation was reached. Similarly, qualitative tools were used to assess the impact of the intervention on counseling and nutrition supplementation provided to target beneficiaries.

The results from quantitative data show that majority of the mothers in their last pregnancy had undergone ANC check-ups (>98%) in both the groups. However, the place of ANC was Primary Health Centre (PHC) (65.5%) in the Intervention blocks compared to the control blocks (51.6%) suggesting an increase utilization of government health care services. A higher proportion in the control blocks was visiting a private facility (45.6%) compared to the Intervention blocks (32.2%). The number of ANC visits was more or less similar in the Intervention and control blocks and the majority of them were attending at least 4 ANC visits. In general counseling on health and nutrition was higher in the intervention blocks compared to the control blocks during the ANC visits. A higher proportion of mothers in the intervention blocks (98.2%) were consuming extra food during pregnancy, compared to the control blocks (95.4%). Similarly, a higher proportion of mothers in the intervention blocks (95.2%) were receiving Take Home Ration (THR) during pregnancy, compared to the control blocks (88.7%). A higher proportion of mothers in the control blocks (8.9%) did not receive Tetanus Toxoid (TT) injection, compared to the intervention blocks (2.7%). The number of iron folic acid tablets received and consumed was not different between the groups.

Morbidities in children were in general lower in the Intervention blocks compared to the control blocks during the preceding 15 days. A higher proportion of the Intervention blocks received THR food compared to the control blocks. Similarly, more children received 2 doses of Vitamin A and deworming in the Intervention groups than in control blocks. A higher proportion of mothers said they would visit a private doctor in case of illness to the child in both the intervention blocks and the control blocks. About 70% of the mothers said they would give Oral Rehydration Salt (ORS) during diarrhea but was not different between the groups. About 1 in 4 mothers said that their mother in law would take care of the child, when she goes to work. A higher proportion of the mothers in the intervention group (96%) compared to the control group (58.1%) washed hands with soap before feeding the child, suggesting the impact of counseling on Water, Sanitation and Hygiene (WaSH) practices.

There was a higher proportion of mothers and adolescent girls in the Intervention block compared to the control blocks, who were aware of basic nutrition and health related issues. There was a higher proportion in the Intervention blocks were beneficiaries in the Mid-day Meal program at schools and those receiving Iron folic acid (IFA) tablets in the past one year. With respect to the nutritional status of the beneficiaries, mean Height for age Z scores, an indicator for chronic malnutrition was significantly better in children in the intervention block, while Mean Weight for height Z scores, an indicator of acute malnutrition was lower in the control blocks but not statistically significant. Stunting in children of below 3 years of age was about 6% lower in the intervention blocks (46%) compared to the control blocks (52.1%) and was statistically significant ($P < 0.05$). The overall thinness (an indicator of chronic energy deficiency) in adolescent girls was similar in the intervention blocks (30.2%) and control blocks (28.2%) and was not significant ($P=0.45$). The overall prevalence of stunting in adolescent girls was 34.3% and was similar in the intervention blocks (35.6%) and the control blocks (33.0%) and was not significant ($P=0.34$). The overall prevalence of anemia was 84.8% and was significantly lower ($P=0.001$) in the intervention blocks (81%) compared to the control blocks (89.5%) suggesting an improvement in the nutritional status of adolescent girls of the intervention blocks compared to the control blocks.

Qualitative results show that majority of the mothers and adolescent girls reported regular counselling, group meetings, house visits, and growth monitoring and food supplementation was given by Village Nutrition Volunteers (VNVs). Mothers of under 3 children reported that they found both counselling and nutrition supplementation useful. They could see a perceptible influence in their child nutritional status like weight and also a feeling of well-being. Adolescent girls also reported that nutrition education and nutrition supplementation was useful and felt an overall well-being in addition to a increase in weight. Mothers of under 3 children as well as adolescent girls reported good acceptability of shakti vita. Mothers of under 3 children and

adolescent girls felt that counseling alone was also beneficial as it has impacted their behavior change in terms of hygiene, sanitation and dietary intakes. Both, mothers of under 3 children as well as adolescent girls requested for the continuation of VNVs and Shakti vita as they found both to be helpful and have asked for the continuation of the program. Mothers of under 3 children as well as adolescent girls felt that VNVs were complementary to the services provided by AWW (Anganwadi workers).

In conclusion, there was a significant difference in the intervention blocks compared to the control blocks in the nutritional status as indicated by lower stunting of children and lower anemia in adolescent girls in the Intervention group compared to the control group. There was a significant difference in the intervention blocks compared to the control blocks on awareness of nutrition, health and sanitation related issues and utilization of various government programs, which were better off in the Intervention blocks compared to the control block. There were an overall well-being and dietary intakes after the implementation of the program in children and adolescent girls in the Intervention group as assessed by qualitative methods in these blocks.

2. Development of easy to use R programming package "ICMR_NIN_STAT" for commonly used statistical tests in medical research

In the age of evidence based medicine, data analysis is an integral component of medical research. Data analysis requires specialized software that is both cost prohibitive and difficult to learn. Commonly used software/programs in medical research include SAS, SPSS, Stata, graph pad, Epi-info and Excel. Most of the above programs are cost prohibitive to purchase for researchers from developing countries. While software such as Epi-info is free, the list of statistical tests is not exhaustive.

R programming language or Environment has become the lingua franca of statistical software over the last two decades. The R Programming Environment is a widely used open source system for statistical analysis and statistical programming. It includes thousands of functions for the implementation of both standard and advanced statistical methods and it is probably the most popular system in the academic world for the development of new statistical tools. The primary reason for its wide adoption is being an open source software and large active user community. Though it is being used in other sciences, its use has not become popular among medical researchers. An important reason for being not popular among medical researchers is, it is hard to learn and hard to use. The learning curve for R is steeper, compared to other statistical software. While there exists documentation on how to use it, in most cases it is terse enough to be not understood by medical researchers without adequate statistical knowledge. For example it is unclear for a beginner to import data into R using simple functions and then export the results back to their favourite windows based office programs such as Microsoft word and excel.

However, R is a functional programming language, where new functions can be easily created that are intuitive in nature and easier to understand by medical researchers. In this regard, packages such as Epi-calc (now renamed to Epi-Display) and Table-One are of great help to R medical user community as they were created by medical doctors. Though, these packages help in the ease of analysis, there is a felt need for a better package for medical researchers for commonly used statistical tests with proper documentation on how to use them.

Objective

- To develop easy to use R programming package for commonly used statistical, medical and nutrition diagnostic functions in medical and nutrition research.

Methodology and Results

R software version, 3.1.0 and above was used for creating the package. R Studio Desktop, an integrated development environment (IDE) for R programming language was used for simplifying package development. Intuitive functions that are understood by medical researchers have been developed for carrying out data analysis. For creating methods, S3 classes were used. The following functions developed have been classified under the following headings, which represent the common workflow of data analysis:

1. ***Data importing/exporting:*** Wrapper functions have been created around existing functions that import data into R environment. Functions on copy and paste which copies data (importing) from spreadsheets to R environment and back to spreadsheets (exporting) from R environment.
2. ***Data screening*:** Functions has been created to screen the dataset which provides information on dimensions of data (no. of variables and no. of records), factors, numeric, logical, characters, missing values, unique values, top 5 ascending and descending variables, a summary of each variable including mode and SD are displayed for the end user.
3. ***Data cleaning*:** Functions for cleaning character variables to numeric variables using regex expressions have been created. Code for factors is also generated to simplify labelling.
4. ***Data preparation:*** Functions for simpler recoding of variables have been created using the mirror vectors approach. Commonly used functions in nutrition disorders such as dietary intakes, anthropometric variables have been created that simplifies the workflow.
5. ***Descriptive statistics*:** Functions have been created based on the nature of the dependent variable and independent variable. For univariate and bivariate descriptive statistics, functions were created for normal, non-normal continuous and categorical variables after normality testing. The major focus is on the presentation style reported in medical journals. Graphs will accompany tables as objects or separately. Compare by the group have also been created for means and frequencies.
6. ***Inferential statistics*:** Functions have been created on existing R functions for inferential statistics for basic tests. Intuitive functions will be created that are understood by medical researchers. For example, parameters are set to independent and dependent variables, instead of x and y.
7. ***Reporting:*** Reporting of tables can be done either in CSV files (Excel) or in HTML files or Word documents. Reporting of graphs will be either in TNG files or PDF format.

III. BASIC STUDIES

1. Technology development and efficacy testing of extruded rice fortified with iron, folic acid and vitamin B12

Multiple micronutrient deficiencies are widespread in the Indian population. A low density of micronutrients in a staple vegetarian diet could be a major etiological factor for the widespread prevalence of the nutritional inadequacies. Though therapeutic nutrient supplementation is being practiced to correct micronutrient deficiencies, due to operational and logistic hurdles, food based approaches such as food fortification with micronutrients are preferred over therapeutic supplementation. The National Plan of Action on Nutrition, under National Nutrition Policy envisages the fortification of foods with micronutrients as an important strategy to tackle the problem. The popular vehicles for fortification include wheat flour and breakfast cereals with multiple micronutrients, common salt with iron and iodine, sugar with vitamin A, and more recently micronutrient sprinkler and micronutrient enriched beverages. However, fortification of whole cereals, grains such as rice, a major staple food in India remained a challenge until recently.

The recent technological improvements in the extrusion process enabled the fortification of rice flour with minerals and vitamins where in, a premix of rice flour having either single or multiple nutrients was extruded into reconstituted-rice kernels appearing similar to that of natural rice grains. These fortified rice grains in turn will be mixed with normal rice, at a fixed proportion to achieve mandatory fortification levels set by regulatory agencies. Earlier, the fortified ultra rice improved the iron status and reduced the prevalence of anemia in Indian children when tested as part of mid-day-meal program. Further, studies in other South-Asian countries also successfully demonstrated the efficacy of such rice fortification technology on improving the nutritional status in different physiological groups. There are no indigenous technologies available for rice fortification in the Indian context. Further, technology development and efficacy testing of rice fortified with multiple nutrients particularly iron along with folic acid and vitamin B12 needs to be attempted and tested. In this context, it is imperative to partner/ provide guidance to the interested Indian firms to develop such rice fortification technologies, based on draft guidelines recently set by the food safety standards authority of India.

Objectives

- To develop reconstituted rice kernels fortified with iron, folic acid and vitamin B12.
- To test stability, retention of fortified nutrients and bioavailability of iron from the reconstituted rice kernels.
- To test the organoleptic properties of fortified rice compared to natural rice in human subjects.
- To study the efficacy of fortified rice supplementation as part of mid-day-meal in school children for improving anemia and status of iron, folic acid and vitamin B12.

Materials and Methods

***Production of Fortified Rice Kernels*:** The extruded rice kernels were produced by a hot extrusion technology. The target nutrients, citric acid (0.5%), maltodextrin (0.5%) and other stabilizing ingredients were added to the rice flour, the dough was prepared through a pre-

conditioning process, and extruded in a Twin-Screw Extruder machine in to rice-shaped kernels. The extruded rice kernels contained 1200 mg Iron as micronized ferric pyrophosphate (MFPP, mean of particle size range from ~3.5), 13 mg folic acid and 100 µg vitamin B_{12}/100g.

Fortification: Fortification was done at a ratio of 1:100 (for testing the fortification levels, acceptability and iron bioavailability) and at 1:10 (to aid in testing the retention and stability of folic acid and vitamin B_{12}). Briefly, either 22.5 or 24.75 kg of normal rice in a steel ribbon blender (fabricated locally) was mixed with 2.5 kg (1:10 ratio) or 0.25 kg (1:100 ratio) of fortified extruded rice kernels, respectively for 3 min. The fortified rice was divided in to six equal portions and stored separately in air-tight plastic containers. The levels of nutrients in fortified rice were estimated as described below.

***Rinsing of rice*:** Six independent portions of fortified rice (0.5 kg each) were soaked in 1 L milli-Q water, swirled and left for 15 min. The water was then discarded followed by two quick rinsing cycles with 1 L water. After the third rinsing, three portions were cooked as described below while the other three portions of rice were made to paste, lyophilized to dryness and powdered using a kitchen blender.

Cooking of rice: The rinsed rice was suspended in 1 L of de-ionised water and cooked with the following three methods: 1. in an electric cooker; 2. in a pressure cooker; 3. 0.5 kg of rinsed rice was cooked with excess water (1.5 L) until the rice was cooked 80%, and after decanting the excess water, was further cooked until done. The cooked rice was then made to paste, lyophilized to dryness and powdered in a kitchen blender.

***Determination of iron*:** Iron content in powdered unfortified, premix, fortified (1:10 and 1:100 ratio) raw, rinsed or cooked rice samples were estimated as described previously (). Briefly, powdered rice fractions (0.5 g) were weighed into digestion vessels, followed by addition of 4.5 mL of 0.1 N HCl to each vessel, 2 mL of 65% HNO_3 and 1 mL of 33% H_2O_2 were added to facilitate the digestion The digestion vessels were sealed and subjected to microwave digestion (MARS XPRESS, CEM Corporation, USA). After cooling, the vessel contents were filtered, and the iron content in the digest was estimated by atomic absorption spectrometry (Shimadzu AA7000, Japan).

***Extraction and HPLC analysis of folic acid*:** Powdered rice fractions were mixed with 0.1 M phosphate buffer (pH 6.0) in a conical flask. The solution was incubated in a sonicator bath for 45 min. The suspension was centrifuged at 10,000 rpm for 15 min at 4°C, and clarified by filtration through 0.22 µm syringe filters. The folic acid content of this filtrate was analyzed by HPLC as described previously '—(). Folic acid in the sample was identified by the retention time and quantified by comparing peak area with an authentic standard. The recovery of folic acid from spiked powdered rice samples using the above extraction method was always found to be 90-103%.

***Extraction and HPLC analysis of vitamin B_{12}*:** Powdered rice fractions were suspended in milli-Q water in a conical flask. The solution was agitated in a sonicator bath for 45 min, centrifuged at 10, 000 rpm for 15 min at 4°C and filtered through 0.22µm syringe filters. The vitamin B_{12} in the filtrate was concentrated by solid phase extraction using Strata C-18-E (55 µm, 70A) columns, pre-equilibrated with 2% acetonitrile. The bound vitamin B_{12} was eluted from the column using 50% ACN and concentrated in a centrifugal vacuum evaporator, reconstituted in the mobile phase, and analyzed by HPLC as described previously (). Vitamin B_{12} in the sample was identified by the retention time and quantified by comparing peak area with an authentic standard. The recovery of vitamin B_{12} from spiked powdered rice samples using the above extraction method was always found to be 87-98%.

Assessment of in vitro dialyzability: The simulated gastrointestinal digestion was performed with minor modifications. Powdered rice samples (0.625 g) were hydrated with 10 mL of normal saline in 50 mL tubes for 30 min. The pH of samples was adjusted to 2.0 with 6 N HCl and the

final volume was made up to 12.5 mL with normal saline. 2 mL aliquot of this digesta was transferred to six-well plates in triplicates. A 0.1 mL pepsin solution (40 mg/mL in 0.1 N HCl) was added to each well, covered and incubated for 2h at ambient temperature on an orbital shaker. At the end of this incubation, a Transwell insert fitted with a 12-14 kDa molecular weight cut-off dialysis membrane (Spectra/ Por-7 dialysis tubing, Spectrum laboratories, Europe) was housed in individual wells of 6-well plate, thus creating an apical and basolateral chamber. The apical chamber was filled with 2 mL PIPES buffer (pH 6.5; the buffer diffused through the membrane and raised the pH of the samples to 6.5). After 30 min, 0.5 mL of a pancreatin (2 mg/mL) and bile salt (12 mg/mL) mixture in 0.1M $NaHCO_3$ was added to each well, and incubated for further 2h. Aliquots of the dialysate from the apical chambers were collected, and the iron content was estimated immediately using the Atomic Absorption Spectrophotometer.

Ferritin expression in Caco-2 cells: The coupled *in vitro* digestion/ Caco-2 cell model was used for measuring the ferritin induction as described previously (;). Briefly, either 1 mL saline (reagent blank) or 1g of rice samples were subjected to *in vitro* gastric and intestinal digestion in the presence and absence of 250 µmol/L ascorbic acid (freshly prepared in 0.1N HCl), followed by feeding the digesta to differentiated Caco-2 cells for a period of 24 h. At the end of incubation, the cells were washed, lysed and ferritin content in the cell lysate. A human ferritin sandwich ELISA kit was used for ferritin estimation in cell lysates, as per the manufacturer's instructions. The colour intensity was measured using an ELISA plate reader.

Sensory evaluation: The study is approved by the Institutional Ethical Committee of the ICMR-National Institute of Nutrition, Hyderabad, India (IEC Protocol Number: 10/I/2017) and is registered with the Clinical Trials Registry, India (CTRI Trial Registration Number: CTRI/ 2017/ 11/010655). Informed written consent from the respondents was taken before conducting the sensory study. A sample size of 84 respondents has been calculated considering an α=0.05 significance level, β=0.10 (90% power) and a P_D = 0.25 (the chance of detecting a difference is less than 25%).

The triangle tests () were performed among apparently healthy adult men and women (n=84; mean age=30.4, 54% men and 46% women) to test if they could distinguish the fortified rice from unfortified rice both in the uncooked and cooked form, evaluated separately. Equal quantities of fortified and unfortified rice were prepared simultaneously using a traditional recipe (rice to water ratio of 1:2), in identical electric cookers. The sensory test was conducted immediately thereafter. The study was conducted at 11.00 AM in a dedicated facility under uniform lighting conditions. The three rice samples (30 g each), of which two were identical and one different were served in polyethylene cups, identified by a three digit random code, simultaneously, to the respondents. The samples were presented in a randomized block design; i.e. the six possible order combinations were randomized across the respondents and presented for sensory evaluation. The respondents were asked to evaluate the samples from left to right and identify which one among the three samples differed from the other two and also describe how it differed based on sensory properties. The uncooked and cooked rice was tested in separate sessions. Each session included 12 respondents. The respondents were blinded and were informed only about the test procedures at the beginning of the session, while specific information about the type/identity of rice was revealed only after the study was completed.

Statistics: All experiments were carried out in triplicate and replicated at least once to generate 6 observations. The mean and standard deviation (SD) were computed using Microsoft Excel (2007). One-way ANOVA followed by Tukey's post hoc test was performed to compare the means (Version 19, SPSS Inc, Chicago, US). The sensory data was analyzed using the binomial test with an expected probability of correct identification of 1/3 in the triangle test. The differences were considered significant at $P<0.05$.

Nutrient content of extruded rice premix, fortified and unfortified rice: The representative images of the premix (extruded rice kernels), fortified and unfortified rice are shown in Fig 1.

The mean (±SD) iron (1200±43.2 mg/100g), folic acid (12700±16.7 µg/100g) and vitamin B_{12} (94±4.75 µg/100g) content in premix were at expected levels (Table 1). In the unfortified rice, iron content was 0.27±0.03 mg/100g, while folic acid and B_{12} were undetectable using the HPLC method. The iron and folic acid content of fortified rice (1:100) were 12.1±0.84 mg/100g and 121±1.01µg/100g, respectively. However, the B_{12} levels remained undetectable even in the fortified rice (1:100), possibly due to very low levels of this nutrient in the sample (~1µg/100g).

Fig 1. Representative images of the fortified rice kernels (left), unfortified rice (middle) and fortified rice (right)

Table 1. Iron, folic acid and vitamin B_{12} content of fortified rice premix, fortified and unfortified rice

Nutrient	Premix	Fortified Rice (1:100 ratio)	Fortified rice (1: 10 ratio)	Unfortified rice	FSSAI Fortification standards[$]
Iron (mg/100g)[#]	1200±43.2	12.1±0.84	136±9.8[#]	0.27±0.03	2
Folic acid (µg/100g)	12700±16.7	121±1.01	1443±117	ND	130
Vitamin B_{12} (µg/100g)	94±4.75	ND*	10.2±0.7	ND	1

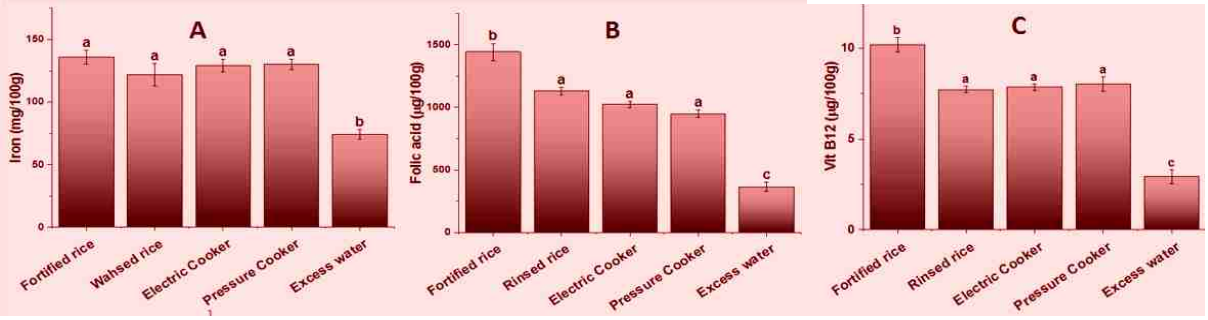
[#] *Iron fortification was kept at 120 mg/kg levels purposefully to facilitate further clinical trial to provide 12 and 18 mg iron/day with 100 and 150g ration of rice/day.*
**ND; not detectable*
[$] *FSSAI draft guidelines on rice fortification, 2016.*

Sensory evaluation of fortified rice in adult human volunteers: In a sensory panel of 84 adult volunteers, about 24 and 33 respondents correctly identified the odd rice sample in the uncooked and cooked form respectively, which is less than the critical number of correct responses required (n_c=36) to detect a significant difference at 5% level of significance. This indicates that the fortified and unfortified rice were sufficiently similar, both in the uncooked and cooked form.

Retention and stability of nutrients during rinsing and cooking: Due to methodological limitations the B_{12} levels in fortified rice (1:100 ratio of blending) were not detectable. Therefore, retention and stability analysis was performed using 1:10 fortification ratio to aid in analysis. The iron (136±9.8 mg/100g), folic acid (1443±117µg /100g) and vitamin B_{12} (10.2±0.7 µg/100g) levels of fortified rice are also are at the expected fortification levels (Table 1). The mean (±SD) iron content of rinsed fortified rice (122±15.4 mg/100g) was similar to the fortified rice. However, folic acid and vitamin B_{12} content significantly ($P<0.05$) reduced by ~25% during the rinsing of fortified rice (Fig 2 A-C). Interestingly, cooking of rice in an electric or pressure cooker led to a small additional decline in folic acid (~10-16%), but not vitamin B_{12} compared to rinsed

rice, but the differences were not significant. The cooking of fortified rice in excess water followed by decanting led to significant loss of iron (45%), folic acid (75%) and vitamin B_{12} (71%). Thus the cumulative retention and stability of nutrients during rinsing and cooking in an electric or pressure cooker were 100% for iron and ~70% for folic acid and vitamin B_{12}.

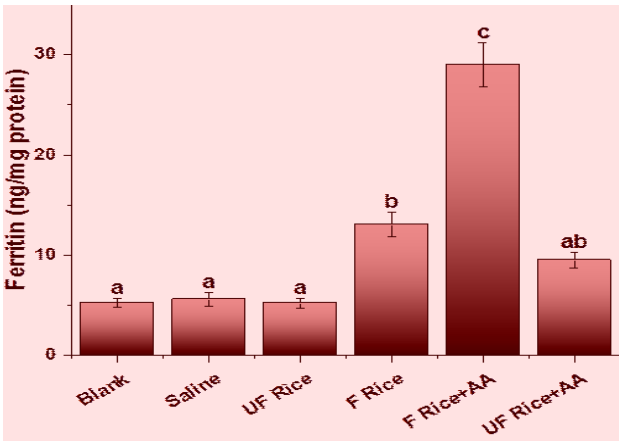
Fig 2. Retention of nutrients during rinsing and cooking of fortified rice: Fortified rice (1:10 ratio of blending) rinsed with excess water followed by 3 different cooking methods followed by measurement of nutrients as described in methods. The bars represent mean ± SE of nutrient levels and the bars that do not share a common superscript differ significantly ($P < 0.05$).



Bioavailability of iron from fortified rice:

The % dialyzable iron from unfortified rice (16.66±1.0%) is significantly higher compared to fortified rice (4.54±0.28%). However, the absolute dialyzable iron content from the fortified rice (0.55± 0.06mg/ 100g) was found to be much higher than that of unfortified rice (0.045± 0.006 mg/100g). The Caco-2 cell ferritin levels exposed to fortified rice digesta was significantly higher ($p<0.01$) compared to blank (untreated cells), saline (reagent blank) or unfortified rice (Fig 3). Further, ferritin response with unfortified rice remained similar to that of either blank or saline control. Inclusion of ascorbic acid induced ferritin synthesis from both fortified and unfortified rice digests compared to its absence, but it was significant ($P<0.01$) only from fortified rice.

Fig 3. Caco-2 cell ferritin formation: The ferritin content of differentiated Caco-2 cells unexposed (Blank- MEM) and exposed to saline (reagent blank), unfortified (UF) and fortified (F; 1:100 ratio of blending) rice digests in the absence and presence of ascorbic acid (AA; 250 µmol/L). The bars represent mean±SE of cell ferritin (ng/ mg cell protein) and the bars that do not share a common superscript differ significantly ($P < 0.05$).



Conclusions

The results suggest that it is possible to achieve target nutrient levels through fortification of rice by blending with extruded fortified rice kernels without changing the sensory properties of the rice. Further, the minimal losses of B vitamins observed during the rinsing and cooking of rice, can be adequately compensated by adding overages. Further the bioavailable iron content from fortified rice is higher compared to unfortified rice, and it can be further improved by the inclusion of vegetables and fruits. However, poor retention of nutrients during cooking with excess water followed by decanting, warrants educating the target population and school kitchen personnel on desired cooking methods to be adopted to reduce the nutrient losses. The clinical study for testing the efficacy is concluded and data analysis is in progress.

2. Effect of zinc supplementation prior to iron on iron absorption, and iron status in deficient rats: Report of *in vitro* studies

The prevalence of zinc deficiency is being increasingly recognized in the general population in India. Inadequate dietary intake coupled with poor bioavailability of iron and zinc from vegetarian staple foods is the major etiological factor for the widespread prevalence of anemia in India. Studies in animal and humans have reported negative interactions of iron and zinc during absorption. Zinc inhibits the intestinal iron absorption when supplemented with iron in intestinal cells was demonstrated. However, iron has no effect on zinc absorption. Interestingly, pre-treatment of intestinal cells with zinc markedly enhanced the iron uptake via stimulation of divalent metal ion transporter 1 (DMT1) and ferroportin (FPN1) expression. Moreover, intestinal DMT1 expression reported being determined by intestinal zinc content in rat pups. It is also possible that zinc offsets the inhibitory effect of hepcidin on iron absorption. Therefore, it appears that intestinal zinc status is a major determinant of iron absorption. From these observations it is logical that zinc status modulates the iron absorption and metabolism, and that supplementation of zinc prior to iron increases the intestinal zinc status and thereby promotes the iron absorption. In the current report, we present the results of in vitro studies using Caco-2 cells.

Objectives

- To elucidate the mechanism of zinc induced modulation of intestinal iron absorption in human enterocyte like Caco-2 cells.
- To study the effect of iron alone, iron and zinc combined or alternate (zinc followed by iron with a time gap) on iron and zinc status parameters in iron and zinc deficient rat models.

Methods

***Caco-2 cell culture*:** Caco-2 cells were grown at 37°C in an atmosphere of 5% CO_2 and 95% humidity in Eagle's Minimum Essential Medium (MEM) supplemented with 10 % (v/v) heat inactivated foetal bovine serum (FBS), 1% (v/v) penicillin/streptomycin. For experiments, cells were seeded into 6-well plates and grown for 21 days to allow cells to fully differentiate. The cells were incubated in serum-free MEM for 12h and treated with $ZnSO_4$ (100 µmol/L) for the times indicated. LY294002 (25 µmol/L), where present, was added 30 min prior to the addition of zinc.

***Iron uptake*:** The measurement of iron uptake by Caco-2 cells has been described previously. Briefly, following zinc treatment, media was removed and replaced with 2mL of 2-(N-morpholino) ethanesulphonic acid (MES)-buffered salt solution (pH 6.5 containing: 140 mmol/L NaCl; 5 mmol/L KCl; 1 mmol/L Na_2HPO_4; 1 mmol/L $CaCl_2$; 0.5 mmol/L $MgCl_2$; 5 mmol/L glucose). Uptake was initiated by the addition of 10 µmol/L Fe^{2+} complexed with 1 mmol/L ascorbic acid (freshly prepared prior to the start of each experiment) and 37 kBq/mL $^{59}FeCl_3$. The reaction was terminated after 15 min, and cell monolayers were washed 3 times in ice-cold transport buffer containing a 10-fold excess of iron to remove non-specifically bound iron, solubilised overnight in 200 mmol/L NaOH. The cell associated ^{59}Fe radioactivity was determined by counting in an Auto Gamma Counter.

Real-time PCR: Total RNA was isolated from cultured cells using TRIzol. Following cDNA synthesis, expression levels of DMT1 (+IRE and -IRE), IRP2 and β-2 microglobulin mRNA (used as a housekeeping gene) were analysed by real-time quantitative PCR using an ABI Prism 7500 FAST Sequence Detection System and a Power SYBR Green PCR master mix kit (New

England Biosciences, UK). Quantitative measurements of target genes relative to the housekeeping gene were derived using the ΔCt method. Data are normalized to the untreated control group in each experiment and are presented as the mean±S.E.M.

***Isolation of polysomes*:** IRP-1 and IRP-2 levels, after the incubations, were assessed in the polysomal fraction following treatments as described previously. Briefly, cells were washed in ice-cold PBS and scraped into 3 mL of digitonin buffer (20 mmol/L Tris–Cl, pH 7.4; 250 mmol/L sucrose; 0.007% digitonin; 1× protease inhibitor cocktail). Cells were manually homogenized using 21G and 26½ G needles and kept on ice for 15 min. The homogenate was subjected to sequential centrifugation at 1500 g (10 min), 10,000g (10 min) and finally at 100,000g for 60 min. The pellets from the latter two steps enriched in polysomes were pooled and suspended in TX-100 buffer (20 mmol/L Tris–Cl, pH 7.4; 250 mmol/L sucrose; 1% TX-100; 5% protease inhibitor cocktail). The IRP levels in polysomal fraction were assessed by immunoblotting as described below.

***Immunoblotting*:** The cell monolayers were washed (3X) with 10 mmol/L phosphate buffer saline pH 7.2 and lysed in RIPA buffer (Thermo Fisher) supplemented with protease inhibitor cocktail (1X), EDTA (1 mmol/L), Sodium orthovanadate (1 mmol/L), NaF (10 mmol/L). The protein content was estimated using the micro-BCA kit method. An Equal amount of protein (20-30 µg) was fractionated on 10% SDS-gels under reducing conditions and transblotted on to the PVDF membranes. The blots were blocked with 5% non-fat dry milk or BSA and probed with primary respective primary antibodies followed by respective commercially available secondary antibodies. The blots visualized using an enhanced chemiluminescence detection kit and Hyperfilm ECL or images were acquired using G-box imaging system. The blots were re-probed with β-actin, used as a loading control. The images were quantified using Image-J software (NIH, USA) and normalized to respective loading controls.

***Transient transfection of Caco-2 cells with siRNA*:** Caco-2 cells were seeded at a density of 1.0 X 10^5 cells/mL in complete media in 12-well plates and allowed to adhere for 10 days. The spent media was aspirated, and the cells were washed once with pre-warmed Dulbecco's Phosphate Buffered Saline (DPBS). Next, the cells were supplemented with OptiMEM containing 5% FBS without any antibiotics. One hour following the addition of OptiMEM, the Caco-2 cells were transfected with 10 nmol/L of either DMT-siRNA, IRP2-siRNA or a non-targeting scrambled siRNA, using Lipofectamine 3000 according to the manufacturer's protocol. 48 hours after transfection, the media was aspirated from each well, the cells were washed once with pre-warmed DPBS, and supplemented with fresh OptiMEM containing 5% FBS for 72 hours.

mRNA stability: The Caco-2 cells were incubated either in the presence or absence of zinc (100 µmol/L) for a period of 4h, followed by the addition of actinomycin-D (10µg/mL). At 0, 2, and 4h after addition of actinomycin D, cells were harvested; qPCR analysis of DMT1 was performed as described above. The mRNA remaining is expressed as a percentage of mRNA levels at t = 0h.

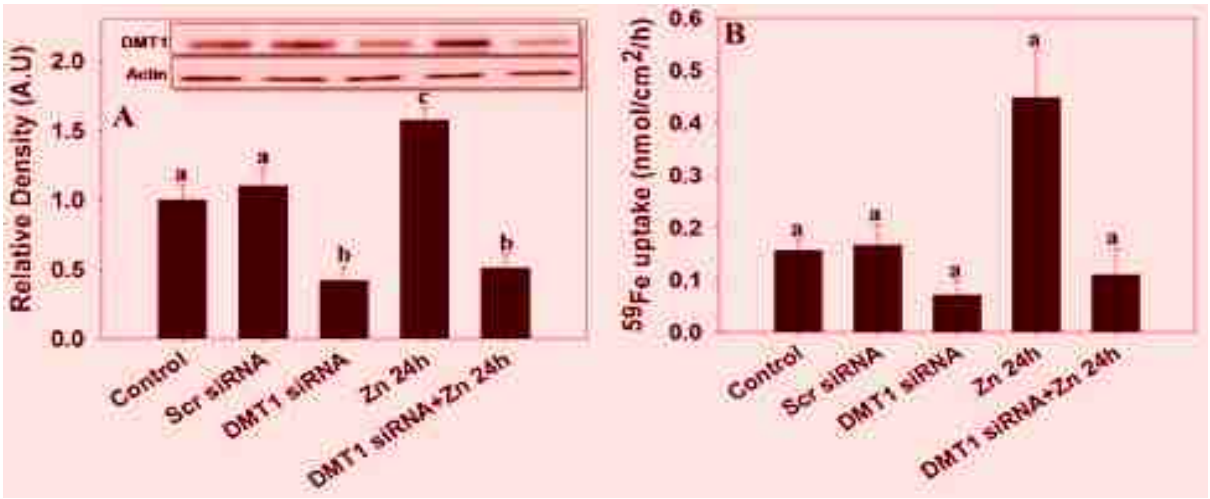
Statistics: All data are expressed as means±SEM. Statistical analysis was carried out using Sigma Plot. One-way ANOVA followed by Tukey's post-hoc test was used where appropriate to detect statistical differences ($P<0.05$) between control and test groups.

Results

Zinc treatment for either 4 or 24h significantly induced DMT1 (+IRE) mRNA ($P<0.01$) and DMT1protein expression and iron uptake. To confirm that the effects of zinc on iron uptake were mediated through DMT1 we performed siRNA knockdown of DMT1. There was no significant difference in the values obtained for DMT1 protein and iron uptake between the un-transfected control group and cells transfected with scrambled siRNA. We therefore used the un-transfected control group for subsequent analysis. DMT1 protein levels were increased in un-transfected

cells following exposure to zinc (Fig 1A). Treatment with DMT1 siRNA led to significant down regulation of DMT1 protein expression compared to control cells and levels remained significantly suppressed in DMT1 siRNA cells treated with zinc. DMT1 silencing also significantly inhibited the basal and zinc-induced iron uptake compared to controls (Fig 1B).

Fig 1. Effect of DMT1 silencing on zinc induced iron absorption in Caco-2 cells: Differentiated Caco-2 cells grown in 6-well plates were transfected with DMT1 or scrambled control siRNA followed by Zn (100 μmol/L) treatment for 24h (A) DMT-1 protein (B) ^{59}Fe uptake; The iron uptake experiments were performed in triplicate and repeated twice to generate 6 independent observations. The immunoblots were repeated thrice, and the same blots were reprobed with β-actin. The densities were normalized to the respective housekeeping gene. The bars indicate the mean+SEM, and bars without common superscript differ significantly ($P<0.05$).



Zinc treatment increased phosphorylation of Akt (pSer-473) in a time-dependent manner without changes in total Akt protein expression, and this was blocked completely by LY294002, a potent inhibitor of PI3K. This prompted us to investigate the role of the PI3K pathway in more detail. Pre-treatment of Caco-2 cells with LY294002 significantly inhibited zinc-induced iron uptake, DMT1 protein and mRNA expression. Interestingly, LY294002 treatment alone also significantly inhibited ($p<0.001$) the DMT1 mRNA expression.

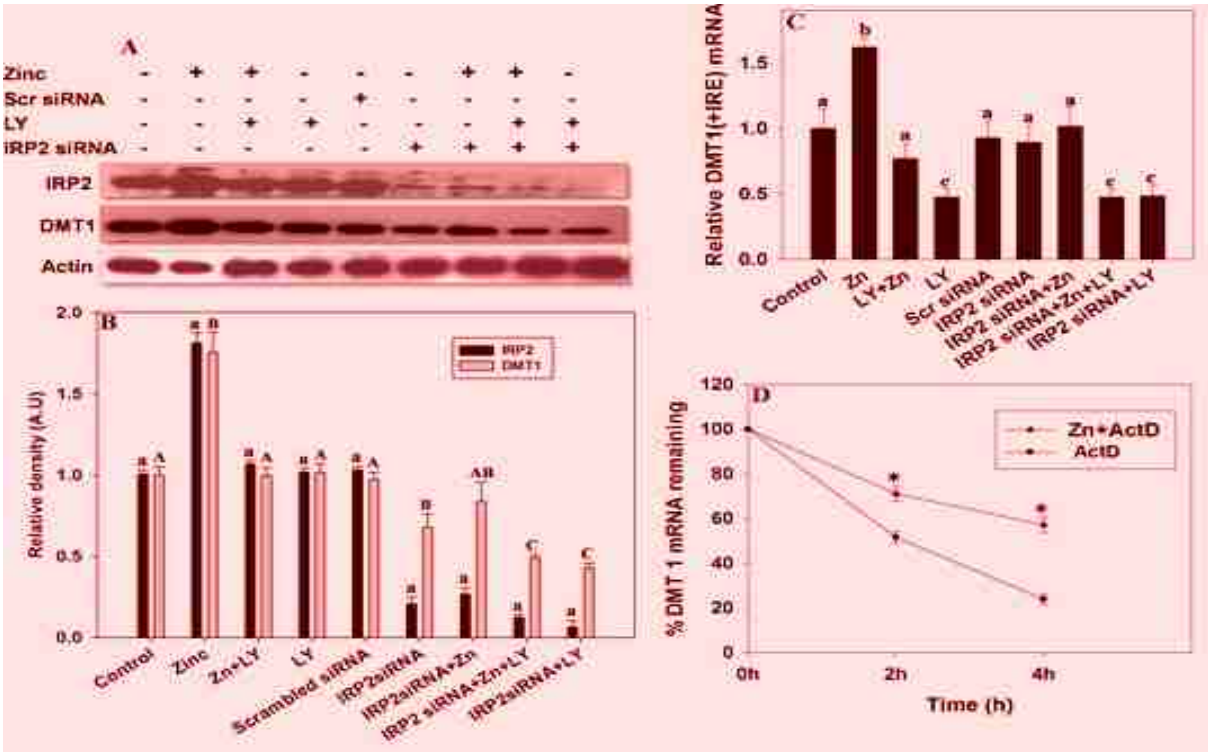
Incubation with zinc significantly increased IRP2 protein expression in a time-dependent manner reaching maximum abundance between 0-2h, and levels remained elevated in the presence of zinc thereafter. However, zinc had no effect on IRP1 expression. Zinc concurrently induced IRP2 levels, but not IRP1, in polysomal fractions (this represents active IRPs bound to IREs in target mRNAs), as a function of time. Zinc did not affect IRP2 mRNA levels over the time course of this study. The effect of zinc on IRP2 protein expression was significantly inhibited by Ly294002.

To determine whether zinc-induced changes in IRP2 expression mediated the regulation of DMT1 siRNA knockdown of IRP2 was carried out. There was no significant difference in IRP2 protein levels between the un-transfected control group and cells transfected with scrambled siRNA (Fig 2A lane 1 and 5). Transfection with IRP2 siRNA, resulted in significant down regulation of IRP2 protein expression compared to un-transfected control cells (Fig 2A and B). IRP2 silencing also significantly reduced ($P<0.001$) the basal DMT1 protein (Fig 2A and B), but not DMT1 mRNA expression (Fig 2C) compared to control cells. Furthermore, zinc failed to induce DMT1 protein (Fig 2A and C) or mRNA expression (Fig 2C) in IRP2-silenced cells

compared to control. LY294002 further inhibited (P<0.01) the IRP2 and DMT1 expression in IRP2-silenced cells (Fig 2A, B and C), either in the presence or absence of zinc compared to respective controls.

To assess whether the zinc-IRP2 axis increased DMT1 (+IRE) mRNA stability we treated cells with the transcription inhibitor actinomycin D in the presence or absence of zinc. DMT1 (+IRE) mRNA decreased with time; however, the rate of decrease in DMT1 mRNA levels was significantly lower (P<0.01) in cells treated with zinc+actinomycin-D compared to cells treated with actinomycin-D alone (Fig 2D).

Fig 2. Effect of IRP2 siRNA silencing on zinc induced changes in DMT1 protein and mRNA expression: Differentiated Caco-2 cells grown in 12-well plates were transfected with IRP2 or control scrambled siRNA followed by Zn (100 µmol/L) and/or LY294002 (25µmol/L) treatment for 24h. (A) IRP2 and DMT-1 and immunoblots (B) densities of IRP2 and DMT1 (C). DMT1 (+IRE) mRNA expression (D). DMT1 mRNA levels in Caco-2 cells incubated either in the presence or absence of Zn and/or actinomycin-D (10µg/mL). The immunoblots were repeated thrice, and the same blots were re-probed with β-actin. The qPCR was performed in triplicate and repeated thrice to generate 9 independent observations, and the data is normalized to the housekeeping gene, the β2-microglobulin. The bars indicate the mean+SEM, and bars without common superscript differ significantly (P<0.05). The asterisks in figure C indicate significant difference at respective time points.



These results demonstrate that zinc stimulates intestinal iron absorption by induction of DMT1 expression via a PI3K/IRP2 dependent mechanism. This is the first demonstration that PI3K pathway is involved in regulating the intestinal iron absorption via modulation of IRP2 and could be potentially exploited to improve iron nutrition and metabolism. Given the likely co-existence of iron and zinc deficiencies in populations subsisting on phytic acid-rich vegetarian diets, consideration should be given to improving the zinc status to augment the efficacy of iron supplementation.

3. Status of micronutrients and its influence on molecular mechanisms in diabetic nephropathy: A nutrigenomics study

Uncontrolled or poorly controlled diabetes can lead to various micro- and macrovascular complications. Diabetic nephropathy (DN) is one of the most significant complications of diabetes and the most frequent cause of end-stage renal insufficiency. Multiple factors are likely to be involved in predisposing diabetic subjects to complications; therefore there is a need to understand susceptible factors that predispose the diabetic subjects to complications. In addition to genetic factors, other environmental factors such as nutritional factors might also play a role in the development of DN. However, studies that evaluated the role of nutritional factors, particularly micronutrients in DN are meager. Several disparate hypotheses of protein glycation, polyol pathway, activation of protein kinase, and hyperinsulinemia are increasingly being seen as part of interrelated biochemical processes. Of the specific metabolically driven glucose-dependent pathways which are activated within diabetic renal tissues, polyol pathway flux and accumulation of advanced glycation endproducts (AGE) have been extensively studied for the progression and development of DN. Evidence is also being accumulated that these processes are susceptible to nutritional modulation. For example, vitamin B1 has been shown to influence the rate-limiting enzyme of the polyol pathway, aldose reductase (ALR2). Similarly, cofactors of vitamin B6 are reported to act as AGE inhibitors. Therefore, it is likely that deficiency of vitamin B1 and B6 may modulate these biochemical pathways either directly or in synergy with the genetic variation of the genes involved in these pathways. Further, the role of the polymorphisms of the genes involved in these metabolic pathways and more importantly gene-nutrient interactions could give a better understanding of the progression of the disease. In the above background, we hypothesize that inadequacy or deficiency of micronutrients may predispose diabetes patients to DN through modulation of molecular processes involved in DN.

Objective

- To evaluate the status of the micronutrientes vis-à-vis the status of major biochemical pathways in DN. The genetic variations of genes involved in polyol and AGE pathways also investigated.

Methodology

A hospital-based case control study was conducted. Subjects were recruited from the patients who visit the Departments of Nephrology and Endocrinology of Osmania Medical College & Hospital, Hyderabad. Four groups of clinically diagnosed subjects were recruited for this study viz. DN: diabetic nephropathy comprising of T2D subjects with nephropathy (n=176), DNC (diabetic no complications): Type 2 diabetic group without any diabetic complications (n=200), C: control group comprising of subjects without diabetes or any other systemic diseases (n=166), non-diabetic CKD (NDCKD) group comprising of subjects without diabetes but with CKD (n=78).A blood sample was collected from the subjects after obtaining their consent. Spot urine was collected for checking the urine albumin to creatinine ratio**.**

Estimation of vitamins, minerals and total homocysteine in human blood and plasma samples: Vitamins A, B1, B2, B6 and plasma total homocysteine (tHcys) were measured using the HPLC method. Plasma levels of vitamin B12 and folic acid were measured by a solid phase RIA. ELISA was used to quantify 25-hydroxyvitamin D and transcobalamin II (active B12). Analysis of plasma minerals (Ca, V, Cr, Mn, Fe, Co, Cu, Zn, Se) was carried out by a high resolution inductively coupled plasma mass spectrometry at Geochemistry, CSIR-National Geophysical Research Institute, Hyderabad.

Diet survey: Dietary intake was estimated by conducting a validated raw-food based quantitative food frequency questionnaire (RFFFQ). FFQ was done in a sub-sample (one-fourth) of the subjects in each group.

Aldose reductase (ALR2) activity assay: ALR2 activity was assayed in RBC by spectrophotometric method.

Sorbitol estimation in RBC: Sorbitol was estimated by a fluorimetric method.

***Advanced glycation end products (AGEs) Index*:** Serum samples were diluted and the intrinsic AGE-specific fluorescence was monitored spectrofluorimetrically. The slope of the regression line was the AGE index.

Genetic analysis: Primers and restriction enzymes were designed for genotyping SNPs of the RAGE gene namely, G82S, −374T/A, −429T/C, 1704G/T, 2184A/G, 2245G/A and 63-bp deletion polymorphisms and rs759853, rs5053 in aldose reductase gene, by the method of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Genotyping was carried out by incubating the PCR product with a specific restriction enzyme overnight followed by electrophoresis. SNPStats was used to determine odds ratios (ORs), 95% confidence intervals (CIs), and p values adjusting for age and gender as co-variables. The frequencies of the marker alleles were estimated by the allele counting method and tested for Hardy–Weinberg Equilibrium.

Statistical Analysis

As most of the data were skewed, the population characteristics were reported using medians, 25^{th} (P25) and 75^{th} (P75) percentiles, and comparisons for the same were carried out by Kruskal-Wallis test. Comparison of the probability of adequacy (PA) and mean PA (MPA) was done by ANOVA and F test with post hoc test of Tukey's multiple comparisons. A Spearman rank correlation analysis was carried out to evaluate the correlation. The level of significance was considered at $p<0.05$.

Results

The median values of blood/ plasma concentrations of the seven vitamins by disease status are shown in Table 1. Blood/ plasma concentrations of Vit A, Vit D, Vit B2, Vit B6, and folate are significantly higher in the CKD groups (DN and NDCKD) compared to the control and DNC groups. In the case of Vit B1 and total B12 and active B12, the blood/ plasma concentrations are significantly higher in the DN group. Higher plasma/blood concentrations of all the vitamins in the CKD groups indicated their connection with kidney dysfunction. These results intrigued us to further validate our findings by stratifying the study population by CKD classification by albuminuria and GFR categories, the predictors of kidney damage and function. By albuminuria categories, except Vit B6, plasma/blood concentrations of all other vitamins are significantly different among the categories. Blood/ plasma concentrations of Vit A, Vit D, Vit B2, and vitamin B12 (total and active) are significantly higher in the A2 and A3 categories compared to A1. The vitamin concentrations are significantly associated with kidney damage, and their values increased with the increasing UACR values. By GFR, the blood/ plasma concentrations of all the vitamins are significantly higher in the CKD categories (G3a to G5) compared to the G1 and G2 categories and are increasing with the declining GFR. By disease status, median (P_{25}-P_{75}) percentiles of plasma Hcys concentrations are significantly higher in the NDCKD group compared to the remaining three groups. By albuminuria categories, plasma Hcys concentrations are significantly higher in the A3 category compared to the A1 and A2. By GFR categories, plasma Hcys concentrations are significantly higher in the G5 category compared to the remaining categories (Fig 1). The blood/plasma concentrations of all the vitamins showed a

positive correlation with increasing plasma creatinine, UACR and a negative correlation with the declining GFR. Compared to the control group, plasma levels of Ca, Mn, Cu, Fe, and Zn were significantly lower in the diseased groups (DNC, DN, NDCKD). Plasma Fe levels were found to be significantly lower in the groups with CKD (both DN and NDCKD groups) compared to DNC and C groups. Compared to the C group, plasma Co levels are significantly lower in the DN group which further lowered in the DNC and NDCKD group (Table 2).

Table 1. Median (P_{25}-P_{75}) values of blood/plasma concentration of vitamins by disease status

Parameter	C	DNC	DN	NDKD	p value
Vit A (µmol/l)	1.4 ^{c} (1.0 -1.7)	1.5 ^{bc} (1.2 - 1.9)	1.7 ^{ab} (1.2 - 2.2)	2.1 ^{a} (1.3 - 3.0)	<0.001**
Vit D (nmol/l)	34.2 ^{c} (24.5 - 44.4)	44.9 ^{b} (31.7 - 57.9)	56.4 ^{a} (34.4 - 78.6)	55.4 ^{a} (33.7 - 85.9)	<0.001**
Vit B1 (nmol/l)	119 ^{c} (102 - 144)	138 ^{b} (114 - 175)	186 ^{a} (142 - 237)	145 ^{b} (101 - 201)	<0.001**
Vit B2 (nmol/l)	202 ^{b} (167 - 296)	251 ^{b} (183 - 299)	311 ^{a} (251 - 450)	308 ^{a} (225 - 382)	<0.001**
Vit B6 (nmol/l)	19.8 ^{c} (13.0 - 32.8)	24.3 ^{bc} (11.8 - 36.7)	25.1 ^{ab} (12.6 - 57.5)	34.8 ^{a} (17.0 - 65.6)	0.005**
Folate (nmol/l)	11.7 ^{c} (8.1 - 19.0)	13.9 ^{b} (8.8 - 26.7)	18.8 ^{a} (11.3 - 45.3)	24.6 ^{a} (12.7 - 43.6)	<0.001**
Total B12 (pmol/l)	174 ^{c} (125 - 280)	276 ^{b} (161 - 398)	420.0 ^{a} (221 - 811)	243.9 ^{b} (154 - 452)	<0.001**
Active B12 (pmol/l)	36.8 ^{d} (22.5 - 63.0)	48.2 ^{c} (33.1 - 80.4)	116.6 ^{a} (72.4 - 180.)	68.3 ^{b} (41.1 - 148)	<0.001**

Vit A, vitamin A; Vit D, vitamin D; Vit B1, vitamin B1; Vit B2, vitamin B2; Vit B6, vitamin B6. **Significantly different at p<0.01. Values represent medians, 25thand 75th percentiles. Significant differences (p<0.01) of median values among the groups are indicated by different superscript letters (a, b, c, d).

Dietary intake of individuals with diabetic nephropathy: The median intakes of 12 food groups and 23 nutrients in the study population are analyzed by disease status, albuminuria and GFR categories. By disease status, the median intakes of almost all food categories except animal foods and fats & oils are significantly different among the groups. The Majority of the median intakes of foods (green leafy vegetables, spices and condiments) are significantly low in the CKD groups (DN and NDCKD) compared to C and DNC groups.

By disease status, the median intakes of all the nutrients except vitamin B12, is significantly different among the groups. Energy and protein were found to be significantly lower in the DN group compared to other groups. The intakes of the following nutrients are significantly lower in the DN group: carbohydrates, fiber, niacin, vitamin C, vitamin B6, folate, and potassium. The intake of fat is significantly lower in the NDCKD group. The intake of vitamin A, calcium and sodium is significantly lower in the CKD groups (DN and NDCKD). The intakes of thiamine, riboflavin, and iron are significantly higher in the DNC group compared to the other groups.

Table 2. Median (P_{25}-P_{75}) values of mineral levels by disease status

Parameter	C	DNC	DN	NDCKD	P value
Ca (mg/dl)	16.2 [a] (12.8 - 19.8)	11.6 [b] (10.6 - 14.4)	8.2 [b] (7.2 - 18.5)	6.6 [c] (5.9 - 10.4)	<0.001**
V (ng/ml)	1.6 [a] (1.2 - 2.0)	0.8 [b] (0.6 - 1.4)	1.1 [a] (0.9 - 1.6)	0.8 [b] (0.6 - 1.0)	<0.001**
Cr (ng/dl)	40.5 [a] (35.8 - 41.7)	40.7 [a] (23.7 - 42.2)	27.3 [a] (26.8 - 44.3)	26.3 [b] (22 - 26.8)	<0.001**
Mn (µg/dl)	0.3 [a] (0.2 - 0.3)	0.2 [b] (0.1 - .2)	0.2 [b] (0.1 - 0.2)	0.1 [c] (0.1 - 0.1)	<0.001**
Fe (µg/dl)	206 [a] (185 - 209)	201 [a] (183 - 210)	165 [b] (160 - 209)	160 [c] (157 - 166)	<0.001**
Co (µg/l)	1.1 [a] (1.0 - 1.3)	0.8 [c] (0.5 - 0.9)	1.0 [b] (0.9 - 1.0)	0.9 [c] (0.5 - 1.0)	<0.001**
Cu (µg/dl)	177 [a] (152 - 206)	155 [b] (102 - 173)	130 [b] (116 - 171)	112 [c] (97 - 123)	<0.001**
Zn (µg/dl)	241 [a] (155 - 435)	96.5 [b] (37.6 - 155)	48.0 [b] (38.3 - 165)	28.0[c] (23 - 36)	<0.001**
Se (ng/ml)	97.0 [b] (92.0 - 105)	97.7 [b] (90.1 - 115)	105 [ab] (89.8 - 117)	111[a] (106 - 114)	0.008**

**Significantly different at p<0.01. *Significantly different at p<0.05. Values represent medians, 25th, and 75th percentiles. Significant differences (p<0.05, p<0.01) of median values among the groups are indicated by different superscript letters (a, b, c).

Aldose reductase activity and sorbitol levels: By disease status, the erythrocyte ALR2 activity is significantly higher in the DN group compared to the C and DNC groups. In the case of sorbitol levels, no significant difference is found among the groups (Table 3). In the NDCKD group, ALR2 activity has a moderate positive correlation with plasma creatinine and UACR and a moderate negative correlation with declining GFR. Similarly, ALR2 activity has a moderate positive correlation with plasma creatinine in A2 and A3 categories and a moderate negative correlation with declining GFR in A2 and A3 categories of albuminuria.

Table 3. Median (P_{25}-P_{75}) values of erythrocyte ALR2 activity and sorbitol levels by disease status

Parameter	C	DNC	DN	NDCKD	p value
ALR2 activity (u/g Hb)	13.4[c] (4.5 - 16.9)	13.5[bc] (8.8 - 17)	14.8[a] (11 - 18)	14.4[ab] (11- 16.8)	0.001**
Sorbitol (µg/ml)	5.0[ab] (3.4 - 7.0)	5.1[b] (3.7 - 6.5)	5.5[a] (4.1 - 7.3)	5.0[ab] (4.1 - 6.2)	0.128

ALR2, Aldose reductase. **Significantly different at p<0.01. Values represent medians, 25th and 75th percentiles and percentages. Significant differences of median values among the categories (p<0.01) are indicated by different superscript letters (a, b).

Advanced Glycation End-products (AGE) Index: The plasma AGE index by disease status is significantly higher in the CKD groups (DN, NDCKD) compared to the C and DNC. Based on albuminuria, plasma AGE index is significantly higher in A2 and is further higher in the A3 category, and it increased with the increasing UACR. By GFR, the AGE index is significantly different among all the categories, except for G3b and G4 and it increased with the declining

GFR. Irrespective of the categories, the plasma AGE index has a strong positive correlation with plasma creatinine, UACR levels and a negative correlation with GFR levels (Fig 2).

Genetic analysis: The RAGE gene promoter region polymorphisms were found to be associated with the development of DN emphasizing the role of the promoter on RAGE gene. 2245G/A RAGE polymorphism, situated at intron 8 region, was found to be highly prevalent among DN suggesting that there could be alternative splicing in sRAGE. The 1704G/T polymorphism and 2184A/G RAGE polymorphism which are responsible for alternative splicing that produces endogenous secretory RAGE were also found to be associated with DN. The exon 3 polymorphism Gly82Ser in RAGE gene which regulates RAGE function was not associated with DN. The polymorphisms in aldose reductase were not associated with DN.

Fig 1. Plasma homocysteine concentrations of the study population by disease status, albuminuria and GFR categories. Box plot represents the 25-75th percentile; the median is shown as the heavy dark horizontal line. Vertical lines extend to the minimum and maximum values. Significant differences ($p<0.05$) of median values among the categories are indicated by different letters (a, b) above the bars.

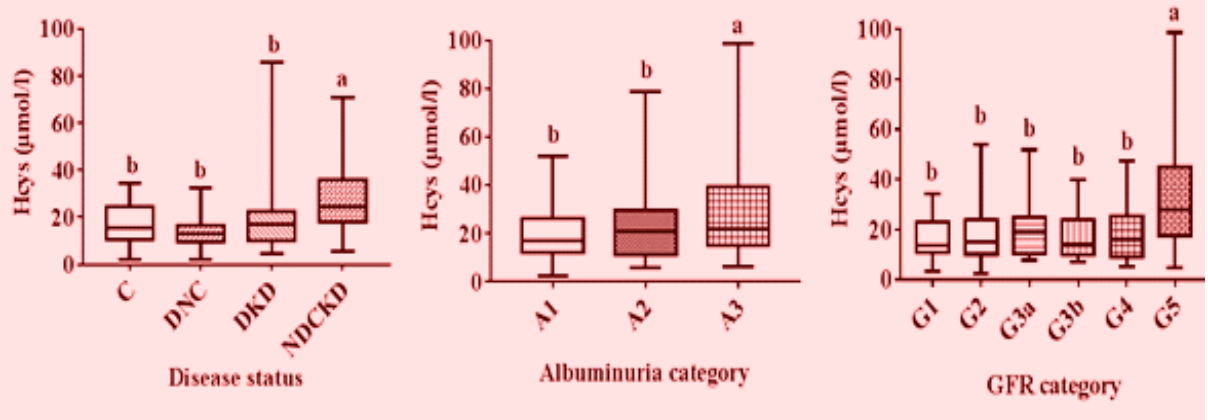
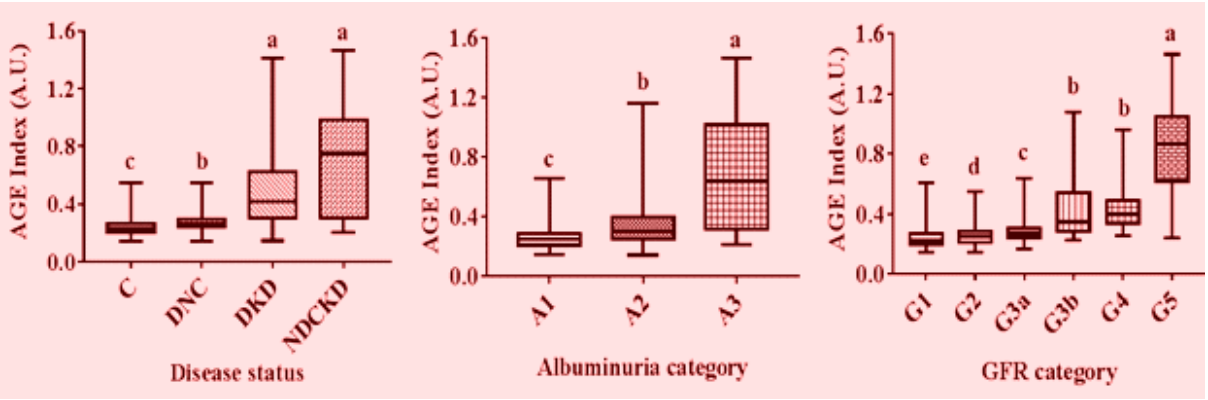


Fig 2. Plasma AGE index of the study population by disease status, albuminuria and GFR categories. Box plot represents the 25-75th percentiles; the median is shown as the heavy dark horizontal line. Vertical lines extend to the minimum and maximum values.



Conclusion

Blood/plasma levels of vitamins might not necessarily represent the adequacy status in DN patients because cellular nutrition depends on the normal uptake of nutrients at the cellular level followed by effective utilization at the tissue level. Despite high levels of vitamins in the circulation, the inability to biologically access these vitamins leads to a functional deficiency of vitamins which might lead to comorbid conditions in DN patients. tHcys emerged as an independent risk factor at a higher degree of renal damage in CKD patients. AGE index can act as

a biomarker of CKD as it increased severity of the kidney disease. A strong positive association of the AGE index with B12 could probably explain the retention of plasma B12 levels in the circulation. High levels of tHcys in the presence of elevated levels of vitamin B12, folate and B6 indicate the functional deficiency of these vitamins which is further supported by their high dietary inadequacies. Association of RAGE promoter polymorphisms would suggest the role of protein expression in these patients and its interaction with AGEs which is leading to vascular complications of diabetes. The polymorphisms in aldose reductase werenot associated with DN.

4. A non-invasive nanoparticle mediated delivery of triamcinolone acetonide for diabetic retinopathy

Diabetic retinopathy (DR) is a multifactorial late stage manifestation in diabetic patients and is the leading cause of preventable blindness. DR progression occurs through two stages, the early non-proliferative DR (NPDR) stage and the advanced proliferative DR (PDR) stage. The NPDR stage is characterized by the enhanced production of various inflammatory mediators such as tumour necrosis factor α (TNFα), nuclear factor κB (NF-κB), and intercellular adhesion molecule-1 (ICAM-1) in the retina. A persistent hypoxia and sustained production of these inflammatory mediators leads to the development of PDR. PDR is characterized by the enhanced secretion of angiogenic mediators such as hypoxia inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF), disruption of the blood retinal barrier and formation of microvascular tufts in retinal capillaries. The production of inflammatory and angiogenic mediators causes a stress response in retinal glial cells which is characterized by enhanced production of the glial fibrillary acidic protein (GFAP) and compromised photoreceptor cell function or photoreceptor cell death which can eventually lead to blindness. Hence, DR causes structural and functional abnormalities in retinal tissue which demand therapeutic interventions to enable control of disease progression.

Since both inflammatory and neovascular mediators are involved in the pathogenesis of DR, therapeutic agents that show anti-inflammatory and/or anti-angiogenic activity have been widely used for the alleviation of DR associated complications. Triamcinolone acetonide (TA) is a corticosteroid that has been demonstrated to be efficacious for NPDR and PDR due to its anti-inflammatory, anti-angiogenic, anti-apoptotic and neuroprotective properties. Hence, intravitreal injections of TA are clinically used for the treatment of DR. However, intravitreal injections are invasive in nature and are associated with various complications such as intraocular bleeding, pain and discomfort resulting in poor patient compliance. Moreover, the chronic nature of DR can demand frequent intravitreal injections that can cause vitreous haemorrhage and retinal detachment. These shortcomings are making it imperative to explore eye drop based-non-invasive routes of drug administration. The complex anatomy and physiology of ocular tissue layers act as barriers and disallow the diffusion of topically administered drugs to the retina. The cornea, conjunctiva, sclera, Bruch's membrane and retinal pigment epithelium act as anatomical (static) barriers, whereas tear fluid, lymphatic vessels, and conjunctival and choroidal blood flow act as physiological (dynamic) barriers, which together cause low bioavailability of topically administered drugs at the retina. The approaches that have been explored thus far to overcome these barriers and enhance the bioavailability of topically administered drugs in the retina include the use of penetration enhancers, cell penetrating peptides and drug delivery systems like cyclodextrin–drug complexes, liposomes, and nanoparticles. However, penetration enhancers and cell penetrating peptides are known to be

cytotoxic above a certain concentration, whereas cyclodextrin–drug complexes and liposomes are known to have short half-lives and release the drug only for a few hours. Therefore, there is a need to develop a non-invasive drug delivery system, which not only crosses the ocular barriers to reach the retina but also provides the encapsulated drug for longer durations.

Objective

- To formulate a non-invasive polycaprolactone (PCL) core and a Pluronic® F-68 (PF68) shell nanoparticle (NP)-based sustained release TA delivery system and to evaluate its *in vivo* efficacy for the treatment of diabetic retinopathy.

Methodology

Blank PCL-PF68 NPs (NPBs) and TA loaded PCL-PF68 NPs (NPDs) were fabricated using the nano-precipitation method as reported previously (Mahaling B, 2016). Three-month old male SD rats were used in the study. The control group of rats received 0.1 M sodium citrate buffer (pH 4.5) as a vehicle, whereas the experimental groups of rats received a single intraperitoneal injection of streptozotocin (35 mg kg−1) in sodium citrate buffer to induce diabetes. At the end of 10 weeks, the animals were randomly divided into two groups for the two time points (20 days and 40 days) of the study. Each of these groups was divided into three sub-groups {sub-group-I [diabetic rat controls – left eye was provided with PBS (PBS), the right eye was provided with TA in PBS (D)], sub-group-II [diabetic rat experimental – left eye was provided with placebo NPs (NPB), the right eye was provided with TA loaded NPs (NPD)], and subgroup-III [non-diabetic rat controls – both left and right eyes were provided with PBS as eye drops (control)]}. Approximately 25 µL of the respective eye drop was administered twice a day – morning 6 AM and evening 5 PM. The treatment was continued for 20 and 40 days. On day 20 of eye drop administration, electro-retinography (ERG) was performed on live animals which were then sacrificed on the day. The sections were stained with hematoxylin and eosin (H&E) and images were captured. Retinal microvascular abnormalities were visualised on the whole mount of isolated rat retina using an endothelial cell specific fluorescent marker, isolectin-B. The sections were then washed with PBS and incubated with Alexa Fluor 594 tagged isolectin-B for 36 hours at 4 °C. After incubation, the samples were washed with PBS and mounted on a glass slide. The slides were then screened for the presence of vascular tufts. For immunohistochemistry and immunofluorescence, the sections were incubated with primary antibodies of anti-NFκB, anti-TNFα, anti-VEGF and anti-GFAP for 10 hours at 4 °C. Slides were then washed 3 times with PBS and incubated with Alexa Fluor 488 conjugated secondary antibody (for GFAP); Alexa Fluor 594 conjugated secondary antibody (for NF-κB and VEGF) for 1 hour. Finally, the sections were mounted in an anti-fade reagent containing DAPI and visualized using a microscope.

Results

Electroretinography (ERG): The physiological function of retinal cells was recorded in vivo non-invasively using ERG to ascertain the efficacy of the developed PCL-PF68 NPs in treating diabetic retinal complications. The ERG results indicated that the amplitudes of b-waves and OPs for diabetic animals were less than those observed for non-diabetic animals (Fig. 1) which corroborated with previous reports. After 20 and 40 days of treatment, the treatment group that received NPDs showed higher mean values for photopic, scotopic b-waves and OPs compared to other diabetic groups treated with PBS, NPBand free drug (Fig 1). Overall, the results indicated that after 40 days of NPD treatment, there was an improvement in both b-wave amplitudes and OPs which suggested that an improved retinal drug bioavailability with NPD treatment caused a reduction in retinal inflammation and as a consequence improved retinal cell function.

Histopathological analysis: Based on histology, the thickness of the retinal layers was not significantly different for the treatment groups after 20 days of eye drop treatment (Fig. 2).

However, after 40 days of treatment it was observed that the non-diabetic controls and NPD treated eyes showed a significantly greater thickness as compared to other diabetic groups (PBS, NPB and free drug administered eyes) (Fig 2). Furthermore, it was also observed that the thicknesses of NPD treated eyes and non-diabetic control animal eyes were comparable with no statistical significance. These results indicate that treatment with NPDs showed improved structural integrity of the retina. This could be possibly attributed to the drug delivery system presenting higher concentrations of TA to the retina. This in turn would have led to reduced inflammation, VEGF secretion and endothelial cell proliferation and as a consequence, offered an improved therapeutic effect.

Fig 1. ERG plots after 40 days of different eye drop treatments. (a) scotopic b-waves, (b) scotopic OPs, (c) photopic b-waves and (d) photopic OPs. Control (non-diabetic rats administered with PBS eye drops), other groups were diabetic rat eyes treated with PBS (PBS), placebo NPs (NPB), free drug (D) and TA loaded NPs (NPD) as eye drops.

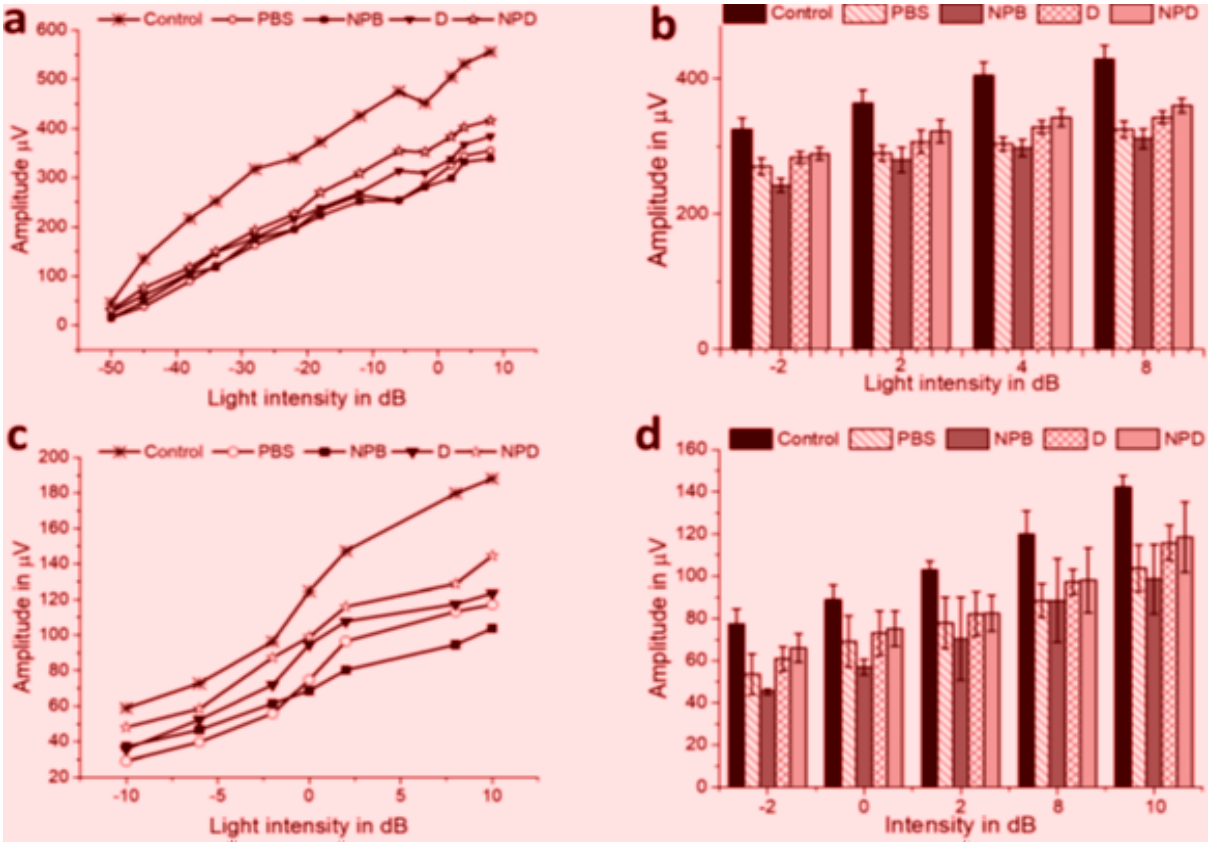
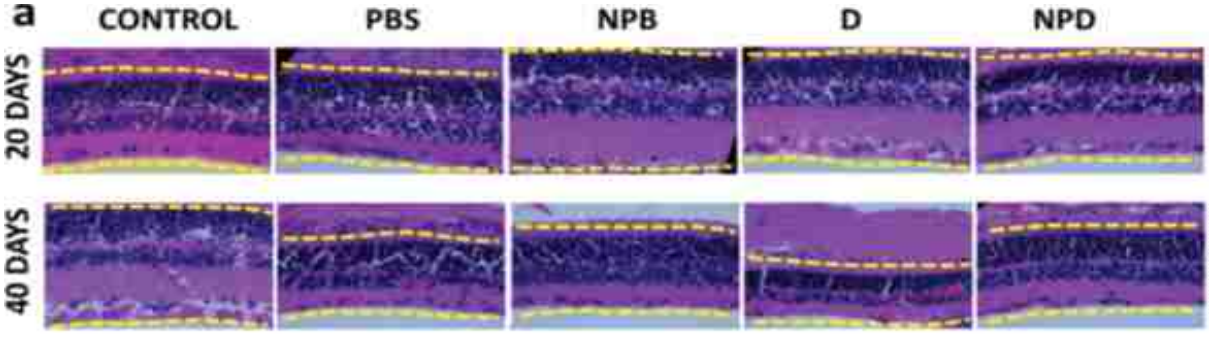
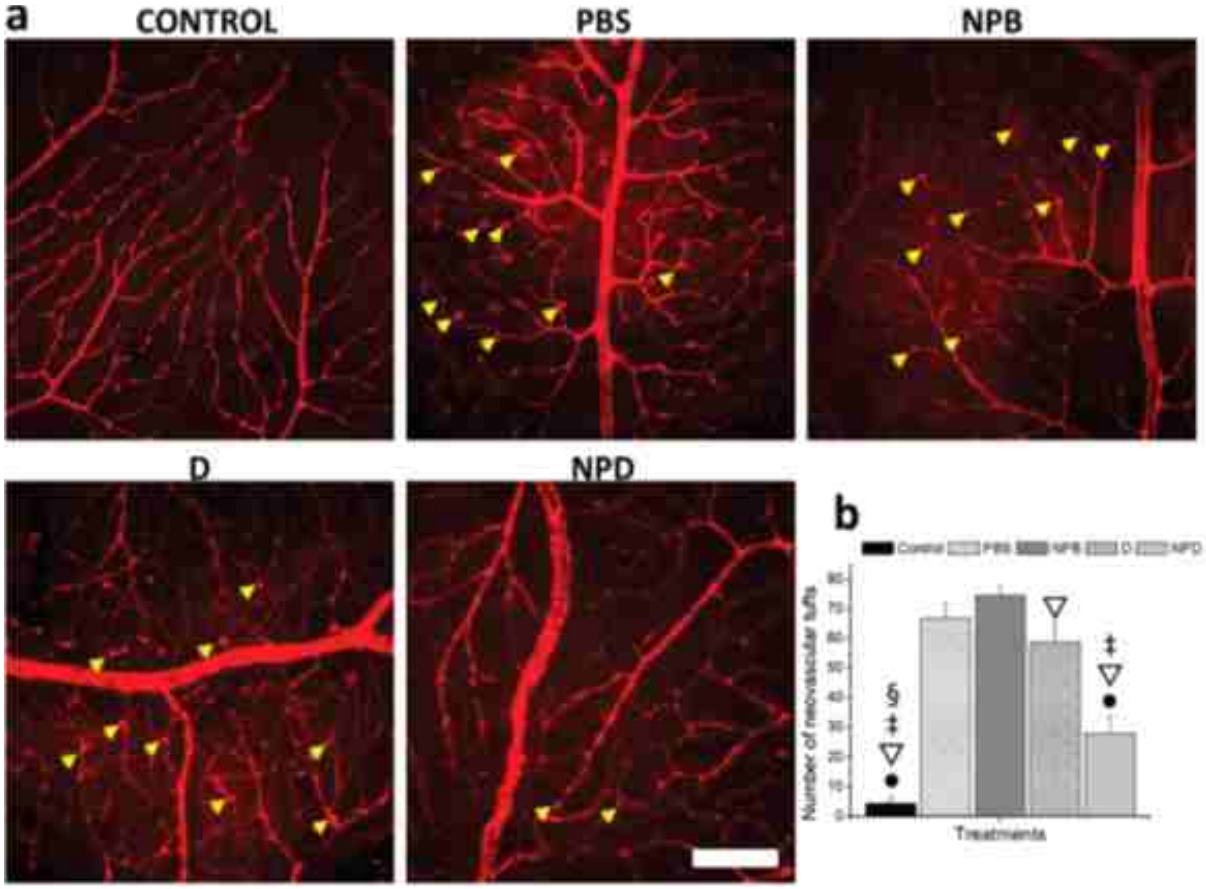


Fig 2. Histopathology (H&E staining) of rat retinae after 20 and 40 days of different eye drop treatments. Control (non-diabetic rats administered with PBS eye drop), other groups were diabetic rat eyes treated with PBS (PBS), placebo NPs (NPB), free drug (D) and TA loaded NPs (NPD) as eye drops.



Visualization of vasculature by retinal flat mounts technique: From the retinal flat mount images, it was observed that the vascular tufts were absent in non-diabetic control eyes. However, in diabetic eyes, vascular tufts were observed and the number of vascular tufts (indicated by yellow arrows) in eyes that received PBS, NPBs and free drug was significantly higher compared to the eyes that received NPDs after 40 days of treatment (Fig 3a and 3b). A reduction in vascular complications in the NPD treated group could be due to the anti-inflammatory, anti-proliferative and anti-angiogenic effects of TA. These results demonstrate that the delivery of TA in a nanoparticulate delivery system probably offered higher bioavailability at the retina thereby inhibiting retinal capillary endothelial cell proliferation and vascular tuft formation and as a result showed improved structural integrity of retinal vasculature.

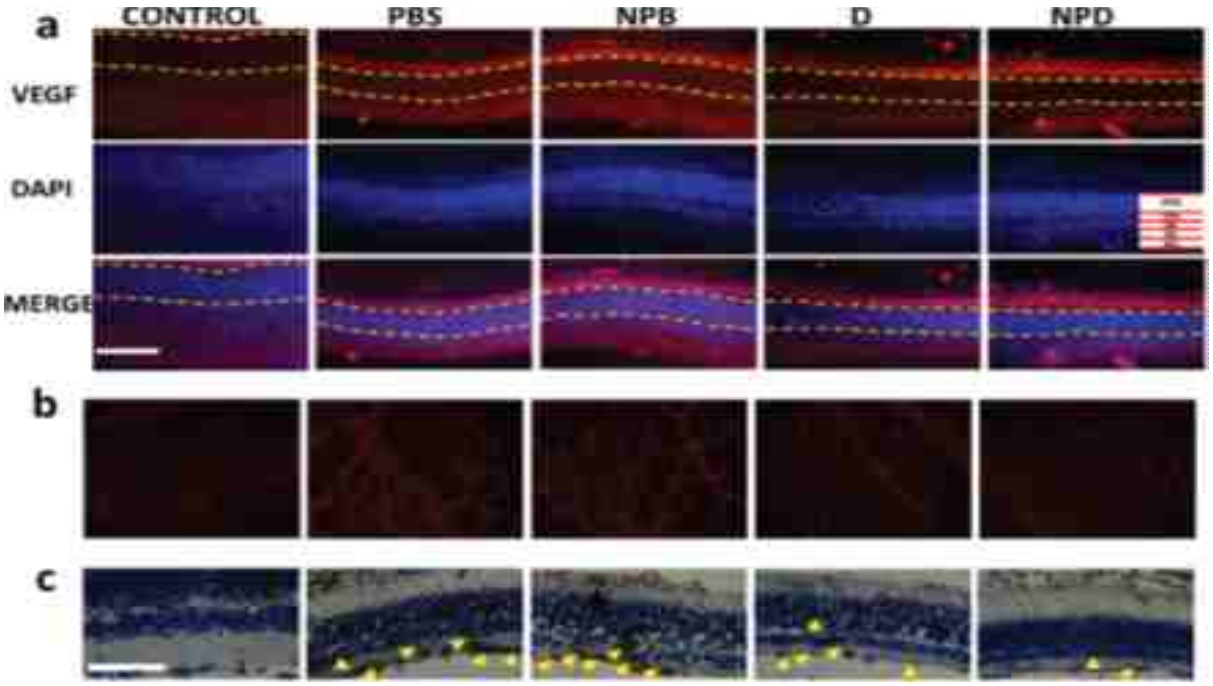
Fig 3. (a) Isolectin-B staining of retinal vasculature after 40 days of different eye drop treatments. Yellow arrows point to vascular tufts, (b) quantification of a number of neovascular tufts in retinal flat mounts.



Immunohistochemistry of inflammatory markers: The immunofluorescence results indicated that there was an increase in expression and translocation of NF-κB in PBS, NPB and free drug treated groups when compared to the control group. A reduction in expression and nuclear translocation of NF-κB in the outer nuclear layer of NPD treated eyes was observed when compared to other treatment groups suggesting a reduction in inflammatory responses with NPD treatment (Fig. 4a and d). Since high blood glucose levels in diabetes cause enhanced oxidative stress, glycation of proteins and immune activation, the translocation of NF-κB takes place. While the anti-inflammatory properties of TA are known to reduce the expression of pro-inflammatory transcription factor NF-κB, for the free drug administered group, the levels of TA available in the retina were probably insufficient to cause a reduction in NF-κB. However, the NPD treated group showed a decrease in expression and nuclear translocation of NF-κB as compared to free drug and had levels that were comparable to control. This indicated that NPDs

crossed the ocular barriers and released therapeutic amounts of TA to cause a reduction in inflammation. The results also indicated that the expression of ICAM-1 in retinal blood vessels was higher in PBS and NPB treated groups when compared to non-diabetic control (Fig. 4b and e). However, when compared to PBS and NPB, a reduction in the expression of ICAM-1 was observed in free drug and NPD treated eyes, with the expression level of NPDs being comparable to non-diabetic control (Fig. 4b and e). These results demonstrate that the sustained delivery of TA, an anti-inflammatory drug, via PCL-PF68 NPs offers improved efficacy and reduced expression of ICAM-1 in retinal vasculature. Furthermore, immunohistochemistry results showed a reduction in the expression of TNFα in inner plexiform, outer plexiform and ganglionic cell layers for animals treated with NPDs when compared to other diabetic treatment groups including free drug treated eyes. These results indicated a greater extent of retinal inflammation in PBS and NPB treated eyes, a moderate inflammation in free drug treated eyes and reduced inflammation in NPD treated eyes. Furthermore, TNFα levels for NPD treated eyes were comparable to those observed in the non-diabetic group. These results demonstrated that NPDs crossed ocular barriers and released therapeutic amounts of TA in the retina, whereas the administration of TA free drug did not offer higher retinal drug bioavailability.

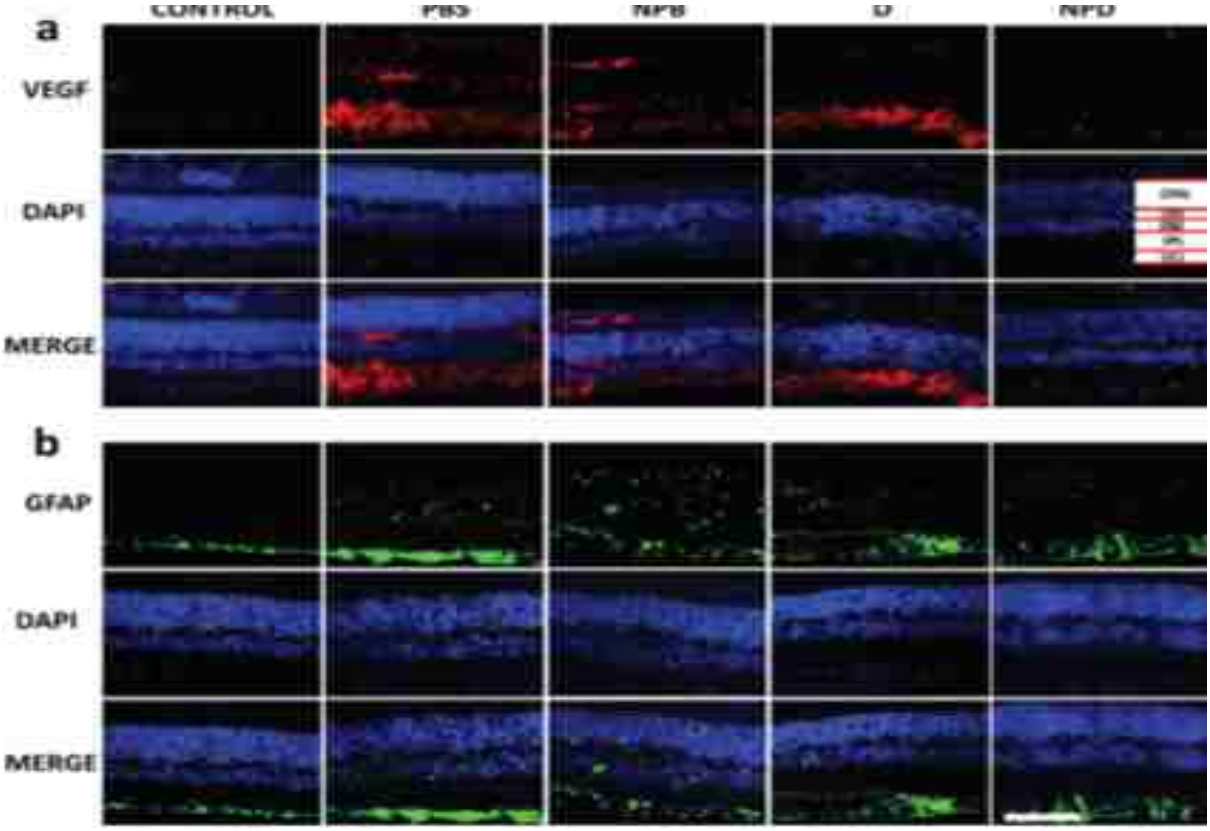
Fig 4. (a) Immunohistochemistry of inflammatory markers after 20 days of different eye drop treatments. Row-1: NF-κB antibody staining, row-2: DAPI staining, row-3: merged images of rows-1 and 2. Yellow dotted lines represent the zone of NF-κB nuclear translocation. (b) ICAM-1 antibody staining on retinal vasculature. (c) Immunohistochemistry of TNFα along with hematoxylin counterstaining. Yellow arrows represent TNFα expression.



Immunofluorescence of VEGF and GFAP: It was observed that non-diabetic controls and NPD treated eyes expressed trace amounts of VEGF only in the ganglionic layer. However, for diabetic controls treated with PBS and NPB, the expression was observed in the ganglionic cell layer, inner plexiform layer and the outer plexiform layer of the retina in higher amounts, whereas in free drug treated eyes, the VEGF expression was lower than PBS and NPB treated eyes (Fig. 5a). Fluorescence intensities from immunofluorescence images were found to be non-significant between the control group and NPD treated group, whereas the fluorescence intensities were significantly less than PBS, NPB and free drug treated diabetic eyes (Fig. 5c). The observed higher levels of VEGF in the ganglionic cell layer, inner plexiform layer and the

outer plexiform layer of the retina for PBS and NPB treated eyes at both 20 and 40 days of treatment were due to higher secretion of VEGF by hypoxic retinal cells due to DR associated ischemia (Fig 5a). The expression of GFAP was observed throughout the retina in PBS and NPB treated diabetic eyes, whereas in healthy control animals, the GFAP expression was limited to the ganglionic cell layer. Furthermore, a higher GFAP expression was observed in diabetic eyes compared to healthy eyes. In the case of diabetic eyes, the GFAP expression of free drug treated eyes was less than that seen in PBS and NPB treated groups, however, it was expressed not only in ganglionic cell layers but also in other retinal layers (Fig 5b), whereas for NPD treated rats the GFAP expression was limited to the ganglionic cell layer and the extent of expression was lesser than other diabetic groups and comparable to the non-diabetic control rats (Fig 5b). The fluorescence quantification data for different treatments were compared and it was found that the expression levels for control and NPD treated eyes were not significantly different from each other. However, the GFAP expression levels in NPD treated eyes were significantly less than those seen in PBS, NPB and free drug treated eyes after 40 days of treatment. These results indicated that the NPDs had the ability to cross the ocular barriers and decrease retinal cell hyperplasia.

Fig 5. (a) Immunofluorescence of neovascularization marker – VEGF after 40 days of different eye drop treatments. Row-1: VEGF antibody staining, row-2: DAPI staining, row-3: merged images of rows-1 and 2. (b) Immunofluorescence of GFAP after 40 days of different eye drop treatments. Row-1: GFAP antibody staining, row-2: DAPI staining, row-3: merged images of rows-1 and 2.



Conclusion

TA loaded PCL-PF68 core shell NPs delivered TA to the retina when administered as eye drops, the most noninvasive and safe mode of drug administration to the posterior eye that circumvents the drawbacks associated with intravitreal injections. This ability to transport TA to the retina in therapeutic concentrations not only led to reduced inflammation but also improved structural and functional activity of the retina in DR rats.

5. 4-PBA prevents diabetic muscle atrophy by modulating ER stress response and ubiquitin-proteasome system

Diabetes is one of the disorders that adversely disturb skeletal muscle health. Diabetic myopathy is characterised by diminished muscle mass, physical capacity, and strength. Reduced muscle quality in diabetes, undesirably affects muscle function; the capability to perform daily activities, quality of life and ultimately might increase the chance of premature mortality. This vital, yet frequently ignored complication is accepted to add to the movement of further diabetic problems because of the indispensable significance of skeletal muscle for physical and metabolic prosperity. Lack of insulin and/its action, elevated glucocorticoids, myostatin and inflammatory cytokines in diabetes deleteriously affects muscle homeostasis by increasing myofibrillar protein degradation while inhibiting its synthesis leading to muscle atrophy/ myopathy. Atrophy of skeletal muscle has been revealed to be an independent risk factor foreseeing diminished survival in numerous maladies. Accordingly, much consideration has been devoted to elucidating the molecular mechanisms underlying this process. The ubiquitin-proteasome system (UPS) plays a significant role in muscle health as a key proteolytic system as well as regulating several cell signaling pathways. Previously we reported the involvement of UPS and Endoplasmic Reticulum (ER) stress in triggering myocyte apoptosis in the skeletal muscle of diabetic rats that prompted us to investigate whether an ER stress inhibitor could control myocyte apoptosis and assist in improving muscle health in diabetes.4-Phenylbutyric acid (PBA) is an aromatic fatty acid with the abilities of histone deacetylase inhibition, ammonia sink and chaperone. Further, PBA is a well-known ER stress inhibitor demonstrated as advantageous in several disorders accompanying ER stress including diabetes and its complications. Presently it is used in urea cycle disorders and is under investigation in clinical trials for numerous other diseases. In the present study, we investigated the efficacy of PBA in ameliorating diabetes-induced muscle atrophy using the streptozotocin-induced diabetic rat model.

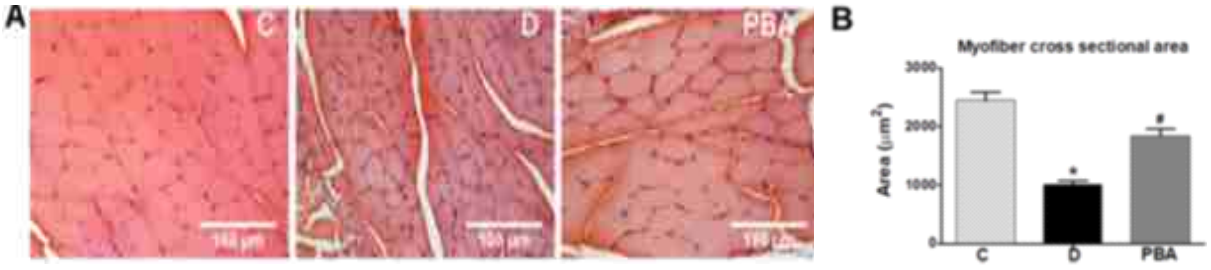
Methodology

A group of 2-month-old male SD rats received a single i.p. injection of streptozotocin in citrate buffer for inducing diabetes while another group of rats received buffer as a vehicle and served as control. After 72 h, rats with fasting glucose levels of ≥150 mg/dL were retained in the diabetes group, and after two months duration of diabetes, half of the diabetic rats were intervened (intraperitoneal) for two more months with PBA at a dose of 40 mg/kg/day. All three groups of animals were fed with AIN-93 diet *ad libitum*. Fasting blood glucose levels and body weight of rats was measured weekly. Four months after diabetes induction, overnight fasted rats were sacrificed to collect gastrocnemius muscle for analysis. The conduct of the experiments was in accordance with the Institutional Animal Ethical Committee of the National Institute of Nutrition. At the end of the experimental period, gastrocnemius muscle of rats was collected, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The muscle fiber cross-sectional area was measured in transverse paraffinized muscle sections, stained with H&E. visualized under microscope and images were obtained. The mean of muscle fiber cross-sectional areas was determined. Total RNA was extracted from the gastrocnemius muscle and reverse transcribed to cDNA. Quantitative RTPCR was performed with cDNA template using gene-specific primers. Data was compared between samples according to a comparative threshold cycle (2-ΔΔct) method and expressed as fold change over control. An equal amount of protein from the muscle homogenates of all the experimental groups was subjected to immunoblotting with specific antibodies. The proteasomal activity in the muscle was assayed using the Biovision Proteasome Activity Assay Kit. To determine apoptosis in the muscle, we performed TUNEL assay using a Kit.

Results

Diabetes adversely affected muscle health as indicated by the decreased cross-sectional area of myofibers. Interestingly, PBA prevented the diabetes-induced decline in the myocyte area as shown in Fig 1. UPS is a primary proteolytic system, excessive activity of which leads to muscle atrophy due to a shift in the balance between anabolic and catabolic processes. Hence, the protein levels of certain UPS components in the rat muscle were examined. The ubiquitin-activating enzyme- E1 is the first enzyme in the ubiquitination process. Atrogin-1/MAFbx is a muscle-specific E3 ligase that targets specific proteins to the proteasome for degradation. UCHL1 and UCHL5 are deubiquitinating enzymes. Fig 2 shows increased expression of E1 enzyme, atrogin-1, UCHL1 and UCHL5 proteins in the diabetes rat muscle. Immunoblot for ubiquitin showed accumulation of ubiquitinated proteins, especially of higher molecular weight in the diabetes group. Further, we observed the increased proteasome activity in the muscle of diabetic rats (Fig 2G). Nevertheless, PBA intervention prevented alterations of the UPS components and their activity in diabetic rats.

Fig 1. Rat skeletal muscle cross-sectional area. (A) Representative cross-sections of gastrocnemius muscle stained with H&E, from control, diabetes and PBA treated diabetes group after four months of experimentation. (B) The cross-sectional area of skeletal muscle fibers. Data are means ± SEM (n=4). C-control group; D-diabetes group; PBA-diabetes rats treated with PBA.



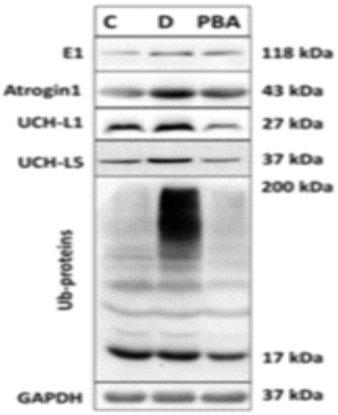
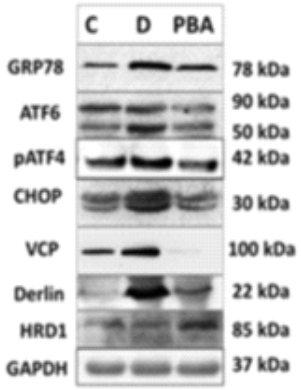


Fig 2. Effect of PBA on rat skeletal muscle ubiquitin-proteasome system. Representative immunoblots of ubiquitin-activating enzyme-E1, Atrogin-1/MAFbx (muscle-specific E3 ligase), deubiquitinating enzymes (UCHL1 and UCHL5), and ubiquitinated proteins. C-control; D-diabetes; PBA-diabetes rats treated with PBA.

Fig 3. Effect of PBA on ER Stress markers and ERAD components in the rat skeletal muscle. Representative immunoblots from whole muscle lysates probed for ER stress markers (GRP78, ATF6, pATF4 and CHOP) and, ER-associated protein degradation (ERAD) components (VCP, Derlin and HRD1). C-control; D-diabetes; PBA-diabetes rats treated with PBA.



The protein expression of ER stress markers and ER-associated degradation (ERAD) components in skeletal muscles of rats in response to diabetes was next investigated. Fig 3 illustrates elevated expression of GRP78, cleaved ATF6, and pATF4 indicative of ER stress in diabetic rat muscle. Further, the higher expression of CHOP confirms the maladaptive phase of ER stress. PBA, a classical ER stress inhibitor, prevented the increased expression of ER stress

markers in diabetic rats. ERAD components that play a vital role in maintaining ER homeostasis were altered during diabetes. HRD1, an E3 ubiquitin ligase (elemental of UPS) of ERAD is declined, while VCP and Derlin1, other ERAD components were increased in diabetes. PBA treatment remarkably prevented these variations (Fig 3).Since chronic ER stress triggers apoptosis, we further examined the status of apoptotic mediators such as p53, BAX and cleaved caspase-3 in rat skeletal muscle. As shown in Fig 4, PBA lowered the protein expression of p53, BAX and cleaved caspase-3, which are increased in diabetes.

Fig 4. Effect of PBA on apoptosis mediators in the rat skeletal muscle. (A) Representative immunoblots from whole muscle lysates probed for P53, BAX and cleaved caspase-3. (B-D) Quantification of the corresponding densitometry data. C-control; D-diabetes; PBA-diabetes rats treated with PBA.

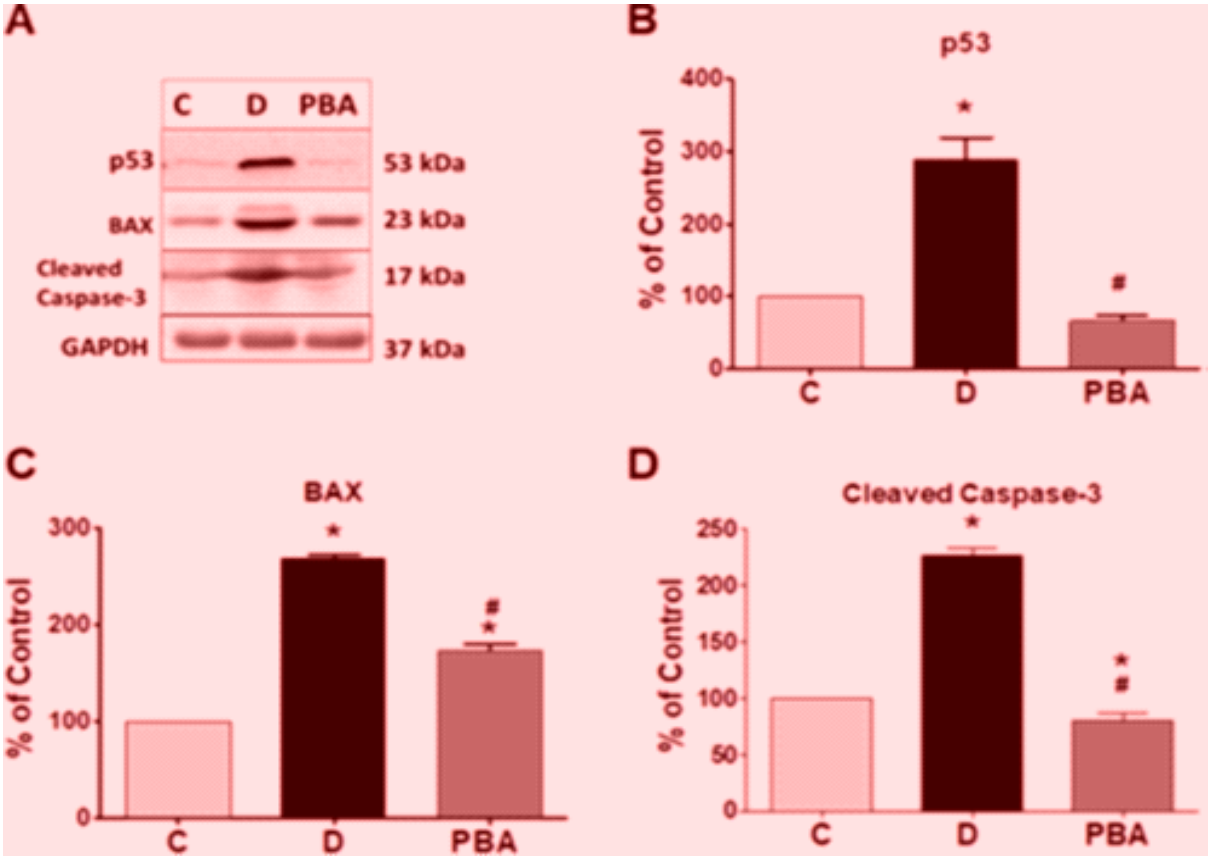


Fig 5. Proposed schematic illustration portraying the beneficiary outcome of PBA

Conclusion

Collectively, these results uncover a novel beneficial effect of PBA in diabetic muscle atrophy by preventing ER stress and alterations in UPS in STZ-induced diabetic rats (Fig 5), highlighting the therapeutic potential of PBA for the treatment of diabetes muscle wastage. Further, PBA is safe and well tolerated and is currently available in the market for various other human ailments.

6. Proteasome inhibitory potential of cinnamon extract in prostate cancer: *In vitro* and *in vivo* studies

Cancer is the second largest non-communicable disease and it has a sizeable contribution to the total number of deaths yearly. The World Cancer Report stated that cancer rates are set to increase at an alarming rate globally. Every year one million new cases of cancer are detected in India and 0.6 million deaths of cancer every year. It has been reported that breast, prostate and colon cancers are on the rise in urban India due to the adoption of a western life style and changing food habits. "Polyphenols" are phytochemicals and are known to have health beneficial effects. A high intake of polyphenol-rich dietary sources is known to reduce cancer risk and also result in tumour growth suppression. Polyphenol-rich dietary sources include tea (green and black), fruits (including dry fruits), spices (such as cinnamon and clove) and vegetables. In recent years it has been demonstrated that human neoplastic cells have very high proteasome activity, which is required for their growth and survival. Proteasome activity is essential for a number of critical cellular processes as described above, hence its inhibition leads to deregulation of signaling cascades ultimately resulting in apoptosis. Since transformed cells have faster proliferative rates and defective cell-cycle checkpoints they are more vulnerable to pro-apoptotic stimuli compared to normal cells. A number of synthetic proteasome inhibitors have been described, and most of them interfere with the proteolytic activity of the β-subunits of the 20S proteasome. Nevertheless, synthetic proteasome inhibitors are associated with some toxicity. Therefore, proteasome inhibitors from natural food sources with minimal or no toxicity can be attractive, potential anticancer agents. Hence there is a great need to identify proteasome inhibitors from natural food sources, displaying either better potency or fewer side effects. The present proposal is aimed at testing the efficacy of cinnamon and its bioactive components for proteasome-inhibitory activity and further its anticancer properties in prostate cancer. This work may lead to identification of proteasome inhibitors from cinnamon bark and development of potential, novel anticancer drugs for cancer therapy.

Objectives

- To test the efficacy of the cinnamon extracts in inhibiting growth, metastasis and inducing apoptosis in cancer cell lines
- To assess the proteasome-inhibitory [Pr-Inb] activity of the cinnamon extract in human cancer cell lines
- To isolate the active component from the cinnamon extract and test its efficacy as a proteasome inhibitor and anticancer agent *in vitro*
- To assess the proteasome inhibitory and anti-cancer potential of other bioactive compounds from cinnamon in cancer cell lines

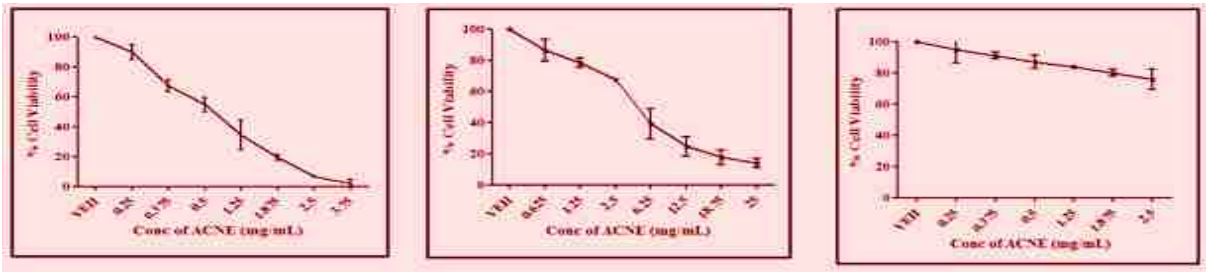
Methodology

- Human prostate cancer cell lines (LNCaP & PC3) were used for all the studies, while rat normal lung stromal cells (NLSC) were used as a control.
- Cell viability was assessed by the MTT assay.
- Cell cycle analysis was done by flow cytometry, while apoptosis was monitored by the Annexin V binding assay.
- Proteasome inhibition was measured in cell extracts prepared from prostate cancer and NLSC using specific fluorogenic substrates to the three enzymatic activities of the proteasome by fluorometry.

Results

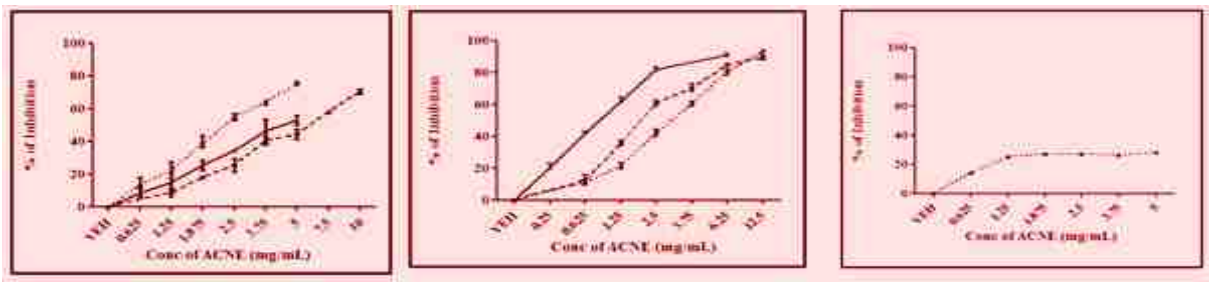
- MTT assay was done in two prostate cancer cell lines namely LNCaP and PC3 with aqueous cinnamon extract (ACNE) to assess the effect of the extract on cell viability/proliferation. There was a dose-dependent decrease in cell viability in both the cell lines treated with the ACNE as shown in Fig 1A & B. However, there was a minimal effect of the ACNE on the viability of NLSC (Fig 1C).

Fig 1. Effect of ACNE treatment on cell viability in LNCaP (A), PC3 (B) and NLSC ©



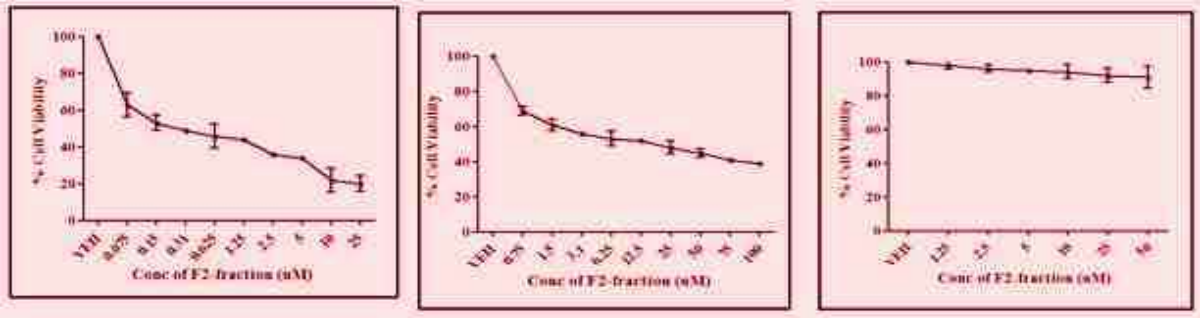
- Further, cell cycle analysis was done to assess the effect of ACNE on the different phases of the cell cycle. Treatment with ACNE led to the arrest of cells in the 'S' phase of the cell cycle.
- There was a dose-dependent increase in the percentage of apoptotic cells after treatment with ACNE. LNCaP cells appeared to be more sensitive to ACNE treatment compared to PC3 cells. 47% of apoptotic cells were seen in LNCaP cells at a dose of 50ug/ml whereas 31% of apoptotic cells were observed in PC3 cells at 375ug/ml.
- 26S enriched extracts were prepared from both cancer and normal cell lines and proteasome inhibition assays were performed. The chymotrypsin-like (Ch-L) and trypsin-like (T-L) and caspase-like (Cp-L) activities of the 26S proteasome were assessed in cell extracts prepared from PC3 and LNCaP cells. There was a dose-dependent decrease in all the three enzyme activities after ACNE treatment which indicates that the proteasomal activities were inhibited. Inhibition of the activities by ACNE in LNCaP cells is shown in Fig 2A, while the results from PC3 cells are depicted in Fig 2B. There was a minimal inhibition of the Ch-L activity in NLSC with ACNE treatment as shown in Fig 2C.

Fig 2. Effect of ACNE on inhibition of the proteasomal enzyme activities in cell extracts from LNCaP cells (A), PC3 (B) and inhibition of Ch-L activity in NLSC extracts ©.



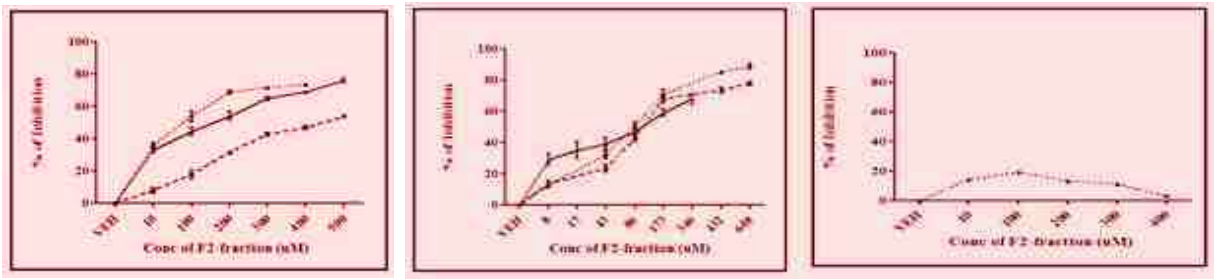
- Procyanidin B2 (PCB2) enriched fraction (F2) was isolated from cinnamon bark powder. The identity of PCB2 compound in the F2 fraction was characterized by HPLC and mass spectrometry. MTT assays were done in both prostate cancer cell lines and normal lung stromal cells with PCB2 treatment to determine its effect on cell viability. As shown in Fig 3A and 3B below there was a dose-dependent decrease in the cell viability with an IC_{50} of 3.81µM and 50.04µM in LNCaP and PC3 cells respectively. Fig 3C shows no change in cell viability in the normal cells with PCB2.
- PCB2 treatment led to growth arrest of LNCaP cells in 'S' phase of the cell cycle and a dose-dependent increase in apoptotic cells as measured by annexin V binding assay.

Fig 3. Effect of PCB2-enriched (F2 fraction) on the cell viability in LNCaP (A), PC3 (B) and NLSC(C)



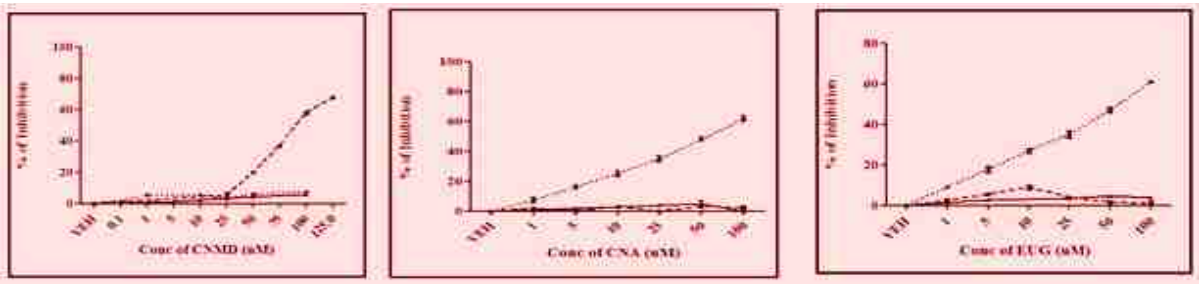
- There was a dose-dependent decrease in all the three proteasomal catalytic enzyme activities of the 26S proteasome after PCB2 treatment in LNCaP cells as shown in Fig 4A-C.
- MTT assays were done in both the prostate cancer cell line, LNCaP and normal lung stromal cells (NLSC) with other bioactive compounds present in cinnamon (i.e. cinnamaldehyde (CNMD) or cinnamic acid (CNA) or eugenol (EUG). Cells were treated with the compounds for 24h. There was a dose-dependent decrease in the cell viability with all the compounds in the LNCaP cell line. The inhibitory concentrations were as follows: CNMD (IC_{50} 37.34µM), CNA (IC_{50} 5.61µM) and EUG (IC_{50} 8.12µM) respectively in LNCaP cells. No change in cell viability was seen in normal cells treated either with CNMD or CNA or EUG for 24h (data not shown).

Fig 4. Effect of PCB2 enriched (F2 fraction) on inhibition of the proteasomal enzyme activities in cell extracts from LNCaP cells (A), PC3 (B) and inhibition of Ch-L activity in NLSC extracts (C).



- Inhibition of the chymotrypsin-like (Ch-L), trypsin-like (T-L) and caspase-like (Cp-L) activities of the 20S proteasome was assessed with all the cinnamon compounds (CNMD, CNA and EUG). Treatment with CNA and EUG inhibited only the Ch-L activity in a dose-dependent manner with IC_{50} values of 67.5 µM, and 67.99 µM, respectively (**Fig 5B &C**) whereas treatment with CNMD inhibited only T-L like activity in a dose-dependent manner with IC_{50} of 95.02 µM (**Fig 5A**).

Fig 5. Effect of treatment with CNMD (A), CNA (B) and EUG (C) on the 20S purified proteasomal enzyme activities in LNCaP cell extracts.



Conclusion

Treatment of prostate cancer cells with aqueous cinnamon extract (ACNE) led to a dose-dependent decrease in viability, arrest of cells in 'S' phase of the cell cycle and apoptotic cell death. ACNE treatment also led to inhibition of proteasome enzyme activities in cancer cell extracts. Further bioactive compounds present in cinnamon bark such as procyanidin B2, cinnamaldehyde, cinnamic acid and eugenol also decreased viability, increased apoptotic cell death and led to inhibition of the catalytic enzyme activities of the 26S proteasome. In conclusion, the results of this project demonstrate that cinnamon and its active components act as proteasome inhibitors and anti-cancer agents.

7. Impact of vitamin D deficiency on the cardiovascular function in a rat model

Vitamin D is a precursor of the steroid hormone calcitriol that is crucial for bone and mineral metabolism. Specific receptors for the hormonal form of vitamin D are ubiquitously present in the different tissues which include the heart. Both the high prevalence of vitamin D deficiency in the general population and the identification of the vitamin D receptor in the heart and blood vessels raised interest in the potential cardiovascular effects of vitamin D. Experimental studies have demonstrated various cardiovascular protective actions of vitamin D. Vitamin D deficiency is widely prevalent in India and worldwide. Although most consequences of vitamin D deficiency involve the musculo-skeletal system, there is a growing body of evidence suggesting that low levels of vitamin D may adversely affect the cardiovascular system. Vitamin D deficiency, which is affected by multiple factors, appears to have an association with diverse cardiac diseases starting with its direct effect on the cardiac cell, its association with coronary artery disease (CAD), and its risk factors such as diabetes and hypertension (HTN); ending at last and probably not least in its relation with congestive heart failure (CHF). The biologically active form of vitamin D 'Calcitriol' is known to be one of the negative endocrine regulators of the Renin-Angiotensin-Aldosterone System [RAAS] which regulates blood pressure. Vitamin D deficiency may be an important factor in the pathogenesis of heart failure. Vitamin D is known to act as an anti-oxidant and increase anti-inflammatory factors. Therefore, vitamin D deficiency is linked to a broad spectrum of cardiovascular disease and its risk factors.

Objectives

- To create a vitamin D deficient rat model.
- To study the role of vitamin D on the oxidant status of rat heart and examine the histo-pathological changes if any in the heart.

Methodology

Sprague Dawley Weanling (21 days old) rats were initially divided into two groups, namely Control (normal Vitamin D), and Deficient (no Vitamin D). Rats were housed individually in wire mesh–bottomed cages, and maintained under incandescent lighting conditions (12h light/dark cycles) to prevent cutaneous vitamin D synthesis. The Control group will be fed on a synthetic AIN-93 based formulation control diet (66kcal% carbohydrate, 20kcal% protein, 15kcal% fat) with 1000IU D3/kg diet), while the Vitamin D deficient group will be given the

same diet but devoid of any Vitamin D_3 for a period of 10-12 weeks. Animals were provided free access to diet and de-ionized distilled water. Food intake and body weights were monitored daily and weekly respectively. The Vitamin D deficient status of the rats was confirmed by measuring the serum 25-hydroxycholecalciferol ($25(OH)D_3$- a valid indicator of vitamin D status) and calcium levels, after which the rats in the deficient group were further subdivided into two groups: one group (Def) were continued on the deficient diet, the second group was shifted onto the control diet containing Vitamin D (1000IU/kg diet) (RD) to assess reversibility of the effects. All the three groups of rats were fed on their respective diets for a further 6 weeks. At the end of the feeding period the serum $25(OH)D_3$ and calcium levels were checked again and rats were sacrificed; blood and heart collected, weighed and snap frozen in liquid nitrogen and stored at -80°C till processed.

- Serum calcium was measured by AAS while the levels of vitamin D metabolites 25(OH)D3 and 1,25(OH)2D3 were estimated by HPLC and ELISA kit.
- Heart lysates were prepared from the different groups and antioxidant parameters such as malondialdehyde (MDA), protein carbonyls, reduced glutathione and antioxidant enzymes (superoxide dismutase (SOD), Catalase and glutathione peroxidase (GPx) were measured using standard protocols. Nitrosative stress was monitored by estimating the nitrate levels in the lysates. Protein was estimated in the lysates by the BCA method.
- Heart sections were prepared from the three groups and stained with Masson Trichrome stain to assess the deposition of collagen (a marker for fibrotic changes).

Results

- Vitamin D deficiency was confirmed by undetectable serum $25(OH)D_3$ levels (an indicator of vitamin D status) and hypocalcaemia. In addition, the serum $1,25(OH)_2D_3$ levels (a biologically active hormonal form of vitamin D3) was significantly ($P \leq 0.001$) decreased (~10pg/ml) in the vitamin D deficient confirming the D-deficient status. On the contrary, the other two groups fed diets containing vitamin D had normal (>40 pg/ml) serum $1,25(OH)_2D_3$. The vitamin D dependent parameters in the different groups, is depicted in Table 1.

Table 1. Vitamin D dependent parameters in the different experimental groups

Parameter	Control (n = 6)	Deficient (n = 6)	Rehab (n = 6)
$25(OH)D_3$ (ng/ml)	22.8- 2.9[a]	ND	19.8- 2.5[a]
$1,25(OH)_2D_3$ (pg/ml)	50.6- 1.64[a]	10.3- 1.48[b]	42.0- 1.67[c]
Calcium(mg/dl)	9.91- 0.37[a]	6.45- 0.25[b]	10.2- 0.59[a]
Phosphorus(mg/dl)	7.54- 0.09[a]	9.30- 0.17[b]	7.75- 0.11[a]
Alk. Phosphatase(U/L)	238- 7.8[a]	364- 8.5[b]	242- 10[a]

- Both protein carbonyls and total nitrate levels were observed to be significantly higher in the deficient heart in comparison to the control heart (Fig 1A & B). Further the activity of the anti-oxidant enzymes namely SOD, catalase and glutathione peroxidase in heart lysates of the different experimental groups was assessed. The Activity of the SOD enzyme was significantly increased, while catalase was decreased in the deficient muscle compared to vitamin D given control group (Fig 2A & B). Rehabilitation with vitamin D appeared to normalize the catalase activity. On the other hand the activity of GPx was not altered in the deficient heart.
- Deposition of collagen in the heart is known to lead to diastolic and systolic dysfunction. Masson Trichrome (MT) is used to stain collagen in tissue sections. Heart sections revealed an increase in the amount of collagen in the vitamin D deficient rat hearts compared to vitamin D sufficient rat hearts (Fig 3). Rehabilitation with vitamin D appeared to reverse the changes.

Fig 1. Levels of protein carbonyls (A), and total nitrate (B) in heart lysates in the different experimental groups

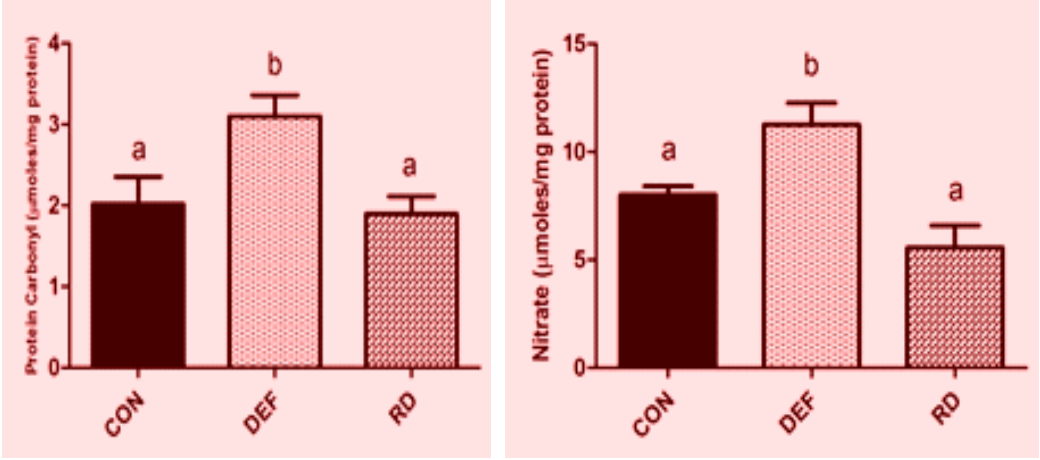


Fig 2. Antioxidant enzyme activities in heart lysates of the different experimental groups

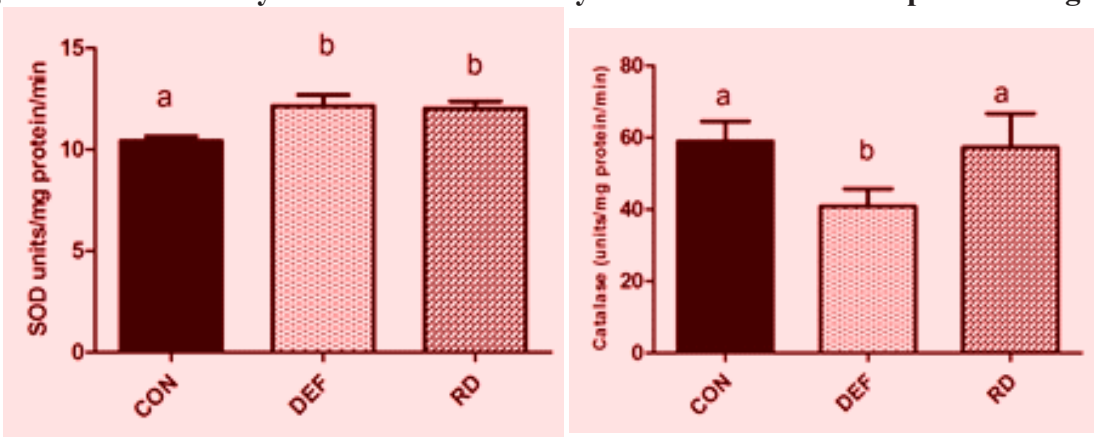
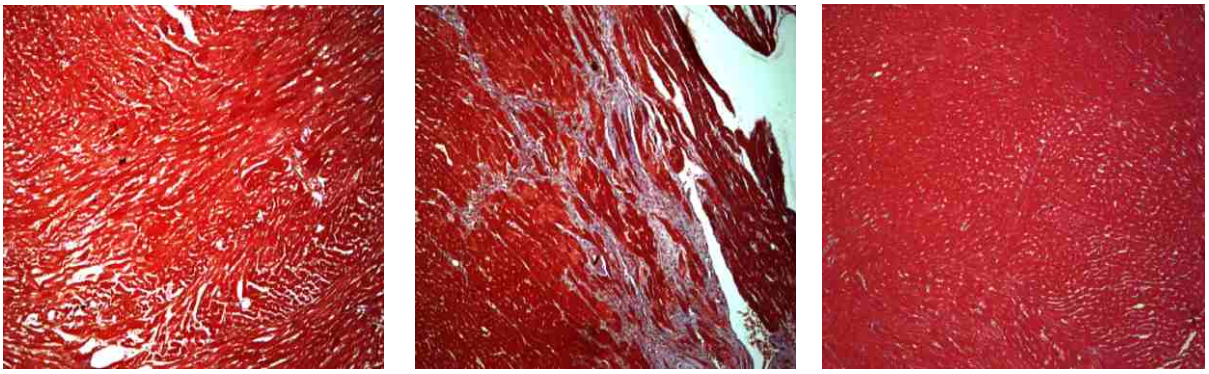


Fig 3. Masson trichrome stained heart sections showing fibrotic changes in the vitamin D deficient heart compared to control heart



CONTROL (+D) **DEFICIENT (-D)** **REHABILITATED (+D)**

Conclusion

Vitamin D deficiency led to an increase in oxidative stress and altered the activity of anti-oxidant enzymes in the rat heart. Further, vitamin D deficiency appeared to lead to fibrotic changes in the heart. Supplementation of the deficient rats with vitamin D corrected all the changes.

8. Application(s) of human derived embryonic stem cells to understand the dynamics of folic acid and vitamin B12 (deficiency/ supplementation) towards vasculogenesis-recapitulation of early embryonic development

The coenzymatic and functional role of micronutrients like Vit B6 (pyridoxine), B9 (Folic acid) and B12 (cobalamin) has been well documented in several metabolic pathways and their requirements in target organs. However, their influence on tissue regeneration, maintenance of red blood cells, stem cells, components of the nervous and immune systems is still obscure and needs to be studied more so on vasculogenesis. It was proposed to address vasculogenesis using in vitro model system of human derived embryonic stem(hESCs) under differential conditions of Vit B6, Vit B9 and Vit B12.

Objectives

- To customize for the deficient media (Vitamin B6, B9 & B12), assess the cultures for maintenance, proliferation and pluripotent state of human embryonic stem cells (hESCs), embryoid bodies (EBs).
- To study commitment/ differentiation potential of hESCs to three germ layer lineages using specific biomarkers in the presence and absence of micronutrients.
- To establish an *in-vitro* embryoid body model mimicking sequential processes of early embryoid vasculogenesis and endothelialisation in the presence and absence of micro-nutrients.
- To assess the role of inflammatory and stress signals (pro-anti-inflammatory responses) on early embryonic vasculogenesis with and without micronutrients
- To analyse the epigenetic out comes–nutrient gene interactions and nutrient-nutrient interactions.
- To evaluate the role of micronutrients on total methylation during vasculogenesis.

Methods

Standardized the culture conditions in four different concentrations (0%, 30%, 70% and 100% supplemented media for Vit B6, Vit B9 and Vit B12. The hPMSCs were procured from the university of Hyderabad, with their ethical approval. All the cells were cultured at 37°C with 5% CO_2. At frequent intervals microscopic examination was carried out for cell morphology, EB formation and Vasculogenesis, MTT assay for viability and senescence associated β gal assay were employed to study the effect of B6, B9 and B12 deficiency/ supplementation.

Conclusion

Among several deficient conditions,10 to 30 % deficiency supported the cells as compared to 70% have shown. The initiation of vasculogenesis was noted in the combination group of Vit B6, Vit B9 and Vit B12 (30%) as compared to per se treatments.

9. Effect of isoflavones isolated from naturally available cowpea as a source for the treatment of osteoporosis in MG-63 human osteosarcoma cells and to assess its synergetic role with vitamin D in bone formation

It is well known fact that the bone volume is maintained by two phases of bone remodeling, namely, bone formation by osteoblasts and bone resorption by osteoclasts. Maintaining the balance between these two processes is found to be a key aspect in reducing the bone related disorders. Genistein and daidzein are natural isoflavones found in *leguminosae*. Genistein has been shown to have an inhibitory effect on protein tyrosine kinases, whereas the biological effect of daidzein has not been clarified fully. It has been shown recently that genistein has an anabolic effect on bone metabolism the isoflavone can increase alkaline phosphatase activity, DNA, and calcium contents in bone tissues. Earlier studies have reported that genistein inhibits bone loss in ovariectomized rats. Further, it has been demonstrated that genistein has a direct inhibitory effect on bone resorption and a stimulatory effect on bone formation in tissue culture system *in vitro*. The effect of daidzein on bone metabolism is unknown. More recently, there is a possibility of daidzein stimulating bone formation and mineralization *in vitro* cell culture systems. The anabolic effect of daidzein and genistein on bone metabolism seems to be equal and comparable. Infact, when daidzein is hydroxylated, its chemical structure is similar to that of genistein. Hence, genistein and daidzein may have similar kind of mechanisms on bone metabolism, and these isoflavones may have pharmacological and nutritional roles in the prevention of osteoporosis. The cellular mechanism by which an isoflavone exerts an anabolic effect on bone metabolism, however, remains to be elucidated. Interestingly, vitamin D also found to play an important role in the bone metabolism, but the mechanism of action through which vitamin D acts remains to be elucidated.

Since, recently concluded study on the diet induced model of osteoporosis in the WNIN wistar rats, indicates that naturally available rich phytoestrogens content foods such as cowpea and other food isoflavones are effectively acting in improving the BMC, BMD, Ca, P and vitamin D, further studies in understating the osteoblastic and osteoclastic mechanism is to be investigated. During the process, several parameters like measurement of osteocalcin synthesis, ALP, expression studies by RT-PCR, Western blot analysis, measurement of Vitamin D levels, cell morphology, collagen synthesis, calcification potential, studying the relative proliferation, transfection and luciferase assay, RNA isolation and Northern blot analysis and other investigations are to be conducted in a cell culture model of MG-63 human osteosarcoma cells. Probably the outcome of this study would add to the knowledge of osteoporosis prevention.

Objectives

- Investigating the effects of treatment of cowpea extract (CPIF), vitamin D (VD) and the combination of CPIF and VD on MG-63 human osteosarcoma cells viability and morphology.
- To study the effects on relative proliferation, osteocalcin secretion and mineralization assays, the formation of bone nodules, calcium (Ca) and phosphorous (P) content and, alkaline phosphatase (ALP).
- To study the effects of CPIF in stimulating the osteogenic gene expression of different bone forming protein markers.
- To find the antioxidant effects of the CPIF, expression of p53 tumour suppressor protein and the DNA content, apoptosis, DNA fragmentation, cell cycle analysis and to study the effects on the other cellular markers like COX activity, the Isoprostane content, Protein kinases (A and C).

Methodology

Extraction and quantification of CPIF using HPLC-DAD analysis

Extraction: Extraction of isoflavones are eluted with ethyl acetate and analyzed by HPLC.

Cell culture and exposure studies: MG-63 cells were procured from NCCS Pune. The cells were cultured as monolayers as per the standardized cell culture protocol. After 24 hrs the cells were exposed with different concentrations of Dz(0.01, 0.1, 1, 2.5, 7.5 and 10µM) Ge(0.01, 0.1, 1, 2.5, 7.5 and 10µM), CPIF extract, V-D(15 to 50µM), Dz+Ge (5 to 30 µM), V+C, [V+C+(D+G)], and further cultured for appropriate periods of time(12, 24, 36, 48 HRS). The viability was tested using MTT.

Scanning Electron Microscopy: MG-63 cells are exposed to EC_{50} Concentrations of all exposure treatments for 48Hrs and then washed with PBS for three times. The cells were scraped and then fixed in 2.5% glutaraldehyde overnight. This is followed by PBS wash and transferring the cells on to the blocks. Finally, the images were captured at 1000X using Scanning electron microscopy.

***Inhibition Studies: BMP Signalling Pathway*:** MG-63 cells were cultured for 48 HRS and exposed to all standardized EC_{50} exposure treatments individually and in combinations with or without Noggin, an antagonist of BMP-2. As per standardized, immunoblot and qRT-PCR protocol the expression levels of, BMP-2, OSX, T-Smad-1/5/8, P-Smad-1/5/8, ALP, OPN and Collagen were identified before and after Noggin treatment.

Statistical data

All the values are presented as mean and standard errors of mean (SEM). Data were evaluated by one way ANNOVA. The significance was calculated by using post a hoc Tukey's test. A p value of < 0.05 was considered statistically.

Results

Fig 1. Represents histograms of A) Expression levels of BMP-2 by FACS. B) Expression levels of OSX by FACS. C) Expression levels of ALP by FACS.D)Expression levels of OPN by FACS. E) Expression levels of Collagen by FACS. Data were analyzed by one-way ANOVA with *post-hoc* least significant difference (*post-hoc* LSD) test. Statistically significant at *P*-value ≤ 0.05 level (a). Significant at *P*-value ≤ 0.01 level (b).

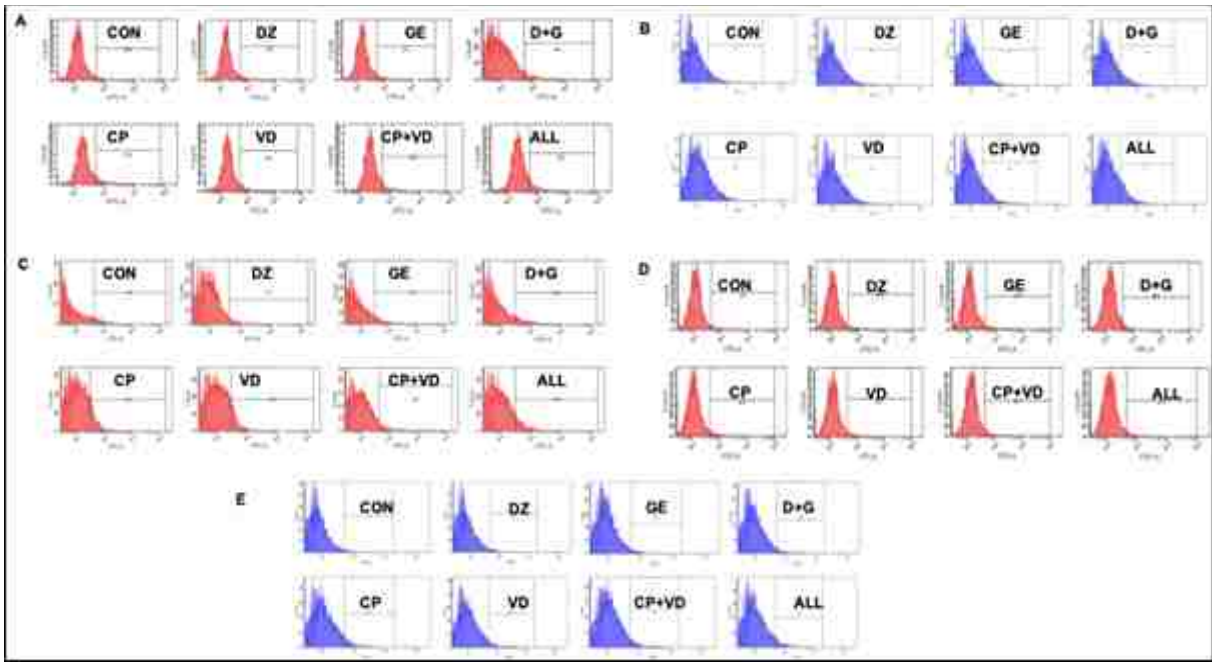


Fig 2. Represents histograms of A) Densitometry analysis of BMP-2. B) Densitometry analysis of OSX. C) Densitometry analysis of T-Smad-1/5/8. D) Densitometry analysis of P-Smad-1/5/8. E) Densitometry analysis of ALP. F) Densitometry analysis of OPN. G) Densitometry analysis of Collagen. H) Immunoblots of ß-Actin, BMP-2, OSX, T-Smad-1/5/8, P-smad-1/5/8, ALP, OPN, Collagen. Data were analyzed by one-way ANOVA with *post-hoc* least significant difference (*post-hoc* LSD) test. Statistically significant at *P*-value ≤0.05 level (a). Significant at *P*-value ≤0.01 level (b).

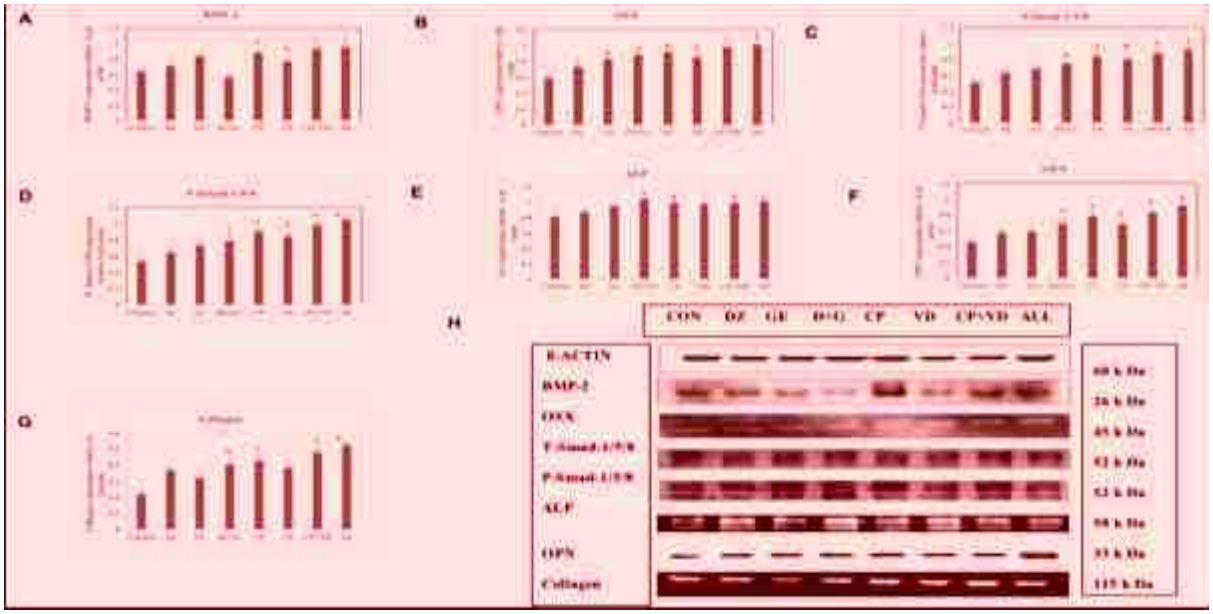


Fig 3. Represents histograms of A) Densitometry analysis of OSX after Noggin treatment. B) Densitometry analysis of T-Smad-1/5/8 after Noggin treatment. C) Densitometry analysis of P-Smad-1/5/8 after Noggin treatment. D) Densitometry analysis of ALP after Noggin treatment. E) Densitometry analysis of OPN after Noggin treatment. F) Densitometry analysis of Collagen after Noggin treatment. H) Immunoblots of ß-Actin, OSX, T-Smad-1/5/8, P-smad-1/5/8, ALP, OPN, Collagen after Noggin treatment. Data were analyzed by one-way ANOVA with *post-hoc* least significant difference (*post-hoc* LSD) test. Statistically significant at *P*-value ≤0.05 level (a). Significant at *P*-value ≤0.01 level (b).

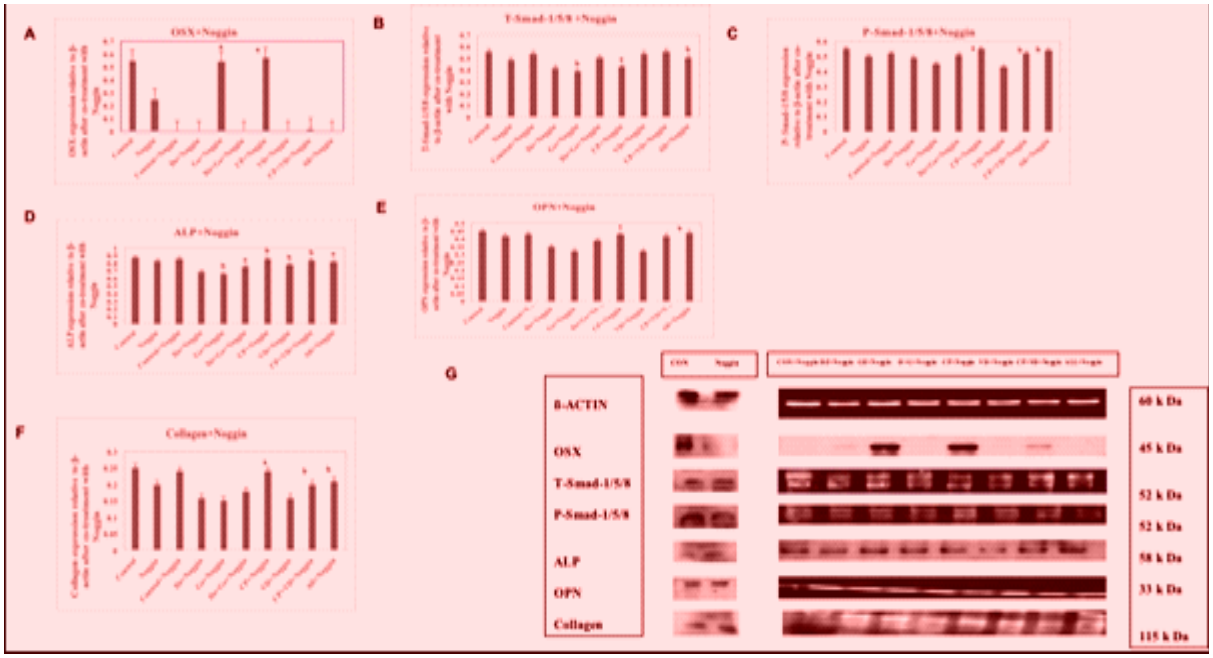
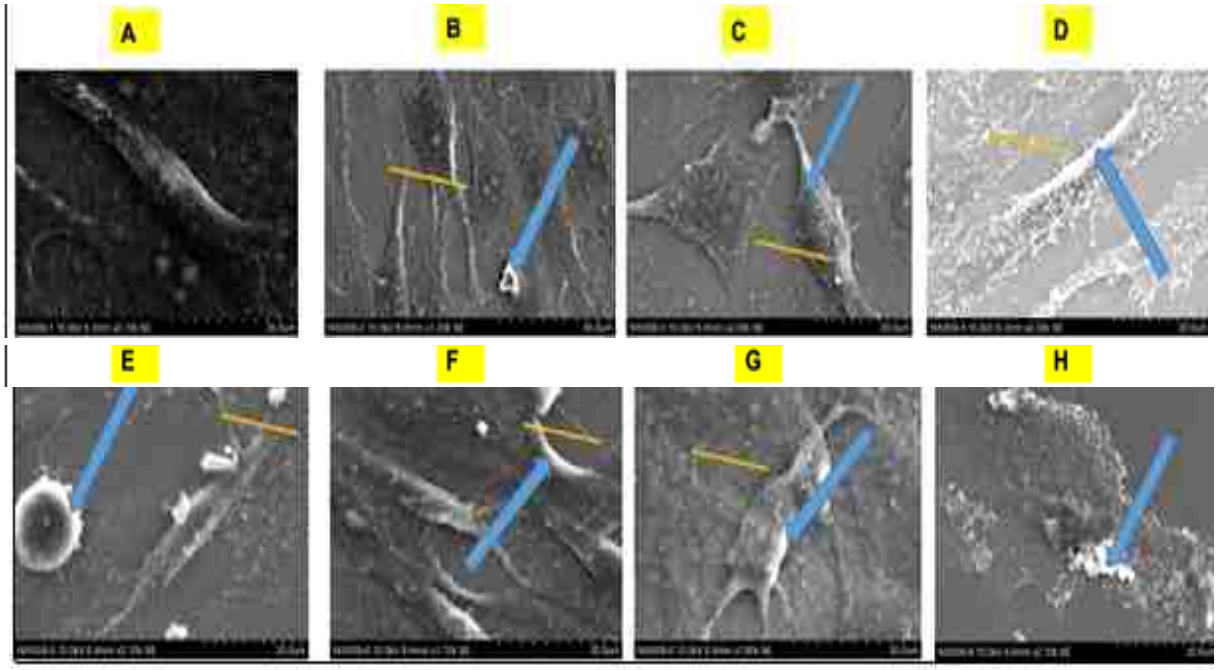


Fig 4. Represents Scanning electron microscopic images of A) Control B) Daidzein C) Genistein D) Daidzein+ Genistein E) Cowpea F) Vitamin D G) Cowpea+ Vitamin D H) All. Blue arrows indicate calcium deposit formation. Yellow arrows indicate collagen fibrils in the respective images at 1000X resolution.



Salient findings

- After treatment, the morphology of the cells showed improved cell to cell connections and an increase in cell number.
- Cell viability increased when compared to control after treating with CPIF extract and V-D.
- Alkaline phosphatase levels (ALP), BMP-2, Osteocalcin (OC), Osteonectin and Osteopontin, RANK-L levels improved significantly after treatment when compared with that of control by all parameters.
- Apoptotic cells and ROS levels, p53, 8-isoprostane, Caspase-3 and PGE-2 decreased after treatment with Cowpea extract and V-D.
- Intracellular calcium and p38 levels increased after treatment.
- OPG and RANKL levels which got increased after treatment, the same expression levels of the markers decreased after co treatment with ICC 182 780 an estrogen antagonist, suggesting that our compounds activate OPG/RANKL pathway and pathway is estrogen dependent.
- Initial increased expression levels of RUNX-2, Smad-3, T and P-p38 MAPK levels and their downstream molecules Osteocalcin, Osteonectin after the treatment with a specific inhibitor of p38 MAPK, i.e. SB203580, attenuated supporting the fact that the compounds activate cbfa1 gene by the involvement of p38 MAPK pathway.
- Similar way, the involvement of our compounds in activating BMP signaling pathway leading to differentiation of Osteoblasts is found to be little paradox since there is a moderate decrease in the expression levels of the BMP-2, OSX, T and P-Smad-1/5/8, ALP, OPN and Collagen after the treatment with Noggin a BMP antagonist, initially in which the gene expression levels were high.
- Considerable increase in calcium deposit and collagen fibrils formation is observed after treatment in MG-63 cells by Scanning electron microscopy studies.

Conclusions

To summarize, with increased activity of cell proliferation, biochemical parameters like (ALP, OC, p38) intracellular calcium levels and Osteocalcin, ALP, Osteonectin and Osteopontin and RANK-L marker levels and antioxidant activities (CAT, SOD, Vitamin C, and GSH) using CPIF and V-D and decreased activity of MDA levels and TRAP activity and ROS levels and different inflammatory markers like PGE2, 8-IP, Caspase-3 showed enhancing effects in stimulating Osteoblastic activity and bone formation after treatment indicating that isoflavones CPIF and V-D stimulates bone formation. The expression levels of the cytokines i.e. RANKL and OPG were upregulated by the action of CP and VD in estrogen dependent/mediated pathway at m-RNA level. CP and VD activate the p38 MAPK pathway and its downstream molecules. Similarly, CP and VD, also activate BMP signaling pathway and help in differentiating the Osteoblasts. Thus, the present study may provide the beneficial role of using natural Isoflavones in protecting the bone mass but whether it can be used in a therapeutic way has to be further elucidated and investigated.

10. Intra-cellular mechanism of naturally available neuro-protective compounds in mitigating the combined toxicity generated by the lead (Pb^{2+}) in combination with amyloid peptides in human brain cells

Environmental exposure to low-levels of lead (Pb^{2+}) is known to exert neurotoxic effects resulting in an impairment of higher functions of the brain in infants as well as in Adolescents. Continuous exposure to Pb^{2+} leads to growth and mental retardation, intellectual impairment, neurobehavioral changes and hyperactivity, Other pathological effects like suppression of cognition; learning and memory functions were also characteristic features of Pb^{2+}-intoxication. Acute high dose of Pb-intoxication can also cause encephalopathy with coma, convulsions and frequent fatal outcome. Environmental toxins are among the risk factors that may contribute to the development of neuro-pathological diseases (Platt, B. 2006). A number of epidemiological investigations have demonstrated that among heavy metals, Pb^{2+} is considered to be an important possible risk factor for the pathology of Alzheimer's disease (AD). AD is an irreversible and progressive neurodegenerative disorder, which ultimately results in progressive loss of cognitive function, dementia and death. It is characterized by loss of neurons, an abundance of intra-neuronal neurofibrillary tangles associated with protein Tau and extracellular deposition of the β-Amyloid Peptide (AP) as amyloid plaques. Though Aβ formation has been considered as a pivotal and crucial step in the pathogenesis of AD, the mechanism by which Aβ induces neuronal death is still unknown. It has been shown that during the progression of AD, the macromolecular oxidative damage and accumulation of reactive oxygen species (ROS) are prevalent. Oxidative stress plays an important role in Aβ-mediated neuronal cytotoxicity by triggering or facilitating neuro-degeneration through a wide range of molecular events, eventually leads to neuronal cell loss. Induction of oxidative stress is involved in mechanisms where the production of excessive ROS can mediate neuronal apoptosis in Aβ-induced neuronal cell death.

The beta-amyloid peptide (AP), derived from sequential proteolysis of the amyloid precursor protein (APP) by β-secretase and γ-secretase has been shown to trigger neurotoxicity, oxidative damage and inflammation . Recent studies from our laboratory reported that Pb^{2+} plays a very important role in the generation of oxidative stress, leading to apoptosis and initiates

inflammatory changes and the protection by a different naturally available neuro-protective compound. In addition to the synergistic role of Pb^{2+} and AP, they found to generate neurotoxicity through induction of apoptosis, inflammation and oxidative stress. Therefore, the toxicity being resulted from the combination of Pb^{2+} and AP needs to be addressed through the exposure of different neuro-protective compounds for their anti-neurotoxic effects.

Objectives

- To study the synergistic effects of Lead and Amyloid peptides on human brain cells in terms of cell viability and proliferation.
- To identify and study the intracellular mechanisms of naturally available Neuro-protective compounds (EGCG, Genistein) in mitigating the combined toxicity generated by Lead in combination with Amyloid peptides in Human Brain cells.
- To study their specific intracellular effects on different biomarkers of inflammation, signal transduction, oxidative stress and apoptosis, DNA fragmentation, COX activity, isoprostane levels, Protein kinase (A and C) and the cell cycle analysis.
- To study the protective effects on the gene expression.

Methods

- SH-SY5Y Human neuroblastoma cells were procured from NCCS Pune. Lead acetate and MTT are purchased from SIGMA. Amyloid peptides of (1-40), (25-35) were procured from Genscript. Other chemicals used are all obtained from standard chemical companies.
- ***Cell culture*:** SH-SY5Y cell lines were grown in RPMI1640 medium containing 10%FBS, 50µg/ml Penicillin-streptomycin and OPI (150µg/ml Oxalo acetate, 50µg/ml pyruvate and 0.2U/ml Insulin) in a humidified air/5% CO2 chamber at 37. Medium was changed every three to four days and passed once they reached approximately 80% confluence.
- ***Exposure of cells to Pb, AP (1-40) and (25-35) EGCG:*** Cells were seeded at 2×10^4 cells per wellin a 96-well plate and grown for two days and pretreated with EC_{50} concentrations of EGCG. Cells were exposed to IC_{50} concentrations of Pb, AP(1-40), and (25-35) individually and in different combinations for 24hrs.MTT assay was performed and values were expressed in terms of percentage of cell viability.
- ***Quantification of Apoptosis and Cell cycle analysis:*** Different combinations of EGCG exposed and unexposed combinations of Pb, AP(1-40) and (25-35), treated SH-SY5Y cells were used for early apoptosis and Late apoptosis/ necrosis by Flow Cytometry, according to the manufacturer's instructions (Chemicon International, ApopNexin™ FITC Apoptosis detection kit, #APT750.Similarly, to the fixed cell lysates, Propidium Iodide was added based on the standard protocol by Flowcytometry.
- ***Determination of caspase-3 activity assay:*** Caspase-3 activity was determined according to the manufacturer's protocol (#k106, Caspase-3 activity assay kit, Biovision).
- ***ThT and Congo red fluorescence:*** Thioflavin T fluorescence and Congored binding intensity of amyloid peptide were determined by the protocol Hyung et al2013.
- ***BACE 1 levels determination:*** The enzymatic activity of β-secretase was measured by a fluorimetric reaction (Promokine-#CA577-K360).
- ***Oxidative stress markers assays:*** *(Glutathione, SOD, catalase, TBARS, VitC, and Nos levels:* All the antioxidant assay markers levels were carried out by standardized protocols.
- ***Gene expression studies:*** Total RNA was extracted from cells cultured in the 25cm2 plastic flasks with 5 × 106 cells using Trizol reagent (SIGMA) as described by the manufacturer.

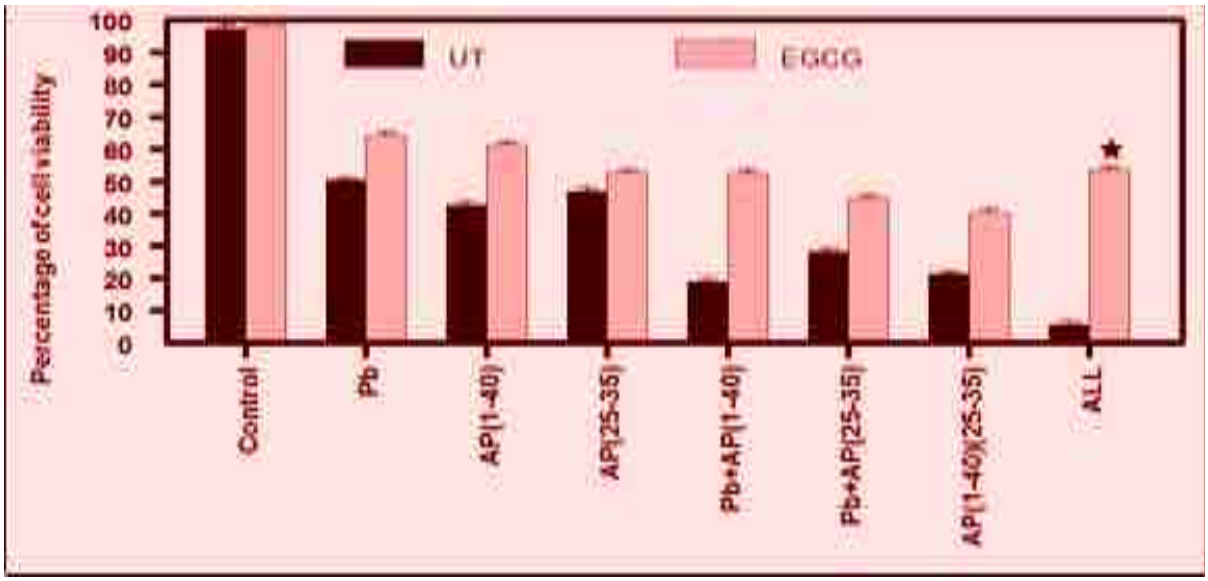
And its reverse transcription to cDNA was performed using Verso cDNA Synthesis Kit (Thermo verso cDNA synthesis kit), according to the manufacturer's protocol. Human *β*-actin, ABCA7(5'-CTGCCCACCGCTAGGTATTC-3', R5'-TAGGCACTCTACCTTGCCCT-3'),BACE1 (5'-TGACCCTTCTTAGCCCTGGA-3', R5'-GTGGAGCTTCACTCTGGACAT-3)', Bax, $Bcl_2$and P^{53} primers were synthesized by IDT according to the following sequences. SYBR Green QPCR kit (F416L) was used for real-time PCR to detect the abundance of PCR products among samples by the Real-time PCR system (AB, Step one plus).

- ***Western blot analysis***: Treated cell lysates were prepared by RIPA buffer with Protease inhibitor cocktail. The Protein samples were quantified by Bradford assay. Then 30µg of protein was loaded and resolved by 12% SDS-PAGE (Biorad), transferred to the nitrocellulose membrane (Bio-Rad Laboratories, Inc.), and incubated with primary anti-rabbit monoclonal antibodies against Bax (1:1000 dilution),Cyt C, and bcl-2 (1:1000 dilution) overnight at 4°C. Subsequently, the membrane was incubated with HRP-conjugated goat anti-rabbit secondary antibodies (1:10,000) for 1 h at room temperature. Images were acquired and quantified by an Image Analyzer (G-Box iChemi XR; Syngene, Cambridge, UK). Images were analyzed and quantified using Image J software.

Results

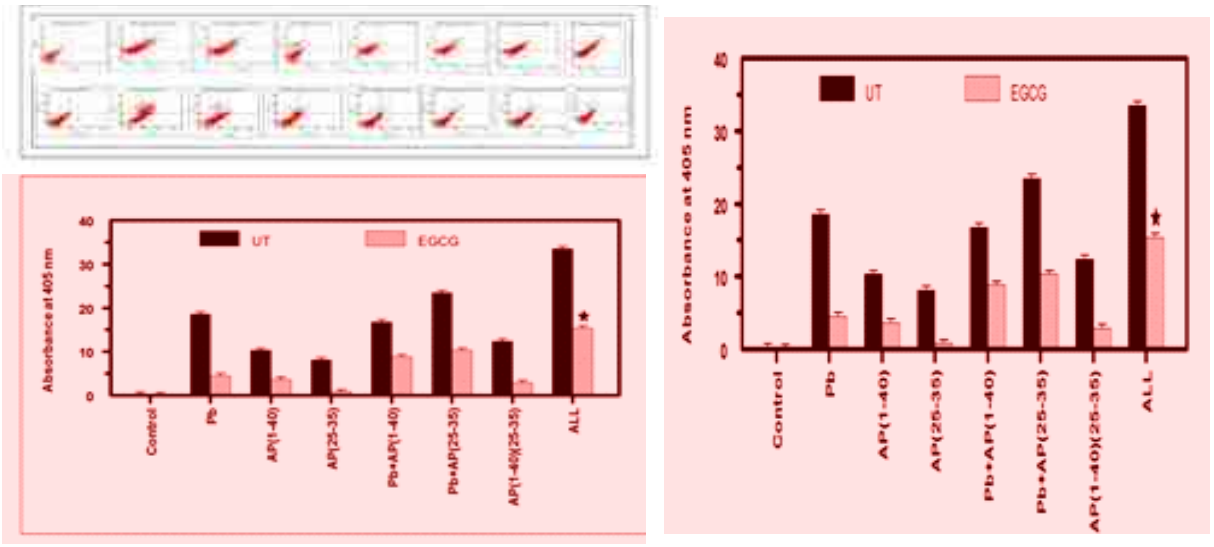
- Cells were treated with EGCG in both time and concentration-dependent manner and effective concentration value for cell viability as 50µM was standardized for 24 hrs by MTT assay. The viability was increased in Lead and Amyloid peptide exposed cells individually and in the combination of All, Lead with Amyloid peptide (1-40) and (25-35) as 64.3%, 61.3%,52.7%, 53.5%, 52.5% 44.7% respectively when compared with control when pre-treated with EGCG (50µM) (Fig 1).

Fig 1. Effect of EGCG on cell viability when treated with Lead and Amyloid peptides individually and in different combinations. The data represented is the mean±SD of triplicates. * represents a significant increase in viability when compared to control at $p<0.05$.



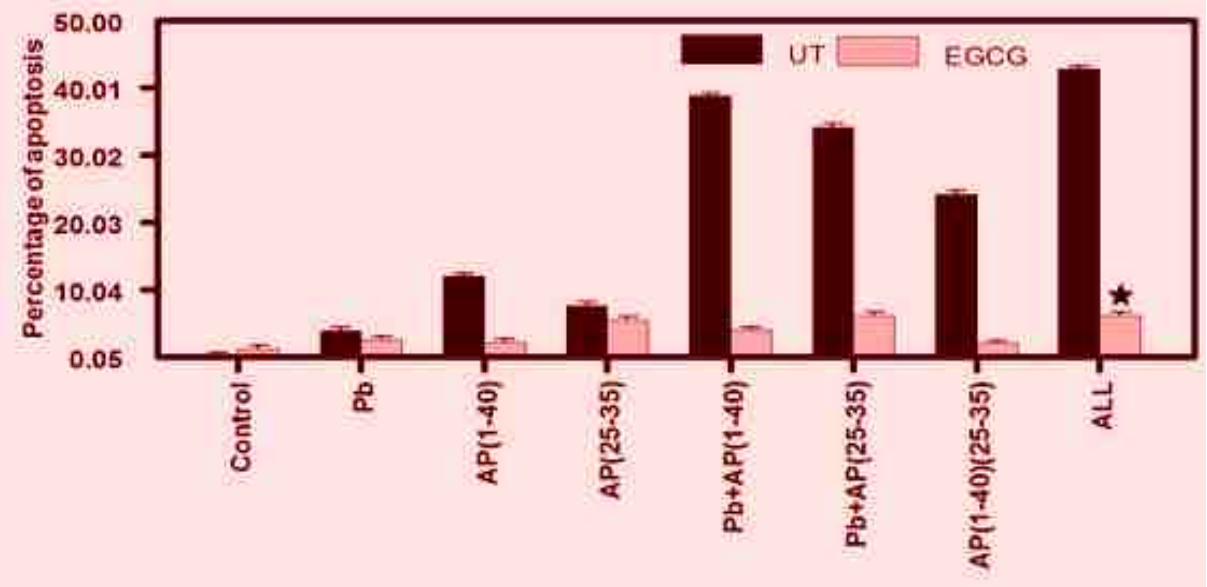
- When observed in apoptosis with the individual exposure of Lead and Amyloid peptides(1-40),(25-35) and in the combination of All, Lead with Amyloid peptide(1-40),(25-35), pretreated cells with EGCG have shown decreased levels of apoptosis(Annexin –v) as 2.6%, 2.2%, 5.5%, 4%, 6.2%,2%,6.2% respectively when compared to the group without EGCG treatment (Fig 2).

Fig 2. a. Flow cytometric quantification of apoptosis in SH-SY5Y cells when exposed to Lead and Amyloid peptides with and without pre-treatment with EGCG, b. The histogram represents the percentage of apoptosis in SH-SY5Y cells. The values are mean ± SD of four different samples from each group.



- Decreased levels of caspase-3 were observed in EGCG treated group when exposed to Lead and Amyloid peptides individually and in combinations when compared with the group without EGCG treatment (Fig 3).

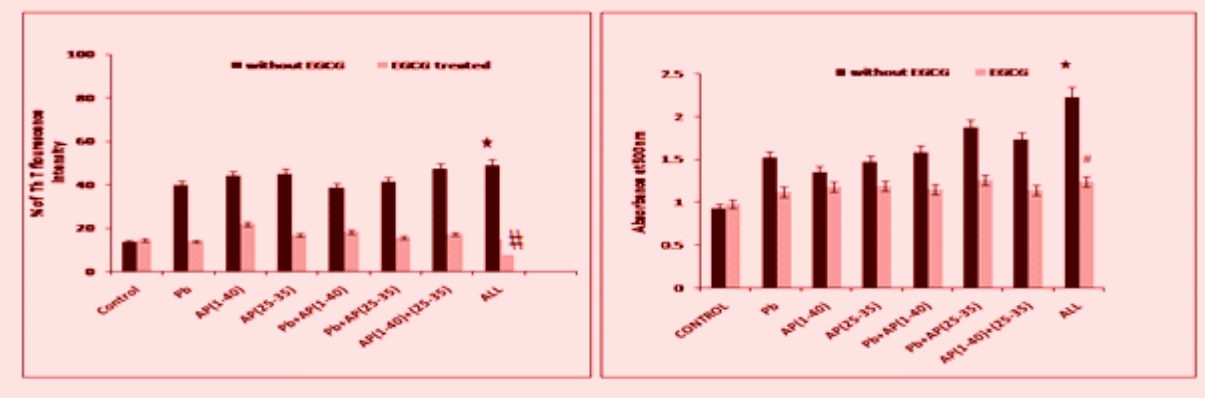
Fig 3. The effect on Caspase-3 levels when treated and untreated with EGCG in human neuroblastoma cells. The data presented is the mean ±SD from four samples from each group



- The amount of fluorescence in EGCG untreated group was Control-13%, Pb-39.9%, AP(1-40)-44.04%, AP(25-35)-44.9%, Pb+AP(1-40)-38.7%, Pb+AP(25-35)-41.2%, AP(1-40)+AP (25-35) -47.3%, ALL(Pb+AP(1-40)+AP(25-35)-49.1% respectively, whereas when treated with EGCG with individually along with the similar combinations of Pb and AP(1-40), AP(25-35), the percentage of ThT fluorescence levels were Control- 14.4% Pb-13.8%, AP(1-40)-21.8%, AP(25-35)-16.8%, Pb+AP(1-40)-18.2%, Pb+AP(25-35)-15.7%, AP(1-40)+AP (25-35) -17.1%, ALL(Pb+AP(1-40)+AP(25-35)-14.7%.Similarly, Congo red assay reported that, the decreased levels of amyloid uptake intensity were observed in EGCG treated group

when compared to the EGCG untreated group. The results were expressed in EGCG untreated group as Control-0.93%, Pb-1.52%, AP(1-40)-1.35%, AP(25-35)-1.47%, Pb+AP(1-40)-1.58%, Pb+AP(25-35)-1.87%, AP(1-40)+AP(25-35)-1.73%, ALL(Pb+AP(1-40)+AP(25-35)-2.23% respectively, whereas when treated with EGCG with individually along with the similar combinations of Pb and AP(1-40), AP(25-35), the percentage of Congo red absorbance levels were Control- 0.98% Pb-1.12%, AP(1-40)-1.18%, AP(25-35)-1.19%, Pb+AP(1-40)-1.15%, Pb+AP(25-35)-1.26%, AP(1-40)+AP(25-35)-1.14%, ALL(Pb+AP(1-40)+AP(25-35)-1.24% (Fig 4).

Fig 4. Thioflavin T and Congo red Assay; Effect of EGCG, Pb, and Amyloid peptides (AP), on Thioflavin T fluorescence levels in SH-SY5Y cell line. The levels of amyloid peptides fibril formation were expressed in terms of percentage compared to control. The data are represented as a mean ± SD from triplicate independent experiments. $P \leq 0.05$ vs. the untreated control group , # $P \leq 0.05$ vs. the EGCG-treated group.



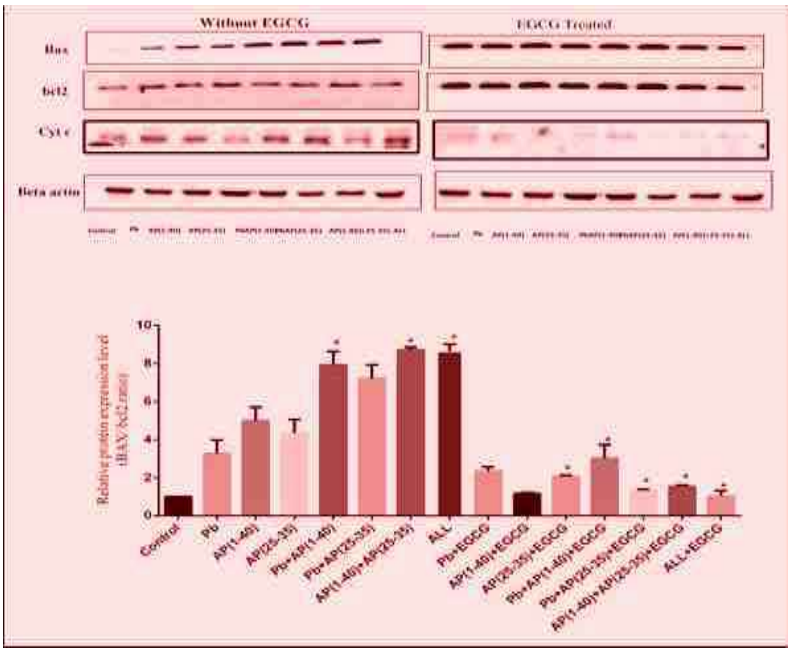
- From the western data analysis we observed that, the combination of Lead and AP, individually and in different combinations has showed significant increased levels of BAX as Control- 0.036%, Pb-0.05%, AP(1-40)-0.94%, AP(25-35)-0.64%, Pb+AP(1-40)-1.78%, Pb+AP(25-35)-1.29%, AP(1-40)+AP(25-35)-1.72%, ALL (Pb+AP(1-40)+AP(25-35)-1.73% where as in the EGCG treated group, the expression levels of bcl2 was significantly decreased as Control- 0.81%, Pb-1.17%, AP(1-40)-1.28%, AP(25-35)-0.82%, Pb+AP(1-40)-1.27%, Pb+AP(25-35)-0.98%, AP(1-40)+AP(25-35)-1.09%, ALL (Pb+AP(1-40)+AP(25-35)-0.71%. Likewise, in group untreated with EGCG, a significant decreased level of bcl2 was observed. The relative bcl-2 expression levels in the EGCG untreated group was Control- 0.32%, Pb-1.32, AP(1-40)-1.13, AP(25-35)-1.14%, Pb+AP(1-40)-0.98%, Pb+AP(25-35)-0.83%, AP(1-40)+AP(25-35)-1.59, ALL (Pb+AP(1-40)+AP(25-35)-0.9%, where as in the group treated with EGCG, the expression levels of bcl2 was Control- 0.8%, Pb-1.11%, AP(1-40)-1.34%, AP(25-35)-0.81%, Pb+AP(1-40)-1.41%, Pb+AP(25-35)-0.79%, AP(1-40)+AP(25-35)-1.01%, ALL (Pb+AP(1-40)+AP(25-35)-0.98% (Figure 5).

Salient findings

- Selected and standardized EGCG as one of the compounds for reducing the toxicity EC_{50} was determined as 50µM in both time and concentration-dependent manner by MTT assay. Increased levels of cell viability in terms of percentage were observed in EGCG treated cells when exposed to Lead and Amyloid peptides individually and in combinations when compared with the cells exposed to Lead and Amyloid peptides without EGCG treatment.
- Decreased levels of Early and late apoptosis and caspase-3 were observed in EGCG treated cells when exposed to Lead and Amyloid peptides individually and in combinations when compared with the cells without EGCG treatment.

- Decreased levels of amyloid peptide concentration in Thioflavin T binding assay and levels of amyloid peptide-binding intensity in Congo red assay was observed in EGCG treated Pb and AP exposed group when compared to the group without EGCG treatment.
- EGCG exposure group has shown upregulation of Bcl2 and downregulation of BAX in Pb and AP exposed group when compared to the group without EGCG treatment.
- Decreased levels of P53 were observed in Pb and AP treated and EGCG exposed group when compared to the group without EGCG treatment.
- Beta secretase levels (BACE1) were significantly increased in Pb and AP exposed group without EGCG treatment .whereas the same group when exposed to EGCG, decreased levels of BACE1 levels were observed. Decreased levels of oxidative stress biomarkers such as ROS and NOS were observed in the group treated with EGCG when compared to the group untreated with EGCG.

Fig 5. Expression of BAX,Bcl2 levels ratio and Cytochrome C levels in SH-SY5Y cells treated with Pb and AP (1-40), AP(25-35) individually and in different combinations for 24hrs, Thirty-microgram aliquots of each cell extract were subjected to SDS-PAGE and western blot analysis. (A) Representative western blotting and densitometry analysis of BAX expression relative to beta-actin. Data represent mean±SD of four different experiments.
* and # are significantly different at P<0.05 versus control and individual exposures of Pb and APs respectively.



- Increased levels of antioxidants such as Glutathione, SOD, catalase, Vitamin c was observed in EGCG exposed group when compared to the untreated EGCG group.
- When observed in the gene expression studies, upregulation of BACE1 levels in the EGCG untreated group, and down regulation of BACE 1 in the EGCG exposed group was observed.ABCA7 levels were relatively increased in the EGCG untreated group when compared to the group with EGCG treatment.

Conclusion

Natural compounds have gained popularity in recent years because of their multiple mechanisms of action with limited side effects. Among them, EGCG is selected as a natural flavonoid abundantly present in green tea based on literature support, having the ability of metal chelate and anti-oxidant. Our study reveals the intracellular mechanism of the protective action of EGCG by showing anti-apoptotic and anti-oxidant properties and by inhibiting BACE and APP enzymatic pathways in Pb-induced Alzheimer's disease pathology. Further *in vivo* reports and clinical studies will prove its efficacy in the prevention and management of Alzheimer's disease either in the form of capsule supplementation or serves as a template for new drug designing to combat Pb-induced alzheimer's disease.

11. Role of micronutrients in cancer stem cell metabolism and therapy

Cancer stem cells (CSCs) can be defined as a population of tumor-initiating cells present in tumors. Upcoming literature suggests that CSCs are responsible for recurrence, resistance, and differentiation into cancer cells. Literature indicates that all the clinically used and investigative new drugs along with strategies are not effective in preventing or reversing the disease. Therefore, a better understanding of the process is a prerequisite to designing successful drugs and better strategies to manage disease progression. One such potential drugs that can be considered for this aspect is metformin, which is a well-established and widely prescribed oral hypoglycemic drug. Apart from metformin, the popular micronutrient Vitamin B6 has been proven to be an anti-cancer agent. The combination of metformin and vitamin B6 might have a synergistic role in combating disease progression. Here, metformin may target to CSCs growth restriction and vitamin B6 may hamper cancer cell proliferation in pancreatic cancer cells (Panc1). This approach may work for a better therapeutic approach, so as the recurrence of tumor and cancer metastasis can be understood and managed for effective survival and cure of cancer patients.

Objectives

- To maintain and isolate the enriched population of cancer stem cells (CSCs) from Panc-1 cell line and their characterization by physicochemical properties.
- To understand the role of metformin in presence of micronutrient vitamin B6, vitamin C, and retinoic acid in a pancreatic cancer cell line (Panc1) with emphasis on cancer stem cell target.
- To study the metformin and micronutrients of vitamin B6, vitamin C, and retinoic acid their metabolism to RNA and protein expressions in CSCs and pancreatic cancer cells.

Various experiments like MTT Assay, cell cycle analysis, and apoptosis were conducted the micronutrient combinations were tested with metformin drug in normal as well as isolated cancer stem cells by using magnetic cell sorter in the lab. The initial yield of 1-3% CSCs wassorted and these numbers were enhanced through passaging of more cells. The important observation was micronutrients of retinoic acid and Vitamin C were induced more apoptosis in cancer stem cells than normal cells. The combination of metformin with all micronutrients was showed better apoptosis activity than other individual micronutrients.

Fig 1. Morphological changes upon treatment of metformin and micronutrient pyridoxal phosphate in pancreatic cancer cells

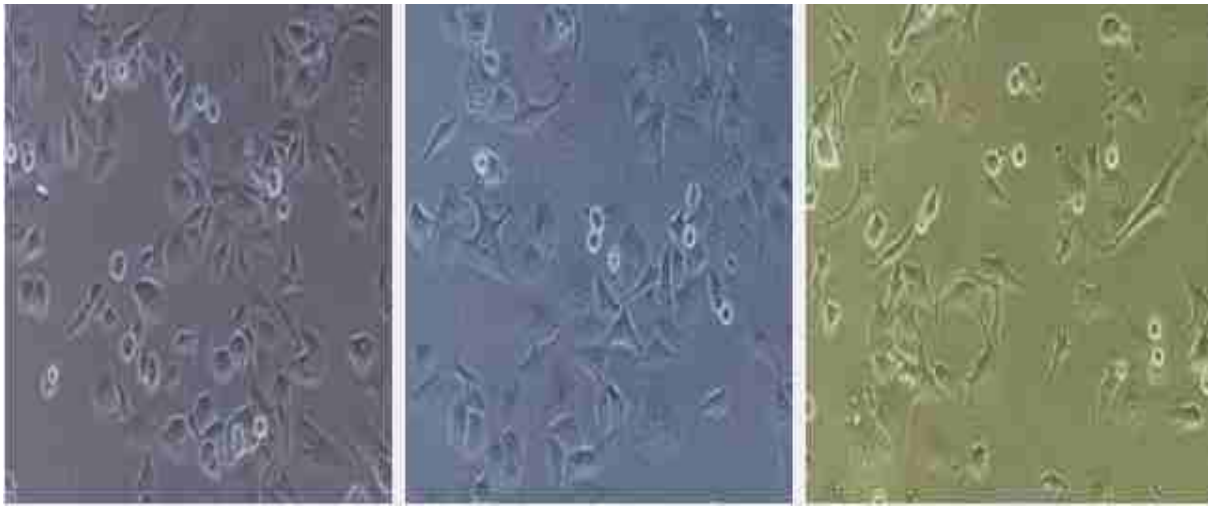
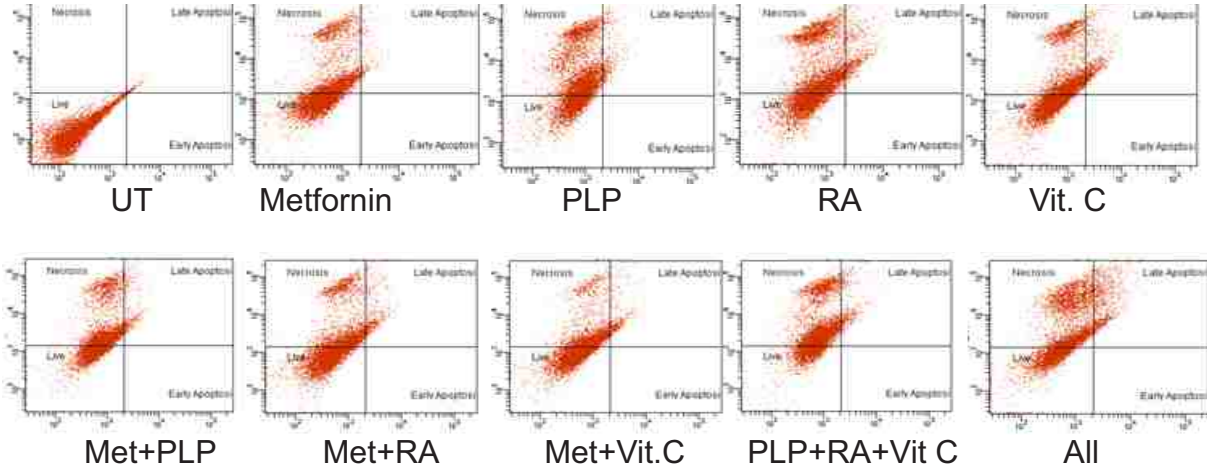
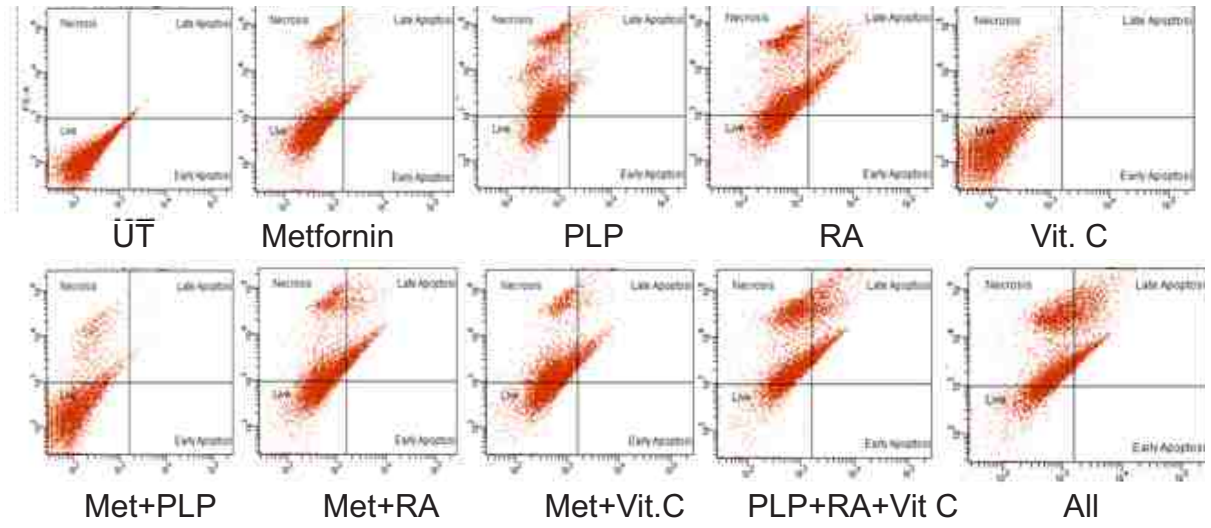


Fig 2. Apoptosis assay and the percentages of cells present in a different phase of cells upon treatment with metformin with micronutrient combinations in pancreatic cancer cells for 24 hours treatments



S.No	Live cells	Early Apoptosis	Late Apoptosis	Necrosis
UT	99.2	0.0	00.7	00.2
Met	65.2	0.0	04.5	30.2
PLP	34.3	0.0	04.8	61.0
RA	28.5	0.0	15.8	55.7
Vit C	86.2	0.0	00.5	13.2
Met+PLP	87.7	0.0	00.7	11.6
Met+RA	40.7	0.0	15.5	43.8
Met+ Vit C	42.3	0.0	09.0	48.7
PLP+RA+Vit C	19.1	0.0	21.0	59.9
All	23.4	0.0	19.4	57.2

Fig 3. Apoptosis assay and the percentages of cells present in a different phase of cells upon treatment with metformin with micronutrient combinations in pancreatic cancer stem cells cancer cells for 24 hours treatments



S.No	Live cells	Early Apoptosis	Late Apoptosis	Necrosis
UT	99.0	0.1	00.8	00.1
Met	58.0	0.0	01.9	40.1
PLP	36.2	0.0	03.8	59.9
RA	39.6	0.0	07.5	52.9
Vit C	54.7	0.0	07.0	38.3
Met+PLP	46.2	0.0	04.1	49.7
Met+RA	57.9	0.0	05.8	36.4
Met+ Vit C	59.3	0.0	04.6	36.1
PLP+RA+Vit C	27.6	0.0	05.4	67.0
All	39.8	0.0	11.0	49.1

Salient findings

- Pancreatic ductal epithelial cancer cell lines (Panc1) were maintained in DMEM medium and treated with metformin and pyridoxal phosphate (PLP) for 24 hours.
- The IC value of metformin is 20.95mM, Pyridoxalphosphate (PLP) is 5.70mM, Vitamin C is 1.65mM and Retinoic acid (RA) is 29.12µM for 24-hour treatment in a pancreatic cancer cell line (Panc1) obtained by MTT assay.
- Upon 5mM PLP treatment cells changed in their morphology and shrunk in shape was observed. Whereas, the combination of metformin and PLP has shown less severity of morphological changes than PLP alone treatment.
- The cell cycle analysis data showed that cells were reduced in the G2-M phase with treatments of 20mM metformin followed by 5mM PLP.
- The apoptosis data revealed that metformin treatment increased necrosis cells to 21.3%, whereas, PLP treatment was showed 16.9% of apoptosis and 28.9% of necrosis. The combination of metformin and PLP decreased necrosis to 9.6% and apoptosis to 13.8% has been indicated that synergistic effect was not found in Panc1 cells.
- Whereas metformin and vitamin C combination also did not synergize apoptosis activity. Vitamin C alone showed 17.4% of late apoptosis and combination decreased it to 14.0% and necrosis also decreased to 16.8%.
- The combination of metformin with retinoic acid further decreased late apoptosis to 8.2% and necrosis to 12.4% was compared with the individual of retinoic acid late apoptosis is 14.6% and necrosis is 22.8% indicates that there is no synergistic effect with the combination.
- The cancer stem cells were isolated from pancreatic cancer (Panc1) cell lines using CD133 positive magnetic beads and expanded further passages for more treatment experiments.
- The apoptosis assay revealed that metformin drug has not induced more apoptosis in cancer stem cells than cancer cells. Whereas, micronutrients PLP and RA were induced more apoptosis in CSCs than normal cancer stem cells.
- But micronutrients vitamin C have induced more apoptosis in normal as well as cancer CSCs in pancreatic cancer cells compared with PLP and RA.

- But, the combination of metformin with vitamin C has been induced more apoptosis in CSCs than other PLP and RA combinations.
- All combinations of metformin with micronutrients induced more apoptosis in CSCs than in normal cells. However, more necrosis was observed in all the experiments.
- Further, some of the experiments will be repeated for reproducibility of the results.

Conclusion

The combinations of metformin along with micronutrients (vitamin B6, RA and vitamin C) have been showing significant apoptosis activity in cancer as well as cancer stem cell populations. Metformin drug has been shown better apoptosis activity in cancer stem cell than cancer cells. Combinations of all micronutrients showed more necrosis in cancer stem cells than cancer cells.

12. Evaluation of physiological efficiency and formulation of energy allowances for different groups of junior athletes from Sports Authority of Telangana State (SATS)

Adolescent athletes are at a stage requiring energy for growth, added to the training. Suitable fuel and nutrition are important at this stage. As suggested that it is best for adolescent athletes to be in a positive energy balance. Very limited research on energy balance and nutritional status of young players has been carried out world-wide with adult recommendations still utilized for adolescent athletes. Researchers have voiced the need for such studies on junior athletes. Even in an Indian setting, there is scanty research carried to understand the energy expenditure or nutrient intake pattern of junior athletes.

Any deviation from energy requirements would reflect on the physical and physiological efficiency. This in turn can affect the health-related fitness of the young athlete. Physical and Physiological characteristics differ among conditioned athletes and non-athletes. The standards developed for non-athlete Indian adolescents will not be suitable for athletes. Therefore, this study also focuses on identifying the physical characteristics of Indian junior athletes and their comparison with international athletes.

In India, studies have focused on understanding the energy and nutrient intake of adult and young athletes. However, the energy expenditure pattern, in terms of determining the resting metabolic rate and energy cost of activities has not been explored. Thus, this study further attempts to understand the energy expenditure, energy intake, and nutrient adequacy of junior athletes. Further, explores its association with physical characteristics across the various phases of training.

Objectives

General Objective: To evaluate physical, physiological profile and to identify energy requirements of different groups of junior athletes during their transition phase (TP), Pre-competition phase, and competition phase (CP) of training.

Specific Objectives:

- To assess the physical characteristics including anthropometry and body composition (lean body mass and fat mass).

- To measure Basal Metabolic Rate (BMR), the energy cost of various day-to-day activities (Training and non- training activities).
- To evaluate cardiovascular and cardio-respiratory efficiency and components of physical fitness (power, endurance, strength, speed, agility).
- To assess the 24-Hours energy balance of junior athletes and its influence on body composition and performance.

Methodology

The subject was briefed about the purpose and scope of the study and written consent was obtained from each of the individuals and their parents. Consent was also obtained from the Director, Sports School for the participation of the students. A test trial of the study protocol was given to each athlete well in advance to acquaint the athlete with the procedure and to avoid stress and anxiety during the actual test, which would interfere with the measurement.

Study protocol

Study design	Prospective and observational study
Study location	Telangana State Sports School (TSSS), Hyderabad
Subjects	Junior athletes (Aged 10-17 years) undergoing training for various events of sports will be recruited for the study.

Four major areas of the study

Areas focused	Methods used
Physical characteristics	Anthropometry and body composition (Skin fold technique and Bio-electrical Impedance Analysis, BIA) and Handgrip strength
Physiological parameters	Basal Metabolic Rate (BMR) and CPET-graded exercise testing
Estimation of Total Daily Energy Expenditure (TDEE)	Time Allocation Pattern (TAP) and Energy cost of day-to-day physical activities
Assessment of 24-hour dietary intake	Direct weighment method

Major Findings

Physical characteristics across training phases i.e. Post competitive (PC-rest: January-February), Preparatory phase (PP: July-August) and Competitive phase (CP: November-December) showed differences only among boys, but not among girls. Across the Initial (PC-rest) vs. Final time point (CP), boys in Athletics (track event) exhibited a decrease in circumferences like neck, waist, thigh, and MUAC, while, Football players showed an increase in circumferences and fat (kg). Further, weightlifters showed an increase in chest circumference and fat-free mass (kg). Among girls, there was no significant difference in physical characteristics on comparing the PP phase to PC-rest and CP. This could be attributed to the differences in training protocol across the events.

Across the phases of training, the height significantly increased in the athletics group compared to other events with no significant difference in body mass at CP. For Weight lifters, most circumferences were higher, compared to athletes and football players, particularly in the PP phase, and waist, hip, and MUAC were higher in the CP phase as well. Further, weight lifters also showed a mean decline in fat mass from CP-rest to PP to CP and a consequent increase in FFM. This may be attributed to the graded resistance training protocol adopted by weight lifters.

The energy cost of activities measured ranged from 0.7 MET for Resting Metabolic Rate (RMR) to 10.8 MET for shuttle run. Further, the measured METs of the majority of the non-training or training activities of junior athletes, irrespective of the sex or event, showed more similarity to the compendium of physical activities (CPA) developed among adults than the Compendium of energy expenditure for youth (CEEY).

RMR prediction models may be developed from the existing data and out of the prevalent prediction models for RMR, the equation Henry (2005) and athlete-specific models were closer to the measured RMR. The measured METs of RMR of Indian junior athletes were significantly higher than the existing compendiums. Therefore, there is a need to develop a compendium specific to junior athletes. However, in the absence of such information, the existing compendiums can be used by applying the RMR correction factor.

The total energy expenditure of athletes (Boys: 59 to 84; Girls: 53 to 68 kcal/kg body mass) in track events were highest, followed by weight lifters (Boys: 51 to 60; Girls: 45 to 58 kcal/kg body mass) in CP and finally football players (Boys: 53 to 68; Girls: 39 to 62 kcal/kg body mass) in PP. Energy deficits and low energy availability was observed among U16 as opposed to U12 players at a one-time point. Indicating that the energy density of diet needs to be improved with increasing age, and not adopting the "one portion-size fits all" approach. Further, the larger number of girls exhibited low energy availability compared to boys. Despite excess consumption of carbohydrates, its intake pre-, during- and post-training were not meeting the requirements of JSPs. An excess carbohydrate intake was observed post-training, mainly through consumption of the main meal. A similar observation was made earlier in 2014. Thus, imparting appropriate nutrition education to junior athletes on the timing of carbohydrate intake is necessary. Further, the percent contribution of carbohydrates to energy intake may be reduced, to incorporate more fat and protein in the diet for optimal distribution of calories.

Coaches observed that the athletes were avoiding fruits and vegetables while consuming more rice. Consumption of high-protein diet, meal timing and fluid and supplement intake was considered essential for international performance, albeit coaches were unaware of the specific requirements. Poor hygiene practices and peer isolation were prevalent and coaches believed that affected food intake.

Based on the observations of coaches and the existing literature, a context-specific knowledge, attitude, and practice questionnaire (KAP) on sports nutrition was developed with an acceptable level of internal consistency and test-retest reliability for 46 Items. The administered questionnaire, included 49 items since 3 questions with lower consistency were important in the context of this study and were retained for scoring. Upon administration, the KAP scores independently or combined did not show any significant difference across event and sex. Thus, indicating its generalisability and suitability for any sport across sex. However, on administering this questionnaire to the study participants, the mean score was 50% lower than the maximum score. The group with the highest scores showed better handgrip strength of the non-dominant hand and higher body mass, however, no other physical characteristics showed differences. The opinions and perceptions of coaches concerning the food habits, eating attitudes, and practices of junior athletes matched with that of the responses of the junior athletes to KAP-Q, suggesting that persons closely monitoring athletes can indeed determine their problems faster.

Conclusions

- The variation in physical characteristics across events seems to indicate that the football players have gained more fat mass at competition season, compared to other events and this needs appropriate intervention time-to-time. This could also be attributed to the higher consumption of fats and sugars among these players, compared to other events.
- The energy cost of activities represented in terms of METs covered a wide range of intensities and may be utilized for arriving at the energy expenditure pattern of junior athletes, after suitable correction for RMR. Even the RMR prediction equations developed in the study can be utilized for regular monitoring of RMR and the same can be utilized in formulating suitable dietary recommendations and menu planning for achieving optimal growth and development among junior athletes.
- It is also evident from the study that energy deficits and low energy availability existed in U16 football players as opposed to U12 at a one-time point while maintaining an overall energy balance. Thus, indicating that the energy density of diet needs to be improved with increasing age, and not adopting the "one portion-size fits all" approach. Despite excess consumption of carbohydrates, its intake pre-, during- and post-training were not meeting the requirements among junior athletes.
- The KAP-Q developed and validated may be used to assess the general nutrition knowledge, attitude, and practices of adolescent athletes, irrespective of the event. Considering the low KAP scores among junior athletes and the findings from In-depth interviews, the modifiable nutrition practices need to be targeted for inculcating healthy eating habits suitable to their sport.

IV. DIETETIC STUDIES

1. Studies on xanthophylls: Dietary sources, processing, bioavailability and biological effects

The impact of food composition and processing on carotenoid bioavailability has been the subject of several investigations. Absorption of xanthophylls occurs primarily in the small intestine, in several steps including release from the food matrix, incorporation into bile-salt mixed micelles, uptake by epithelial cells, and chylomicron packaging and secretion into the lymphatic system. The impact of meal patterns on carotenoid bioavailability was primarily assessed for the carotenoids in the test food including the xanthophylls, lutein, and zeaxanthin. Xanthophylls are ubiquitously distributed in plants and constitute a major fraction of the dietary carotenoids. Lutein is one of the major xanthophyll in green leafy vegetables such as spinach (*Spinacia oleracea*) while zeaxanthin is the predominant xanthophyll in maize (*Zea mays*). Previous studies showed that poor dietary intake or low plasma lutein and zeaxanthin concentrations are associated with low macular pigment density and increased risk potential for age-related macular degeneration (AMD), an irreversible ocular condition that is the major cause of blindness in the elderly.

The xanthophyll uptake by intestinal epithelial cells is a critical factor for xanthophyll bioavailability. Only one part of the accessible xanthophyll is taken up by the intestinal epithelial cells and secreted into lymph as chylomicrons for circulating in the bloodstream. After the chylomicrons are degraded by lipoprotein lipase, carotenoids in chylomicron remnants are taken up by the liver. The carotenoids are stored in the liver or resecreted as very-low-density lipoprotein into the bloodstream, and then delivered as low-density lipoprotein (LDL). Finally, carotenoids are taken up to tissues through the LDL receptor. Highly hydrophobic carotenoids such as β-carotene and lycopene are localized in the inner part of LDL, while less hydrophobic xanthophylls such as lutein and zeaxanthin are equally distributed to LDL and HDL, and localized in the outer surface area of the lipoprotein particles.

The present study was undertaken to evaluate the bioavailability of lutein and zeaxanthin through the ingestion of cooked green leafy vegetables and maize in humans and to measure the levels of these xanthophylls and their metabolites in human plasma before and after 1-week dietary interventions with cooked spinach and cooked maize respectively. The intestinal absorption was studied by the response of xanthophylls and esters in the triglyceride-rich lipoprotein fraction after a single oral dose of lutein and zeaxanthin.

Objectives

- Screening of common plant foods for xanthophylls.
- Study the natural variability in xanthophylls content and identify, if possible high xanthophylls containing varieties of selected vegetables.
- Stability of xanthophylls during food storage and processing and identify the nature of factors related to it.
- Studies on the bioaccessibility of xanthophylls using *in vitro* digestion methods.
- Studies on the bioavailability of xanthophylls in suitable animal/human models.

Studies on intestinal absorption of xanthophylls in human subjects: The response of xanthophylls and esters in the triglyceride-rich lipoprotein fraction after a single oral dose of lutein /zeaxanthin in human subjects were studied in the year 2018-19.

The human study was conducted as follows:

Subjects: About 18 subjects including both men and women in the age group of 22-35 years with normal Body Mass Index and healthy individuals (assessed by medical history and regular checkup) were recruited for the study. Participants were requested to maintain a normal diet schedule but to avoid xanthophyll-rich foods such as green leafy vegetables, vegetables and fruits, xanthophyll-containing dietary supplements, and vitamin-fortified beverages during the depletion period and the intervention study.

Study Design:18 healthy adult volunteers in the age group of 22-35 years (both male and female) were recruited for the study and were advised to avoid xanthophylls rich foods (4 days before experiment).

Experimental day

- Subjects were categorized into three groups, each group consisting of 6 volunteers.
- After fasting overnight, blood was collected from all the individuals.
- Group I was considered as control where the diet was given without lutein/zeaxanthin.
- Group II was given a diet that included lutein from green leafy vegetable (spinach) which was stir-fried with oil (Experimental) and given along with white bread.
- Group III was given a diet which included zeaxanthin from maize which was provided in the form of upma (Experimental).
- After the consumption of a meal, blood (2ml) was drawn by vein puncture every two hours till 8 hours and TRL fraction were separated by ultracentrifugation and analyzed for the xanthophylls contents using High-performance liquid chromatography (HPLC).

(Depending on the group, volunteers were given the test diet as breakfast and later provided with tea and lunch which consisted of a diet low in carotenoids while water was provided ad libitum during this eight hours study period). AUC (area under the concentration-versus-time curve) was plotted for lutein and zeaxanthin content (Trapezoid rule). Compliance was monitored by daily interviews.

Test products

1. Stir-fried GLV (Spinach) in oil, 2. Cooked maize in the form of upma.

Laboratory measurements

Anthropometric measurement of weight and height for BMI, blood pressure and fat%, determination of lipid profile, HPLC determination of xanthophylls and esters in the chylomicrons after ingestion of cooked spinach and maize.

Results

The study was conducted on the healthy human for the bioavailability of lutein and zeaxanthin revealed that after administration of lutein in the form of cooked spinach and zeaxanthin in the form of cooked maize upma there was slightly lesser bioavailability of these components as compared to other studies indicating that these foods when consumed regularly can be used in the prevention of age-related eye diseases in high-risk populations. When compared by individual treatment groups, no significant differences were noted in individual xanthophyll response based on a treatment order. Analysis of TRL $AUC_{0\text{-}8h}$ values revealed that,

although treatment groups II and III, promoted greater absorption of xanthophylls, lutein, and zeaxanthin when compared to the control group yet the bioavailability was quite lesser as compared to other studies due to the cooking effect which led to losses of some amount of xanthophylls. The study further showed that after consumption of the test meal containing lutein or zeaxanthin, the increase in absorption was observed to be at 2-4 hr interval and dropped down after 6 hr and was not detectable at 8hr indicating maximum absorption of these xanthophylls at 2-4hr showing that the relatively polar xanthophylls predominate in the phospholipid-rich emulsion surface of lipid droplets in gastric chyme and is readily transferred to mixed micelles in the duodenum during digestion, and incorporated into the chylomicrons for transportation and circulation to different parts. The xanthophylls lutein and zeaxanthin have shown to be much accumulated in the lipophilic tissues in humans and then carried in the blood similar to cholesterol. In the human blood lutein and zeaxanthin are known to be distributed in LDL and HDL fractions with upto 75% more concentration in the LDL.

Conclusion

Lutein and zeaxanthin are plant carotenoids predominantly present in commonly consumed foods. These molecules have shown the benefits as antioxidants and also prevent age-related macular degeneration (AMD). Therefore, the absorption of these molecules is exponential. We do not have information for Indian foods especially when in cooked form hence we generated the data on the bioavailability of these molecules by studying the response of xanthophylls and esters in the triglyceride-rich lipoprotein fraction after a single oral dose of lutein/ zeaxanthin in human subjects and it will benefit for RDA in future for these molecules. Also, the individual geriatric population can choose foods with these molecules from better-absorbed foods.

2. Prebiotic effect of legume raffinose family oligosaccharides

Obesity is now characterized by a cluster of important chronic metabolic disorders, including insulin resistance, type 2 diabetes, fatty liver disease, atherosclerosis, hypertension, and hypercholesterolemia, and by a low grade of systemic inflammation, is the cause of exacerbation of all the above and leading to increased morbidity and mortality. The International Diabetes Federation (IDF) estimates the total number of diabetic subjects to be around 40.9 million in India and this is further set to rise to 69.9 million by the year 2025.

Several mechanisms have been implicated in the link between intestinal microbiota, increased fatty acid metabolism, and storage of calories as fat. Among the tools to modulate gut microbiota, probiotics and/or prebiotics appear to be the most important, although actual proof is still limited. The effect of prebiotics on metabolic parameters has been examined in animal and human studies.

High fat diet-induced obesity has been considered as the most popular model among researchers due to its high similarity of mimicking the usual route of obesity episodes in humansand hasit is considered as a reliable tool for studying obesity. In a recent study anti-Obese effect of prebiotics such as glucosamine and chitosan oligosaccharide (COS) in high fat diet-induced obese rats showed improved dyslipidemia and prevented body weight gains by inhibiting the adipocyte differentiation in obese rats induced by a high-fat diet.

Legumeshave been investigated as functional foods particularly concerning their potential as prebiotics based on their constituent galacto-oligosaccharides. The -Galactosides include

raffinose, stachyose, verbascose, and higher homologous series. Raffinose family of sugars contains one, two, or three galactose units linked to glucose moiety of sucrose by - (1 6) linkage. These sugars cannot be hydrolyzed and absorbed in the intestine, due to the lack of -galactosidase activity in the small intestine but undergo anaerobic fermentation by bacteria in the large intestine. Oligosaccharide fermentation can also lead to inhibition of diarrhea, a protective effect against infection, a reduction of cancer risk, mainly the gut cancer and proliferation of bifidobacteria.

In India, legumes are widely consumed in the form of *dhal* as an economical source of protein. The role of legume prebiotics in health may include benefits on risk factors for cardiovascular disease, weight management, immune function, and colonic health. Information on the prebiotic effects of legumes with oligosaccharides is scanty.

Earlier animal study demonstrated that the prebiotic potential of legume oligosaccharides in the animal model. The caecum sample analysis of animals fed with legume prebiotic supplement showed an increase in the short-chain fatty acids and colonies of beneficial bacterial counts such as lactobacillus, bifidobacteria, enterobacillus, and bacteroides by decreasing the population of pathogenic or putrificative bacterial counts. The results of the prebiotic potential of legume oligosaccharides were also promising, since there was a decrease in blood glucose level, improved lipid profile, increased mineral absorption, improved body mass composition, and inflammatory markers in all the legumes prebiotic fed animals.

Further research is needed to understand the role of legume prebiotics to prevent or control diabetes, obesity, cardiovascular diseases, irritable bowel syndrome, and other health benefits.

Hypothesis

Consumption of non-digestible carbohydrates of legumes will have a positive impact on management and control of risk factors of obesity.

Objectives

- To assess the prebiotic potential of legume prebiotics in high fat-induced obesity
- To study the role of legume prebiotics in the regulation of obesity

Methodology

Animals and Diet

The mice model was used for this study. Male C57BL/6 mice were obtained from Animal Facility, National Institute of Nutrition (NIN), Hyderabad, India. The experimental protocol of the study was approved (P20F/II-IAEC/NIN/2016/6/S.DEV/C57BL6j-84M) by the Institutional Animal Ethical Committee (IAEC).

Eighty-four (12 week-old) C57 black mice were fed with a standard chow diet for 2 weeks to be acclimatized. After the acclimatization, all the mice were fed with a high-fat diet except the control group for 6 weeks to induce obesity. After inducing obesity 72 mice were randomly assigned to six dietary treatment groups, 12 mice in each group. The control group (G 1) was fed with a standard chow diet. Group 2 (G 2) were fed with high-fat diet, group 3 (G 3) was supplemented with 3% of standard raffinose, group 4 (G 4) red gram prebiotics, group 5 (G 5) green gram prebiotics, group 6 (G 6) bengal gram prebiotics respectively for 18 weeks.

After 18 weeks on these experimental diets, 2 ml of blood was drawn from retro-orbital plexus after 12 hr fasting and the mice were sacrificed by CO_2 inhalation. After sacrificing the mice, the caecal content and other organs were collected and stored at -80°C for further studies.

Parameters studied

- The body composition of experimental animals was determined by DEXA.
- Oral glucose tolerance (OGTT) was also measured.
- The glucose concentration of blood was determined by glucose oxidase method using the multimode detector, cholesterol by oxidase peroxidase method, HDL-C by precipitation method, triglycerides by glycerol phosphate method using the multimode detector, LDL-C, and VLDL-C by calculation**.**
- The caecum content was analyzed for gut bacteria (lactobacillus using MRS agar plate media, bifidobacteria, enterobacillus, and bacteroides by using bifido agar plate media and pathogenic bacteria by MACONKEY agar plate media) using the conventional method.

Results

Effect of commonly consumed legume prebiotic oligosaccharide on high fat-induced obese mice model was studied. The gain in body weight was observed to be more in the high fat-fed group whereas the green gram fed animal group exhibited lower body weights when compared to other legume fed groups (Table 1).

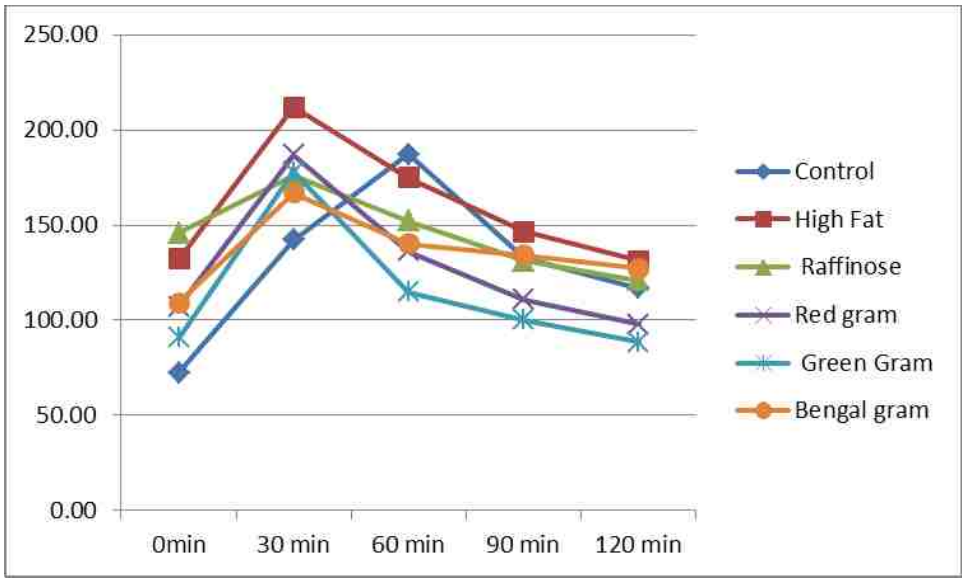
Table 1. Effect of legume prebiotic oligosaccharide on the oral glucose tolerance test (OGTT) on high fat-induced obese animals

Group	0min	30 min	60 min	90 min	120 min
Control	72.33 ± 22.23	242 ± 49	187.3 ± 61.2	132.7± 23.2	116.7± 15.6
HF	132 ± 3.46	212.3 ± 13.57	175 ± 30.80	146.6± 11.71	131± 2
HF raffinose	145.7± 10.50	176 ± 20.7	152.3 ± 9.0	131.3 ± 11.6	120.7± 17.7
HF Red gram	107 ± 10.58	207 ± 66.77	136 ± 20.2	110.7± 7.1	97.7± 15
HF Green gram	90.66 ± 29.36	177.7 ± 39.37	114.7 ± 14.2	100± 1.8	88.3± 4.2
HF Bengal gram	109.3 ± 7.0	167 ± 30.6	140.7 ± 20	134.3± 15.6	127± 15

After 18 weeks of study, an oral glucose tolerance test (OGTT) was carried to see the effect of legume prebiotics on the amelioration of insulin resistance. The results of the oral glucose tolerance test revealed that the high fat-fed group exhibited higher glucose levels for all the time-points i.e 0,30,60,90,120 minutes respectively. The green gram fed group showed lower glucose levels when compared to other legume fed groups (Figure 1).

The results of serum Cholesterol content in all legume oligosaccharide fed groups were decreased when compared to the control group. Also, the triglycerides, LDL, and VLDL cholesterol were decreased and HDL cholesterol levels were improved in legume oligosaccharide-fed animals when compared to the control group. The effect of legume prebiotics on the body mass composition was determined by using DEXA. The results of body mass composition are promising for legume prebiotic-fed animals than that of control and raffinose-fed animals.

Fig 1. Effect of legume prebiotic oligosaccharide on the oral glucose tolerance test (OGTT) on high fat induced obese animals



The results of legume oligosaccharides on the growth of bifidobacteria and lactobacillus and pathogenic bacteria in cecum content. The animals fed with legume oligosaccharides cecum content was showing a higher growth of lactobacillus and bifidobacteria at 10^5, 10^6 dilutions (CFU/ gram of ceum sample) when compared to the control and reference standard (raffinose) fed animals. The pathogenic bacteria counts at 10^4, 10^5 dilutions (CFU/ gram of ceum sample) decreased significantly when compared to control and raffinose fed animals.

Table 2. Effect of legume prebiotic oligosaccharide on the body composition of high fat induced obese animals

Groups	Fat (g)	Lean + BMC(g)	Total mass (g)	% Fat	BMC (g)	BMD (g/cm2)
Group 1 control	1.8 ± 0.62	23.8 ± 1.14	25.6 ± 1.59	6.9 ± 2.10	0.58 ± 0.06	0.07 ± 0
Group 2 high fat	10.26 ± 0.86	25.13 ± 1.46	35.4 ± 2.07	29 ± 1.47	0.80 ± 0.06	0.079 ± 0
Group 3 raffinose	8.6 ± 7.04	22.63 ± 2.07	31.23 ± 7.81	25.23 ± 14.54	0.72 ± 0.16	0.077± 0
Group 4 red gram	1.26 ± 0.21	21.2 ± 1.83	22.43 ± 1.83	5.6± 1.1.4	0.43± 0.03	0.07± 0
Group 5 green gram	2.26 ± 0.38	17.86 ± 0.21	20.16 ± 0.32	11.33± 1.80	0.45± 0.06	0.07± 0
Group 6 Bengal Gram	1.86 ± 0.06	20.4 ± 0.96	22.3 ± 0.87	8.5± 0.66	0.50± 0.09	0.28±0.37

Conclusion

The oligosaccharide fermentation in the caeco-colon by the bacteria can give many positive health benefits as prebiotics. The prebiotic potential of legume oligosaccharides on the control of obesity high fat-induced obesity was carried out in an animal model. The results of the prebiotic potential of legume oligosaccharides were also promising since there was a decrease in blood glucose level, improved lipid profile, and improved body mass composition.

The caecum sample analysis showed an increase in the short-chain fatty acid. The caecum content analysis for gut bacteria by conventional method showed an increase in the colonies of beneficial bacterial counts such as lactobacillus, bifidobacteria, enterobacillus, and bacteroides by decreasing the population of pathogenic putrificative bacterial counts. Further research is in need to understand the many more health benefits.

3. Gluten intolerance in India: Prevalence, food gluten level and intake rates and whether fermented products are a remedy for celiac disease?

Cereals are the predominant form of dietary energy supply in India. Due to changes in lifestyle and dietary patterns many people are switching to wheat and wheat-based food products. Gluten is an important constituent of wheat flour and is physiologically relevant, due to this prevalence of Gluten intolerance or celiac disease is extensively increasing in both developing and developed regions. Celiac disease (CD) is a permanent inflammatory disease of the small intestine developed by ingestion of gluten-containing cereals in genetically predisposed individuals. So CD is a result of both environmental (Gluten) and genetic factors (HLA and non HLA genes) and the distribution of these two factors can probably be used as a marker to identify the areas of the world at the risk of gluten intolerance. Earlier CD was considered as a disorder of European countries; however recent advanced screening technologies resulted in many studies reporting the presence of CD worldwide including many south Asian countries, particularly in India. The CD prevalence ratio was found to be 0.5-1% and this ratio might increase in the next few years. In most situations, the CD is associated with malabsorption, osteoporosis, obesity, type-1-diabetes, and cancer so it might be the major concern for policymakers to prevent CD development. The contribution of wheat to the dietary energy supply increased from 14.9 % in 1969 to 19.8 % in 1989 to 20.8 % in 2009. The consumption of wheat atta was 3.625 and 3.706 kg/30 days in rural and urban regions respectively. The consumption of suji rawa was 0.052 and 0.098 kg/30 days, of bread was 0.023 and 0.091 kg/30 days, of wheat products, was 0.004 and 0.003 kg/30 days and of Barley and products was 0.003 and 0.001 kg/30 days in rural and urban regions respectively (NSS Report No.541: Household Consumption of Various Goods and Services in India). The intake levels of gluten and gliadin can be derived/estimated from the consumption frequency of wheat and wheat products. In India cross-contamination of non-gluten products with gluten during milling and processing might be the major risk factor involved in the prevalence of CD even though the risk individuals try to omitting gluten from their dietary pattern. Gluten-free diet is the primary remedy for CD; however, it has its limitations like cost effect, cross-contamination of oat or rice with gluten, an alternate source for starch in these gluten-free diets. Interestingly some time the gluten levels will be more than the maximum permissible levels of gluten in GFD as per CODEX standards (200ppm). However recent studies were depicting CD symptoms in risk individuals who consume gluten of 50mg/kg of total food. So the major concern of gluten intolerance or CD are,

- Cross-contamination of gluten in non-gluten products.
- Presence of gluten levels more than the permitted levels in gluten-free products.
- The debate in standardizing threshold levels of gluten consumption /day in risk individuals.
- Lack of data regarding the impact of gluten in the general population or risk individuals, who consume a low level of gluten for a longer duration.

Objectives

- Compilation of literature regarding the prevalence of gluten intolerance and % of wheat and wheat products consumption in different parts of India.
- Determination of levels of gluten in non-gluten foods such as oat and rice as well as gluten-free products to measure gluten cross-contamination.
- Estimation of gluten contamination in flour samples collected directly from mills in different rural areas to analyze cross-contamination in non-gluten products. Further co-relation will be drawn between contamination and the ratio of gluten intolerance or celiac disease in that area using regression analysis.
- Assess of minimum threshold levels of gluten required to induce gluten intolerance in genetically susceptible individuals by employing rodent model (BALB/c).
- Optimization of processing methodologies such as fermentation for the reduction in gluten levels in various foods.

Conclusion

The flours represent a higher rate of contamination. Both milling and local market flours samples were highly contaminated with gluten, it might be due to cross-contamination from gluten products like wheat, and might be due to usage of the same milling machine and collecting bags for both gluten and naturally gluten products. Since local markets are depending on local millings, the % and quantity of ppm contaminated in local markets are higher than branded products. Therefore, even though celiac patients are avoiding consumption of gluten products, the cross-contamination might stimulate inflammatory disorders in such individuals. Therefore, either consumption of homemade flour or products which claimgluten-free are safer than local market products.

V. PATHOLOGY AND MICROBIOLOGY

1. Hospital based survey on the prevalence of food allergy (FA) in Hyderabad, India

Food allergy (FA) has become an intercontinental clinical problem. A detailed diversified epidemiological investigation on the prevalence of FA in various regions of India is needed to gain an insight into the extent of the problem in the country. This would require coordination among clinicians, allergists, researchers, diagnostic labs and other working groups regarding diagnostic tools and therapy to be employed to reduce the burden on the community as well as on national resources.

Objectives

- To assess the prevalence of reported food allergy at hospitals, in and around Hyderabad.
- To list the food items causing allergy, as reported by subjects.
- To confirm the reported FA, using skin prick test (SPT), serum IgE, serum histamine, and food-specific IgE estimates.

Methodology

After 'Institutional Ethics Committee' (IEC) clearance from ICMR-National Institute of Nutrition, Osmania Hospital, Govt. ENT Hospital, Govt. Children's Hospital, Gandhi Hospital (GH) and Bhagwan Mahaveer Medical and Research Centre (BMMRC) were obtained, questionnaire modification was undertaken based on the review of literature and suggestions by co-investigators from different hospitals.

Subject recruitment was done (n=1820) and a Questionnaire was administered. Based on the response to the questionnaire, subjects with possible FA were further evaluated by SPT, blood samples were collected and estimations were done for serum IgE, serum histamine, and food-specific IgE.

Based on SPT and serum IgE levels, food-specific IgE estimation was done in a subgroup of 210 subjects for further evaluation.

Results

- Most of the patients were non-vegetarians.
- Based on SPT, 72.1% were atopic: patients were more sensitive to strawberry, orange, papaya, guava, muskmelon, drumstick, brinjal, bean, chickpea, coconut, cashewnut, peanut, egg, prawn, mushroom, cumin and sesame.
- Based on serum IgE levels, 50% of patients were atopic.
- Based onfood-specific IgE levels, 17.1% were atopic: banana, strawberry, brinjal, chickpea, soybean, mutton, egg, prawn, chicken, cumin, sesame were the common food items as allergens in the subjects studied.
- Based on serum histamine levels estimated, it was seen that 23% of the patients were atopic.

2. *Staphylococci* contamination and the risk associated with production of toxin in milk products

Food-borne diseases pose a threat to human health and the economy of individuals, families, and nations. In the Western hemisphere and Europe, *Salmonella* serotype Enteritidis (SE) has become the predominant strain. A review on foodborne diseases in India indicated that the majority of the foodborne disease was caused due to vegetarian foods. Among the foods implicated in India, milk and milk products were predominantly involved in the foodborne disease outbreak.

Staphylococcus aureus food poisoning is an intoxication caused by the ingestion of food containing SEs and is one of the most common foodborne diseases in the world. The primary habitat of *S.aureus* is the nasal passage of humans and the skin and hair of warm-blooded animals. *Staphylococcus aureus* produces a variety of extracellular products including the staphylococcal enterotoxins which have been implicated in humans and animal diseases. Heat stability of *S.aureus* is one of the important properties of SEs concerning food safety. Contamination of foods by S. aureus may occur directly from infected food-producing animals or may result from poor hygiene during the production process or retail and storage of foods or from humans who will carry this microorganism.

The ability to clot blood by producing coagulase distinguishes the virulent pathogen S. aureus from the less virulent coagulase-negative Staphylococcal species. Coagulase positive S. aureus is among the most ubiquitous and dangerous human pathogens for its virulence. Based on this, the majority of the countries laid down the microbiological standards for food products as coagulase-positive S. aureus. As a part of microbiological standards of foods, S. aureus will be analyzed only up to coagulase test. In contrast to this, a pilot study conducted in our laboratory indicated that very few S. aureus isolates (6.3%) were able to produce enterotoxin and both coagulase-positive and negative cultures were able to produce enterotoxins. Hence there is a need for further analysis up to enterotoxin production irrespective of coagulase production to ensure the risk associated with Staphylococcal contamination in foods.

Objectives

- To isolate and identify *staphylococcus* from milk products.
- To evaluate the percentage of enterotoxin producing coagulase-negative and positive strains of *staphylococcus* in milk products.

Materials and Methods

Study area

The study was carried out in Hyderabad which is the capital of Telangana, India. As of now, it is the sixth most populous city and sixth most populous urban agglomeration in India. The twin cities of Hyderabad and Secunderabad come under the ambit of a single municipal unit, the Greater Hyderabad Municipal Corporation. For administrative purposes, Greater Hyderabad Municipal Corporation is divided into many circles with each circle being homogeneous within and different from other circles. Random sampling procedure adopted in the study and the sample required for the study obtained using proportionate representation according to size.

Sample collection

Food samples collected aseptically from retail markets, sweet shops, and households of Hyderabad. Samples were transported to the laboratory in the ice box and transferred to the

refrigerator until further analysis. The interval between the sampling and the analysis will be less than one hour. A total of 420 food samples including khoa (a desiccated milk product), Kulfi (ice cream), paneer (a type of milk curd cheese used in Indian cooking), Dhal (a sauce made from lentils and spices, usually served with cooked rice), non-vegetarian curry (chicken meat and mutton), pine-apple fruit juice, cooked rice, vegetarian curry and sapota/sapodilla juice including 30 households hand washings collected for the analysis.

Isolation and identification of *S. aureus*, coagulase test, and enterotoxin detection were done in all the samples collected.

Statistical analysis

A proportion test has been done to see the differences intoxin production among coagulase-positive and coagulase-negative Staphylococci isolates.

Results

- The incidence, coagulase production and mean of *staphylococcus* in milk products are shown in Table 1. Among 400 milk products that were analyzed for the presence of *S. aureus*, contamination was found to be higher in Khoa (66%) when compared to other milk products like paneer (52%), rasmalai (15%), and doodhpeda (28%).
- Among 161 cultures of Staphylococci, 63 (39%) showed coagulase enzyme production and 98 (61%) isolates were coagulase-negative. The number of *staphylococcus* producing coagulase isolates was found to be less than non-coagulase producing *staphylococcus.*
- Both coagulase-positive and negative cultures of *S. aureus* were showed a positive result for enterotoxin production. The isolates of toxin-producing coagulase-positive and negative *S. aureus* cultures were not significant to each other according to the proportion test.
- Generally it is known that more than 106 cfu/g of S. aureus is likely to produce an enterotoxin, however, in the present study none of the food samples have crossed the above limit but few of them were able to produce enterotoxin.
- A high concentration of S. aureus in milk products was no guarantee of the presence of enterotoxin. Enterotoxin formation requires a high concentration of enterotoxigenic S. aureus to be present, but other factors such as pH, aw (water activity), and the presence of oxygen are also important in determining the extent to which enterotoxin is produced.
- Study on persistence and survival of *S. aureus* at different temperatures (4, 10, and 37°C) for different lengths of time (0-12 days) indicated that *S. aureus* population varied with temperature and showed high population and viability at 37oC. On the 12th day differences in numbers were observed at the lower and higher temperature.

Conclusions

- There is a need to relook at the guidelines for microbiological requirements for different milk products which indicates that coagulase-positive S. aureus could be less than 100 per gram of the milk product. Although enterotoxins are produced mainly by coagulase-positive staphylococci, some coagulase-negative staphylococci are involved in a variety of human and animal infections.
- This study confirms the same findings. Therefore, while assessing the risk of foodborne disease due to *S. aureus* contamination in foods enterotoxin production should be examined irrespective of its coagulase enzyme production. This data will be helpful to set up standards for assessing the microbiological quality of foods.

VI. FOOD CHEMISTRY

Studies on the food system of the Meitei community of Manipur and its nutritional implications

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. A significant proportion of the diverse foods available in our environment have been progressively neglected, thereby narrowing the base of global food security. This has led to food supply crises, hunger, and malnutrition over the years. Despite strides made in reducing global hunger through increases in cereal productivity, the world is still hungry, which also coincides with the erosion of agricultural biodiversity and a reduction in dietary diversity. Furthermore, strategies adopted to address the ongoing food insecurity, hunger, and malnutrition, particularly in developing countries, continue to narrow the food supply base through technological options that neglect indigenous and traditional food systems while focusing on a few staple crops. Increased availability of, and intakes of, cereal and cereal products in developing countries have been linked to decreased intakes of iron and increased incidences of iron deficiency anemia. Enormous resources have been invested globally in the fight against iron and other micronutrient deficiencies, yet there remain formidable health challenges posed by the continuing high prevalence rates of micronutrient deficiencies, increasing rates of obesity, and non-communicable diseases linked to a lack of dietary diversity. These are some of the consequences of the nutrition transition with its attendant simplification of diets.

So, this has resulted in renewed calls for food-based approaches of which deployment of food biodiversity is an important approach that will boost productivity through increased utilization of the genetic resource base of food species. This entails greater use of local biodiversity to ensure dietary diversity for which indigenous and traditional food systems of the poor and rural communities need to be acknowledged.

The North-Eastern region of India which comprises the seven states namely Arunachal Pradesh, Assam, Meghalaya, Manipur, Mizoram, Nagaland and Tripura forms an integral part of the Indo-Burma center of the biodiversity hotspot of global significance. The state of Manipur comprises an area of 22, 327 km^2out of which 17,219 $km^2$is forest-covered area. It is administratively divided into 9 districts, of which four districts (viz. Imphal-East, Imphal-West, Thoubal and Bishnupur) form the lowland valleys while the rest five districts are upland areas. From time immemorial till today the Meiteis which is the dominant non-tribal community in the four lowland districts of Manipur shares an inseparable relationship with its diverse bioresource system for livelihood, religious rituals, foods, medicine, etc. The main occupation of the Meiteis is agriculture and most of them are rice cultivators.

Many of the indigenous foods consumed by the people of Manipur are also traditionally valued for their therapeutic effects, but their nutritional parameters have not been evaluated. Studies have shown the existence of potential plant food in Manipur. *Parkia roxbrughii* which is traditionally being used as a supplementary food source in Manipur appears to be a potential source of protein and fat. Three wild fruits (*Elaeocarpus serratus, Bruceajavanica,* and *Ficus palmata*) of Manipur have been reported to be rich in antioxidants, flavonoids, and phenolic compounds. Therefore, the study aims to investigate the food system of the Meiteis and its role on nutritional and health outcomes.

Objectives

- To assess the availability, accessibility, and consumption pattern of different foods in the food system of the Meitei community in Manipur.
- To identify and analyze the potential foods in the food system of the Meiteis.
- To evaluate the nutritional status of the Meiteis and its relation to the food system of the community.

1. Acquiring the details of foods consumed by Meiteis

For this, two districts were selected randomly and twelve villages were selected by random stratified sampling, thereby dividing all the villages of the two districts into quartiles based on population ranging from least populated to the most populated. As per the guidelines, an informal interview with the *focus group* and *key informants* was conducted in these villages where they were asked open-ended questions (with probing and prompting) on the types of both common and indigenous foods they consume, the availability and seasonality, the cultivars and varieties, the parts used, therapeutically used if any and the frequency of consumption (Table 1 (a)).

Table 1 (a): List of villages selected for the study

Sl.no.	Name of the village	Subdivision	District	Number of Households	Number of population	No. of Household	
						Dietary survey	Nutritional status
1	Wangwookeirap	Moirang	Bishnupur	80	431	3	24
2	Yumnamkhunou	Nambol		251	1253	7	56
3	Potsangbam	Bishnupur		804	3721	23	184
4	Kha thinunghei	Moirang		1858	10107	53	424
5	Yaithibikhunou	Thoubal	Thoubal	115	548	3	24
6	Lourembam	Thoubal		242	1050	7	56
7	Lamding	Thoubal		333	1587	10	80
8	Nungei	Lilong		392	2074	11	88
9	Elangkhangpokpi	Kakching		638	2815	18	144
10	Wangjing	Thoubal		672	2960	19	152
11	Leishangthem	Lilong		1203	6118	34	272
12	Wabagai	Kakching		1810	8578	52	416
					Total	240	1920

A total of 313 foods were recorded out of which 102were less familiar foods (Table 1 (b)). The foods were categorized into sixteen group cereals; pulses and legumes; green leafy vegetables; other vegetables; fruits; roots and tubers; nuts and oilseeds; spices and condiments; mushrooms; sugars; milk and milk products; eggs; poultry; fish; meat; and fat and oil. The foods which are commonly consumed but for which the composition data are not available in the Indian nutritive food tables were taken for analysis (Table 2).

2. Analysis of food samples

Out of all the foods recorded, ninety-five indigenous foods (mostly consumed) were collected for nutrient analysis. Ninety-five indigenous foods of the Meitei consisting of three cereals, seven roots and tubers, forty-two leafy vegetables, fourteen other vegetables, twelve fruits, one nuts and oilseeds, four mushrooms and ten spices and condiments, and one fermented fish were sampled. All the samples were collected separately from each selected village and pooled together into one sample to make single composite samples. The edible portion of food samples was separated cleaned and processed immediately for the nutrient analyses.

Nutrient profile

The methods of the Association of Official Analytical Chemists (AOAC, 2005 and 2012) were used to determine the proximate composition. Moisture content (AOAC 934.01) was derived from the difference of the fresh weight of the samples and their dry weight (dried at 60˚C). Protein values were calculated from the estimated nitrogen (Kjedahl method) in the food using the Jones conversion factor i.e. 6.25 (AOAC 2001.11; Jones, 1941). The total fat content of the samples was determined by the gravimetric method using a mixed solvent of chloroform and methanol (AOAC 963.15). Ash content was determined by the gravimetric method (AOAC 942.05). The total, insoluble and soluble dietary fiber was determined using the enzymatic-gravimetric method (AOAC 991.43). Finally, carbohydrate content was calculated by difference (Greenfield and Southgate, 2003). All the values are expressed on a fresh weight basis.

Table 1 (b): Food count reported through Focus group discussion

Food group	Total recorded	Indigenous recorded	Food analyzed
Cereal	23	7	3
Pulse and legume	15		
Green leafy vegetable	63	44	43
Other vegetable	52	14	14
Fruit	48	13	12
Root and tuber	20	7	7
Spice and condiment	29	10	10
Nut and oilseed	13	1	1
Mushroom	7	4	4
Milk and milk product	4		
Sugar	3		
Fish and shellfish	22	2	1
Eggs	4		
Poultry	5		
Meat	3		
Fat and oil	2		

Among the vegetables, *Albizzia procera* had the highest protein, ash, fat and total dietary fiber content. The vegetables were found to have the ranges of 0.19-25.88, 0.14-8.02, 0.05-5.55, 2.52-33.64, and 0.53-15.72 g/100g for protein, ash, fat, total dietary fiber, and carbohydrate respectively.

Table 3. Proximate composition of the indigenous foods

	Moisture (g)	Protein (g)	Ash (g)	Total Fat (g)	Total Dietary Fiber (g)	Carbo hydrate (g)	Energy (Kcal)
Mushrooms (n=4)	7.58 - 85.67	4.17 – 19.61	0.74 - 9.56	0.13 - 2.32	7.21 - 46.29	2.08 - 29.21	41 – 278
Spices and condiments (n=10)	14.67 - 89.32	1.34 – 14.97	1.62 - 6.09	0.47 - 3.62	5.91 - 56.28	0.99 - 19.69	30 – 242
Fish (n=1)	30.31	49.88	14.58	5.23	-	-	247
Oilseed (n=1)	5.88	23.97	3.82	20.93	12.22	33.17	441
Green leafy vegetables (n=42)	55 – 93.91	0.19 - 13.56	0.14 - 6.22	0.05 - 2.91	1.52 - 19.55	0.22 - 19.49	20 – 197
Roots and tubers (n=6)	55.43 - 80.59	0.85 – 13.29	0.38 - 5.82	0.02 - 1.85	1.72 - 15.26	1.45 - 26.31	60 – 141
Cereal (n=3)	7.95 - 65.28	4.28 – 11.26	0.56 - 1.11	0.88 - 1.62	3.75 – 5.73	24.91 - 75.61	135 – 362
Fruit (n=12)	5.95 - 88.02	0.13 – 7.58	0.41 - 3.05	0.24 - 12.87	1.76 - 36.19	2.1 – 34.35	34 – 356
Other vegetables (n=16)	46.84 - 92.98	1.63 – 7.41	0.26 - 3.87	0.14 - 2.51	1.27 - 23.92	0.53 - 39.32	21 – 195

-values are range (minimum-maximum) in 100g of samples

Garcinia xanthochymus had the highest protein and ash while *Vanguirea spinosa* got the highest carbohydrate and total dietary fiber content among the fruits. Table 3 represents the range of proximate composition of the samples analyzed.

Analysis of water-soluble vitamins such as vitamin C, B6, B5, B9 was carried out using Ultra-High Performance Liquid Chromatography. Individual carotenes and xanthophylls analysis was carried out using the HPLC method and total carotenoids were estimated by taking the absorbance at 450 nm using a spectrophotometer. Vitamin E was quantified by normal phase U-HPLC and the value was expressed as α tocopherol equivalent (WHO/ FAO, 2002).

Table 4 represents the range of water-soluble content of the foods analyzed. The leafy vegetable *Albizzia procera* showed the highest content of vitamin B2 (1.15mg/100g) and C (105mg/100g). *Solanum nigrum* belonging to leafy vegetables had the highest vitamin B3 (2.18 mg/100g) content of all the samples. The highest vitamin B6 and B9 were found in spices and condiments i.e., *Cardamine hirsute* (0.987mg/ 100g) and *Citrus latipes* (561µg/ 100g) respectively. The indigenous plant foods possessed high vitamin B9 content. For fat-soluble vitamins (Table 5), *Eurgale ferox* exhibited the highest vitamin E content (42.7mg/100g of α tocopherol equivalent) while the highest vitamin K and β carotene were found in leafy vegetables namely *Adhatodavasica* and *Allium tuberosum* respectively. Among the cereal, black rice (Chakhaoamuba) got the highest carotenoids content.

Table 4. Water-soluble vitamins content of indigenous food

	Vitamin B1 (mg)	Vitamin B2 (mg)	Vitamin B3 (mg)	Vitamin B5 (mg)	Vitamin B6 (mg)	Vitamin B9 (µg)	Vitamin C (mg)
Green leafy vegetables (n=42)	0.010 - 0.161	0.046 - 1.080	0.025 - 1.850	0.167 - 1.650	0.028 - 0.987	12.87 - 493	0.071 - 105
Spices and condiments (n=10)	0.011 - 0.412	0.007 - 0.256	0.202 - 1.010	0.406 - 1.270	0.013 - 0.921	10.53 - 561	0.674 - 48.41
Fruit (n=12)	0.010 - 0.031	0.006 - 0.086	0.187 - 2.068	0.283 - 0.881	0.016 - 0.403	39.72 - 315	2.891- 63.43
Mushrooms (n=4)	0.010 - 0.021	0.019 - 0.352	0.267 - 1.920	1.140 - 1.750	0.131 - 0.409	51.37 - 305	0 - 1.481
Oilseed (n=1)	0.421	0.086	0.498	1.023	0.915	87.94	0.677
Other vegetables (n=16)	0.01 0- 0.081	0.046 - 0.760	0.094 - 0.892	0.184 - 1.150	0.009 - 0.551	31 - 492	0.627 - 17.28
Cereal (n=3)	0.110 - 0.171	0.010 - 0.107	0.479 - 0.588	0.721 - 1.231	0.061 - 0.281	45.08 - 123	0 - 1.012
Fish (n=1)	0.052	0.084	0.739	1.193	1.219	215	-
Roots and tubers (n=6)	0.010 - 0.061	0.019 - 0.094	0.222 - 1.382	0.202 - 0.900	0.016 - 0.278	11.26 - 238	0.457 - 5.451

-values are range (minimum-maximum) in 100g of samples

Elemental analysis was carried out after wet digestion according to AOAC (968.08) method. Potassium, iron, calcium, copper, manganese, magnesium, sodium and zinc were determined in an atomic absorption spectrometer (flame furnace). Phosphorus was estimated by the Fiske and Subbarow method as described in the AOAC method (931.01).And other trace elements like lithium, chromium, cobalt, nickel, arsenic, selenium, molybdenum, cadmium,

antimony, mercury, and lead were analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (AOAC 2013.06) and expressed in µg/100g of the sample weight. The range of minerals and trace elements is summarized in table 6.

The mushroom *Auricularia delicate* (201 mg/100g) had the highest iron content followed by the leafy vegetable *Trapasnatans* (98.2 mg/100g) and *Centella asiatica* (89 mg/100g). The highest Zn and Cu content were found in spices and condiments namely *Cardamine hirsute* and *Elsholtziablanda*. Leafy vegetables *Trapasnatans* and *Albizzia procera* showed the highest content of Mn and Mg respectively. Some indigenous plant foods showed high micronutrient content in comparison to their counterparts of commonly consumed Indian plant foods.

Phytonutrient profile

Antioxidant activity was measured using three different methods namely, reducing power by FRAP free radical scavenging by DPPH; and ABTS. Total phenolic content was determined by Folin Ciocalteu method and expressed in Gallic Acid Equivalent per 100g of the sample. Water extract (water-soluble) and methanol extract (lipid-soluble) of the sample was analyzed for the antioxidant activity and total phenolic content.

Table 5. Fat-soluble vitamins content of indigenous foods

	a-T.E (mg)	Vitamin K (µg)	Lutein (µg)	Zeaxanthin (µg)	ß – Cryptoxanthin (µg)	ß – Carotene (µg)	Total Carotenoids (µg)	Retinol (µg)
Green leafy vegetables (n=42)	0.221-14.23	0.001 - 1461	0.661 - 727	0.002 - 85.12	0.001- 3.691	56 - 9563	229 - 51535	
Spices and condiments (n=10)	0.031-19.06	0.991 - 95.91	0.909 - 108	0.432 - 4.782	0.022- 0.391	11.08 - 2423	59.36 - 5110	
Fruit (n=12)	0.101-2.942	0.811 - 14.63	0.992 - 24.26	0.944 - 28.78	0.001- 1.941	0.48 - 1091	19.46 - 2297	
Other vegetables (n=16)	0.021-42.72	1.151 - 107	0.001 - 23.66	0.003-9.171	-	2.31 - 863	54.03 - 1626	
Cereal (n=3)	0.082-1.171	1.321-1.611	0.271 - 63.55	0.004-0.671	-	0.992-156	11.85 - 266	
Roots and tubers (n=6)	0.011 - 1.161	1.362 - 26.35	0.003 - 26.91	0.002-0.951	0.032- 2.141	11.64 - 63.21	69.28 - 141	
Oilseed (n=1)	0.306	1.232	0.521	-	-	23.12	185	
Mushrooms (n=4)	0.011-0.671	2.321 - 48.31	-	-	-	-	-	
Fish (n=1)	0.091	19.28	-	-	-	-	-	3.261

-values are range (minimum-maximum) in 100g of samples

Table 6a. Minerals content (range in mg/100) of indigenous foods

	Ca	Cu	Fe	Mg	Na	Mn	P	K	Zn
Green leafy vegetables (n=42)	26.21-957	0.031-1.641	1.261-178	44.81-667	0.421-84.81	0.212-61.07	18.81-723	146-1471	0.142-6.661
Fish (n=1)	3870	0.322	56.14	162	1767	2.131	5043	393	10.55
Mushrooms (n=4)	6.211-342	0.531-1.221	13.93-269	15.41-94.82	5.821-57.92	0.611-25.22	215-1006	125-1442	0.561-4.951
Spices and condiments (n=10)	18.71-688	0.041-3.37	0.621-15.06	20.01-208	0.311-3.112	0.472-16.37	41.71-558	317-1828	0.251-5.732
Other vegetables (n=16)	5.712-583	0.081-2.171	0.181-20.49	4.822-136	0 - 4.721	0.121-14.86	24.22-1386	31-1108	0.251-6.772
Oilseed (n=1)	404	1.452	25.91	311	4.521	6.291	1608	487	3.161
Fruit (n=12)	5.001-197	0.051-0.622	0.081-51.31	4.911-58.8	0.511-8.421	0.021-2.431	7.212-133	115-454	0.021-1.212
Roots and tubers (n=6)	4.921-67.21	0.041-0.451	0.951-35.48	16.62-92.71	1.111-19.31	0.291-3.652	13.21-547	143-922	0.321-1.542
Cereal (n=3)	0.700-11.41	0.051-0.222	0.621-1.7	37.51-125	0.511-2.001	0.361-2.661	344 - 681	132-231	0.611-1.871

Table 6b. Trace elements (range in µg/100) content of indigenous foods

	Cd	Cr	Co	Pb	Mo	Ni	Li	Hg	As	Se	Sb
Roots and tubers (n=6)	0.123-0.431	0.181-0.513	0.149-0.634	0.034-0.123	0.429-1.9.23	0.161-0.519	0.148-3.203	0.001-0.006	0.011-1.519	1.521-5.92	0.042-0.181
Oilseed (n=1)	0.131	0.322	0.421	0.431	0.3571	7.413	0.321	0.001	0.011	0.691	0.049
Other vegetables (n=16)	0.121-0.922	0.981-1.633	0.219-1.033	0.331-1.834	0.1281-1.033	0.281-0.866	0.123-1.034	0.001-0.101	0.081-0.917	0.591-29.28	0.011-0.315
Fruit (n=12)	0.045-0.318	0.542-6.745	0.067-0.539	0.131-0.913	0.281-3.844	0.217-1.212	0.131-1.923	0.001-0.011	0.031-0.912	0.531-15.68	0.012-0.284
Cereal (n=3)	0.111-0.521	0.7231 - 3.612	0.141-0.321	0.811-2.112	0.721-1.725	4.712-30.4	0.002-0.003	0.426-0.625	0.016-0.917	0.507-4.872	0.025-0.052
Mushrooms (n=4)	0.758-9.531	0.122-0.841	0.241-1.634	0.541-2.542	0.122-0.833	0.028-0.241	0.243-1.732	0.001-0.071	0.071-4.22	0.811-8.118	0.085-0.191
Fish (n=1)	0.631	0.142	0.354	0.231	0.231	8.922	0.054	1.661	0.781	171	0.216
Spices and condiments (n=10)	0.034-0.149	1.329-78.3	0.221-2.433	0.139-1.447	0.161-1.533	0.527-4.022	0.371-3.213	0.000-0.012	0.151-7.811	0.421-13.79	0.031-0.232
Green leafy vegetables (n=42)	0.221-1.011	0.0429 - 0.903	0.108-0.951	0.121-1.944	0.012-3.611	0.111-4.222	0.138-1.162	0.001-0.717	0.541-5.519	1.461-55.77	0.061-1.155

-values are range (minimum-maximum) in 100g of samples

3. Dietary and nutritional status survey

A total of 1920 households from the twelve villages were covered for the nutritional status survey and dietary intake and food frequency surveys were carried out every eighth household which came to a total of 240 households (Table 1(a)).

A dietary survey using 24 hours, 7-day food frequency questionnaire and seasonal food frequency (based on the NNMB scheduled with a modification to include the variety/ cultivar) were used to record the consumption of every food in every food group which eventually was used to elicit information on food/nutrient intake and its link to the nutritional status.

Summary and important findings of the study

- Three hundred and thirteen both plant and animal foods were recorded to be consumed by the Meitei community of Manipur. Out of which one hundred and two were identified to be less familiar foods. These recorded foods belong to both wild and cultivated/domesticated types.
- All the less familiar foods were categorized into sixteen food groups, and the green leafy vegetable food group got the highest number of food followed by other vegetable groups and fruit groups.
- A food composition database was generated for ninety-five less familiar foods which were mostly consumed but the nutrient profile was not available in Indian Food Composition Tables.
- Among the vegetables, *Albizzia procera* had the highest protein, ash, fat and total dietary fiber content. It also showed the highest content of vitamin B2 and C.
- *Garcinia xanthochymus* had the highest protein and ash while *Vanguirea spinosa* got the highest carbohydrate and total dietary fiber content among the fruits.
- *Solanum torvum* had the highest vitamin B3 content of all the samples. The highest vitamin B6 and B9 were found in *Cardamine hirsute* and *Citrus latipes* respectively.
- *Eurgale ferox* exhibited the highest vitamin E content (42.7mg/100g of α tocopherol equivalent) while the highest β carotene were found in leafy vegetables namely *Adhatodavasica, Cardamine hirsute, Centella asiatica, Meriandra bengalensis* and *Phlogacanthusjenkinsii*. Among the cereal, black rice (*Chakhaoamuba*) got the highest carotenoid content.
- The mushroom *Auricularia delicate* had the highest iron content followed by the leafy vegetable *Trapasnatans* and *Centella asiatica*. The highest Zn and Cu content were found in *Cardamine hirsute* and *Elsholtziablanda*. Leafy vegetables *Trapasnatans* and *Albizzia procera* showed the highest content of Mn and Mg respectively. Some indigenous plant foods showed high micronutrient content in comparison to their counterparts of commonly consumed Indian plant foods.
- *Elsholtziablanda* and *Albizzia procera* had the highest total phenolic content. High antioxidant activity was shown by leafy vegetables like *Cissus adnata, Nelumbo nucefera,* and fruit like *Euphoria longana.*

VII. FOOD TOXICOLOGY

1. Dietary intake of aflatoxins from spices risk assessment

Spices are important flavouring components in the dietaries of several countries particularly in Asia, Africa, and Europe. Several spices including chilli, black pepper, nutmeg, cinnamon, ginger, and turmeric are important in world trade. India leads in spice production. Spices, like cereal grains and oilseeds, are susceptible to fungal and mycotoxin contamination when conducive conditions are presented such as high relative humidity, moisture levels above 12% during post-harvest storage, presence of insect infestation or mold damaged seeds. Natural occurrence of mycotoxins such as aflatoxins and ochratoxins have been well documented in spices such as dried red chillies, black pepper, and also nutmeg from producing countries such as India, Indonesia, Turkey.

The hepato-toxic and carcinogenic hazards of aflatoxins are well recognized. Hence, several countries have established maximum limits on food commodities to control and reduce dietary exposure in consumers. Aflatoxin tolerance limits in spices established by certain countries ranged between 2-30µg/kg for aflatoxin B1.However, lack of harmonization in these limits has been causing various trade barriers and considerable rejections of export consignments from various producer countries resulting in revenue losses. Although spices are high-risk commodities towards aflatoxin contamination, their relevance to dietary aflatoxin exposure is as yet unclear because of their low level of consumption. Unlike staple cereals, nuts, and oilseeds which are consumed in higher amounts, the use of spices represents less than 2% of the diet as the main role of spices in the diet is mainly to impart flavor and taste. Of considerable importance for risk assessment of aflatoxins is the quantity of food consumed and level of aflatoxin contamination in the food which varies from country to country.

Generation of data on dietary intake of spices becomes important to assess the risk of aflatoxin exposure from these food components that are consumed in far fewer quantities when compared to that of cereals but are susceptible to aflatoxin contamination. The international food standards-setting body namely the Codex Alimentarius Commission (CAC) of the United Nations (UN) is currently deliberating on the fixing of maximum limits (ML) for aflatoxins in spices. However, the modus operandi of fixing the MLs for aflatoxins in spices has yet to take shape in the light of limited availability of data on the occurrence of aflatoxin contamination in various spices, and quantity of dietary intake of spices in different countries both of which required for risk assessment of aflatoxin exposure by JECFA.

The present study was undertaken to i) assess the extent of aflatoxin contamination in various spices and spice blends. Since the presence of ochratoxin mycotoxins have also been reported in spices, selected spices were also assessed for ochratoxin A contamination ii) perform a risk assessment of aflatoxin exposure from spices from available data on aflatoxin levels and dietary spice intake in the Indian context iii) prepare a global review on mycotoxin levels in individual spices and dietary intake of various spices.

Methodology

Collection of samples: A total of 80 spice samples consisting of individual whole and powdered samples (chillies, black pepper, nutmeg, mace, star anise and cumin seeds), and spice blends

(ready to eat spice mixes, spiced tea mixes, and other spice blends) were purchased randomly from local retail markets in quantities of 250g each.

Analysis of aflatoxins and ochratoxins: Analysis was performed on all 80 spice samples using a mycotoxin fluorometer (VICAM Series 4Ex) according to the manufacturer's instructions. Positive samples were verified using HPLC methods. Ochratoxin A (OTA) was analyzed in a total of 36 samples consisting of chilli powder, mace, nutmeg, and spiced tea mixes, using solid-phase competitive inhibition ELISA assay kit from Helica Biosystems Inc.

***Assessment of aflatoxin exposure from spices*:** The probable aflatoxin intake from spices was calculated for individual spices namely, chilli powder, nutmeg, mace, cumin powder, and black pepper powder using published data on spice intake from Siruguri and Bhat (2015) as follows:

Probable aflatoxin intake (ng/kg body weight /day)=

Aflatoxin levels in spices (chilli powder/ nutmeg/ mace/ cumin powder/ black pepper) (ng/g) * Level of spice (chilli powder/ nutmeg/ mace/ cumin powder/ black pepper) intake/ body weight (adult man 60kg).

Results

The occurrence of aflatoxins and ochratoxin A in spice samples

Aflatoxins were detected in a total of 61/80 samples at levels ranging from 2.0-37µg/kg (Table 1). The number of contaminated samples were more in chilli powder, nutmeg, spiced tea mixes, and RTE spice mix samples. All the chilli powder, nutmeg and spiced tea mixes analyzed showed the presence of aflatoxins. Levels exceeded the FSSAI maximum limits of 30µg/kg in one chilli powder and 2 spiced tea mix samples. The highest mean and maximum aflatoxin level detected was in spiced tea mix samples followed by chilli powder samples.

Ochratoxin A was detected in 56% of the samples analyzed in which aflatoxins were also detected (Table 2). The highest mean levels were observed in spiced tea mixes followed by chilli powder samples. The maximum ochratoxin A level observed was 80µg/kg in spiced tea mixes.

Table 1.Aflatoxin contamination in individual spices and RTE and other spice mixes

Spice	Total number analyzed	Number positive (>LOD)	Aflatoxin levels (µg/kg)			Number exceeding MLs*
			Mean of total	Mean of positives	Range	FSSAI
Red chilli powder	15	15	11.4±8.8	11.4±8.8	3.3-37	1
Black pepper powder	7	5	1.6±1.1	2.2±0.3	<LOD-2.5	Nil
Cumin powder	6	5	4.7±4.7	5.7±4.0	<LOD-11.0	Nil
Nutmeg	6	6	4.5±2.1	4.5±2.1	2.0-7.7	Nil
Mace	9	4	1.4±1.8	3.1±1.5	<LOD-5.0	Nil
Star Anise	6	4	8.1±9.9	10±10.3	<LOD-25.0	Nil
Spiced tea mixes	9	9	18.2±11.7	18.2±11.7	4.6-37.0	1
RTE spice mixes	10	7	3.1±3.1	3.6±2.8	2.5-10.0	Nil
Other spice mixes	12	6	1.6 1.5	2.9 1.1	<LOD-5.0	Nil
Total	80	61			<LOD-37.0	

* MLs for total aflatoxins in spices: FSSAI:30 µg/kg.

Dietary aflatoxin exposure from spices

The probable aflatoxin intake was calculated from selected spices namely, chillies, cumin powder, black pepper, nutmeg, and mace based upon their frequency of use and also the level of aflatoxin contamination. The results indicated that the level of aflatoxin intake calculated from mean and maximum aflatoxin levels was maximum from chillies followed by cumin powder, black pepper, nutmeg, and mace (Table 3). The highest and lowest mean aflatoxin intake level was 0.8 and 0.01 ng/ kg bwt/ day from chillies and black pepper respectively. At the maximum level of spice intake, the aflatoxin intake was highest from chillies and lowest from nutmeg. The contribution of mace to aflatoxin intake was negligible. When aflatoxin intakes were calculated from maximum or highest aflatoxin levels obtained in the present study, the levels were highest from chillies followed by cumin and negligible from the rest of the spices.

Table 2. Ochratoxin A contamination in selected spices and co-occurrence with aflatoxins

Spice	Number analyzed	Number positive for AFs and OTA	OTA Levels (µg/kg)	
			Mean	Range
Chilli powder	12	9	10.6±6.37	2.5-20.0
Mace	9	1	2.5	2.5
Nutmeg	6	1	5.0	5.0
Spiced tea mixes	9	9	38.9±28.21	20-80
Total	36	20		2.5-80

Table 3. Aflatoxin intake from spices

Spice intake (g/day)	Aflatoxin intake (ng/kgbwt/d) [Aflatoxin levels in positives (ng/g)]		Spice intake (g/day)	Aflatoxin intake (ng/kgbwt/d) [Aflatoxin levels in positives (ng/g)]	
Chilli powder	Mean [11.4]	Max [37]	Nutmeg	Mean [4.5]	Max [7.7]
Mean:4.2	0.80	2.60	Mean:0.02	0.002	0.003
Min:0.64	0.10	0.40	Min:0.003	0.0002	0.0003
Max:14.8	2.80	9.12	Max:0.06	0.01	0.01
95th perc: 9.3	1.80	5.74	95th perc: 0.04	0.003	0.01
Cumin	Mean [5.7]	Max [11]	Mace	Mean [3.1]	Max [5.0]
Mean:1.1	0.10	0.20	Mean:0.02	0.001	0.002
Min:0.12	0.11	0.02	Min:0.003	0.0001	0.0003
Max:4.7	0.45	0.90	Max:0.08	0.004	0.01
95th perc.:4.0	0.38	0.73	95th perc: 0.08	0.004	0.01
Black pepper	Mean [2.2]	Max [2.5]			
Mean:0.4	0.01	0.02			
Min:0.003	0.0001	0.0001			
Max:3.4	0.12	0.14			
95th perc: 1.4	0.05	0.06			

Conclusions

- Different spices and spice blends were analyzed for aflatoxins and ochratoxin A. Most of the samples analyzed had aflatoxin levels below the MLs established by the FSSAI.

- The results pointed out for increased monitoring to check entry of poor quality spices that may be contaminated in such preparations as spice mixes.
- Although spices are high-risk commodities towards aflatoxin contamination, their relevance to dietary aflatoxin exposure is as yet unclear because of their low level of consumption.
- The levels of exposure as observed in the study suggested that exposure from spices is very low as compared to that from staple cereals and maximum from chillies and minimum from nutmeg and mace.
- The results of the study may help set maximum aflatoxin limits in spices.

2. Assessment of mycotoxin contamination in processed foods containing maize and groundnut

Mycotoxins of toxicological significance namely aflatoxins and fumonisins are not destroyed during food-processing or cooking operations and hence can continue to occur in finished processed foods. In India, various processed foods are marketed in the unorganized sector that often does not comply with mandatory quality specifications. Many of these processed foods are Ready to Eat (RTE) foods such as spice mixes, snack items based on cereals such as those made with cornflakes, cornflour, and various snacks based on groundnut. The Codex CCCF has proposed to evaluate aflatoxin levels in RTE groundnut for fixing aflatoxin tolerance limits (CCCF 2014). The present study was initiated to assess the occurrence of aflatoxins, fumonisins, and ochratoxins in selected processed foods consumed as snacks and dietary accompaniments and based on maize and groundnut, with the following objectives.

Objectives

- To assess the presence of aflatoxins, fumonisins, and ochratoxins in processed foods and snacks containing groundnut, maize, sorghum, rice and wheat.
- To perform a risk assessment of the mycotoxin exposure from the mycotoxin levels and amount of processed foods consumed.

Methodology

Collection of samples: A total of 196 samples consisting of groundnut snacks, processed maize products, sorghum flour, dehusked and polished rice, and products, and wheat flour were procured from different wholesale and retail markets located in different regions in Hyderabad city. Aflatoxins were analyzed by fluorimetry using mycotoxin analyzer (VICAM Series 4EX) and HPLC methods using standard AOAC procedures. In maize and sorghum products in addition to aflatoxins, fumonisins were also analyzed by HPLC and fluorimetry methods.

Analysis of discoloured groundnut kernels for aflatoxins: A total of 34/56 fried and roasted and salted groundnuts were examined for the presence of discoloured kernels which are known to contain high levels of aflatoxins. DKs were segregated from the samples and subjected to aflatoxin analysis either as single DK or pooled DKs using HPLC methods. The aflatoxin content in the RTE samples was computed from the aflatoxin levels determined in the segregated DKs as well as in the non-discoloured kernels (NDKs) and ingredients used for coatings such as flour and spices. Analysis of 77 single DKs indicated the presence of aflatoxins in 31% of the DKs at levels ranging from 0.007 to 1383.4 µg/ g. Analysis of 17 pooled DK samples indicated the

presence of aflatoxins in 13 pooled DKs at levels ranging from 0.142-357.3µg/ pooled DKs. The total aflatoxin content in the RTE samples calculated from the aggregate of aflatoxin levels in DKs, NDKs, and other components ranged from 0.001 to 2.779µg/g sample respectively and was contributed mostly by DKs (90%).

The occurrence of aflatoxins in different groundnut snack products: Out of a total of 103 groundnut snack products, aflatoxins were detected in 51% of the samples with levels ranging from 1.0-660.0 µg/kg. Around 14% of the samples in fried groundnut, chikki, groundnut chocolate bars, groundnut masala powders, and peanut butter had aflatoxin levels that exceeded the FSSAI limit of 10µg/kg in RTE groundnut products. The highest aflatoxin level was observed in chikki and groundnut masala powders. All the groundnut chocolate bars analyzed were positive for aflatoxins.

The occurrence of aflatoxins in cereal and millet products: From a total of 71 products analyzed aflatoxin was detected in around 86% of the samples and 18% exceeded the FSSAI limits of 15 µg/kg in cereal products. High levels were observed in rice flour and sorghum flour. In sorghum flour, in addition to aflatoxin, co-occurrence with fumonisins was observed in all the 14 samples analyzed at levels ranging from 1000-4800 µg/kg which are above the maximum limits fixed by Codex Alimentarius Commission.

Risk assessment of aflatoxin exposure from processed groundnut products

The extent of aflatoxin exposure from various groundnut snack products analyzed were assessed. Aflatoxin intake was calculated as follows:

Aflatoxin intake (ng/ kg body weight/ day) = Aflatoxin levels (median/ maximum) in processed groundnut snack * quantity of groundnut consumed/ body weight (60kg adult man).

Aflatoxin intake from various processed groundnut snacks was calculated based on minimum quantity of the snack assumed to be consumed. Median and maximum aflatoxin levels detected in various processed groundnut snacks were used for calculating aflatoxin intake.

It was observed that the highest aflatoxin intake was through consumption of contaminated fried groundnut snacks especially those with discoloured kernels. At median and maximum aflatoxin levels, aflatoxin intake was observed to be 50 and 1158ng/ kg body weight per day from consumption of approximately 25g of fried groundnut snack. Aflatoxin intakes from other groundnut snacks calculated from median aflatoxin levels were observed to be below 1ng/ kg body weight per day.

Table 1. Aflatoxin levels in single discoloured kernels (DKs) segregated from fried (FG) and roasted and salted groundnut (RSG) samples

Sample	DKs	DKs		Aflatoxin levels			
		No. analyzed	No. positive	µg/DK[1]	µg/ g DK	µg/ g sample	% of total aflatoxin in sample
Fried groundnut	Single	44	12	0.002-253.156	0.007-1383.400	0.002-1.856	94-100
Roasted & salted groundnut	Single	33	12	0.13-7.687	0.026-10.826	0.01-1.0	100
Fried groundnut	Pooled	12	10	2.842-357.3	2.250-99.25	0.001-2.8	78-100
Roasted & salted groundnut	Pooled	5	3	0.14-3.5		0.001-0.014	100

[1]µg/total weight of DK

Table 2. Aflatoxin contamination in other groundnut products

Groundnut product	No. analyzed	No. positive	Levels (µg/kg)	Levels exceeding FSSAI limits (10 µg/kg)
Fried coated groundnut	18	3	71-220	3
Roasted and salted groundnut	28	7	1.0-3.2	Nil
Peanut butter	9	8	2.0-11.0	1
Groundnut chocolate bars	16	16	1.0-13.0	3
Chikki	19	12	1.0-660.0	3
Groundnut masala powder	13	7	2.0-210.0	4
Total	103	53 (51.4%)	1.0-660.0	14 (13.6%)

Table 3 Aflatoxin contamination in cereal and millet products

Sample	Total no. analyzed	No.positive	Levels (µg/kg)	Levels exceeding FSSAI limits (15µg/kg)
Maize products (cornflakes, popcorn)	9	nil	nil	-
Sorghum flour	14	13	2-104	11
Dehusked, polished rice	21	21	2.1-12	Nil
Rice flour	15	15	3.4-20	2
Wheat flour	12	12	0.2-8.1	Nil
Total	71	61 (85.9%)	0.2-104.0	13 (18.3%)

Table 4. Aflatoxin exposure from different processed groundnut products in adults

Groundnut product	Quantity consumed (g)	Aflatoxin levels (µg/kg)	Aflatoxin intake (ng/kgbwt/day)	Aflatoxin levels (µg/kg)	Aflatoxin intake (ng/kgbwt/day)
		Median		Highest	
Fried groundnut	25g/day*	119	50	2779	1158
Roasted & salted groundnut	25g/day*	2	0.83	987	411
Chikki	15g (2 small blocks)	1.5	0.4	660	165
Peanut bars	50g (weight of single bar)	1.1	0.9	13	2.5
Peanut butter	5g (1 teaspoon)	2.1	0.2	12	1.0
Peanut powder	5g (1 teaspoon)	11	0.9	210	17.5

* assuming minimum amount consumed from a packet of prepared groundnut snack

Conclusions

The results of the above study indicated that i) processed groundnut snacks could become important sources of aflatoxin exposure, especially when damaged or discoloured groundnut kernels are used for such preparations. ii) High levels above 1000µg/g were detected in discoloured kernels segregated from fried groundnut snacks. iii) Groundnut snack products such as fried and roasted groundnut, peanut butter, groundnut chocolate bars, and chikki are popular among children and hence carry risk of aflatoxin exposure in this age group. iii) The study reported may gain relevance given the Codex proposal to fix maximum aflatoxin limits in RTE groundnuts. iv) More data needs to be generated on the frequency and quantity of consumption of processed groundnut snack products particularly in children to assess aflatoxin exposure. v) The data generated from the study may help identify risk factors for aflatoxin contamination in processed groundnut snacks and setting maximum aflatoxin limits.

3. Investigation of mycotoxin contamination in herbal and medicinal plants and products to formulate prevention and control strategies

The natural occurrence of mycotoxins is well documented in a variety of foods such as cereals, oilseeds, treenuts, spices, and dried fruits. Among these mycotoxins, aflatoxins have and continue to demand the most attention owing to their hepatotoxic and carcinogenic properties. Fungal and mycotoxin contamination in herbal and medicinal plants gained considerable significance in recent decades owing to their importance in traditional medicine and also high export value. Herbal products are crude preparations of various kinds of medicinal plants and include leaf, stem, root, flower, seed, etc. With the increasing use of herbal medicines safety and quality of herbal preparations as well as the raw materials used have become a major public health concern. Herbal plants and preparations are susceptible to fungal spoilage, particularly during harvesting, handling, transportation, and storage. The Food Safety Standard Authority of India, GOI has proposed food safety and standard regulations for specialty foods containing ingredients of botanical nature that have medicinal properties such as those used in Ayurvedic medicines.The present study was undertaken to investigate fungal and mycotoxin contamination in selected herbal and medicinal plants that are being utilized for health or therapeutic benefits.

Methodology

Samples for collection and analysis of mycotoxins were selected based on the parts used for consumption and included leaves, herbs, roots, flowers, seeds, and bark. Herbal medicinal products and preparations were also collected. A total of 35 samples comprising 15 types of botanicals that are routinely used for health or medicinal benefits and included in the list provided by FSSAI and 51 powdered herbal mixes were collected for evaluating the presence of aflatoxins, ochratoxins, and fumonisins. Methods described by the AOAC and published validated protocols were used for the mycotoxin analysis. ELISA, HPLC, and fluorimetry methods were utilized.

Results

- ***The occurrence of aflatoxins in herbal samples:*** From a total of 35 samples of 15 types of botanicals, aflatoxin was detected in 33 samples at levels ranging from 1.2-40 µg/kg (Table1). Maximum number, as well as high aflatoxin levels exceeding 20 µg/kg, were detected in Daru haldi, Brahmi, Bhojpatra, Amla seeds, and dry ginger and also in Ratan jot and Haldi samples. Among the powdered herbal mixes, aflatoxins were detected in 40 of the 51 samples at levels ranging from 1.0-25.0 µg/kg. The highest aflatoxin level of 47 µg/kg was detected in Triphala churn sample.

- ***The occurrence of ochratoxin A and fumonisin B1 in herbal samples:*** A total of 56 herbal mixes were subjected to Ochratoxin A and fumonisin analysis. Ochratoxin A was observed in 16% (9/56) of the samples while fumonisin was not detected in any of the samples analyzed. A high ochratoxin A level of 210 µg/kg was observed in Brahmi churn sample.

Table 1. The occurrence of aflatoxins in herbal/ botanical samples

S.No	Sample		No. collected	No. +ve	Level of Aflatoxins (µg/kg)
	Whole/raw material				
	Local name	Botanical name			
1	Daru Haldi	Berberis aristata	4	4	6.5-30.0
3	Brahmi	Bacopa monnieri	6	6	20.0-35.0
4	Bhojpatra	Betula utilis	4	3	1.2-31
5	Amla seeds	Emblicaofficianalis	4	4	6-26.0
6	Dry ginger	Zingiber officinale	6	6	27-40
7	HaritakiKarakaya	Terminalia chebula	2	1	12.0
8	Black cumin	Nigella sativa	4	4	11-25
10	Chira root	Achyranthus aspera	1	1	12.0
11	Lodra bark	SymplocosracemosaRoxb	1	1	4.0
12	Jungle kuff	Pithecellobium dulce	1	1	14
13	Ratan jot	Alkanna tinctoria	1	1	24
14	Haldi	Curcuma longa	1	1	25
	Powders				
15	Triphala churn	Terminalia chebula Emblicaofficianalis Terminalia bellirica	18	17	2.0-47.0
16	Amblicachurnam	Emblicaofficianalis	5		
17	Lodhra bark (powder)	SymplocosracemosaRoxb	6	3	3.2-16.0
18	Dry ginger powder	Zingiber officinale	6	5	1.0-25.0
19	HaritakiKarakayachurnam	Terminalia chebula	8	8	2.0-15.0
20	Brahmi churnam	Bacopa monnieri	8	7	1.0-8.9
	Total		86	73	

Table 2. Ochratoxin and fumonisin levels in herbal samples

Sample	No.analysed	No.+ve	Ochratoxin A levels (µg/kg)	Fumonisin B1 (µg/kg)
Brahmi churnam	9	2	11-210	ND
Lodhra bark powder	6	2	14-21	ND
Triphalachurnam	11	1	6.4	ND
Amla powder	6	3	8.1-10	ND
Ashwagandha powder	1	1	18	ND
Yastimadhuchurnam/ Licorice	8	ND	-	ND
Dry ginger powder	6	ND	-	ND
Haritaki/Karakayachurnam	8	ND	-	ND
Bhojpatra	1	ND	-	ND
Total	56	9	-	ND

Conclusions

- From a total of 86 herbal samples analyzed, the majority contained aflatoxins (85%), 18.8% contained ochratoxin A and none showed the presence of fumonisins.
- Herbal mixes/powders may be at high risk as high aflatoxin, as well as ochratoxin A, was detected in these samples.
- Finding the presence of ochratoxin A in some samples may present health concerns as these toxins have been shown to cause toxic effects on the kidney.

The study indicated that the potential for many botanicals that are being consumed for therapeutic or health benefits to getting contaminated with mycotoxins through the use of contaminated raw materials in their preparation.

4. Assessment of children who are helping their parents in agricultural farms of their own and monitoring their health and the health of their mothers with respect to exposure to pesticides

Occupational exposure to pesticides may pose a threat to the health ofthe exposed population directly. The majority of the population (56.11%) in India is engaged in agriculture and farmers are exposed directly to a variety of pesticides. In India, women contribute a large proportion of the farmers and are considered as an important force in the agricultural sector. Further, farm children are often considered as the most vulnerable population to a variety of environmental contaminants.

Though the information is available on the toxicity of pesticides to the farmers engaged in agricultural activities, there is a paucity of information on the toxicity of pesticides in women and children who are working for their farms in India. Therefore, the present study was aimed to study the possible adverse health effects due to pesticide exposure among these vulnerable groups. Further, the study also aimed to study the impact of micronutrient supplementation among children through intervention studies.

Objectives

- To analyze the pesticide residues in the blood samples of farm women and farm children (exposed group) and control group.
- To evaluate the micronutrient status (fat-soluble vitamins viz., Vitamin A, D, and E) and Minerals (viz., Calcium, Copper, Zinc, Manganese, and Magnesium) among both the groups.
- To analyze the various biochemical parameters viz., acetylcholinesterase inhibition activity, oxidative stress parameters. in both the groups.
- To analyze the immunological parameters (by measuring CD3+, CD4+, CD8+, CD16+ and CD19+) in the blood samples of both the groups.
- To determine the levels of reproductive hormone levels viz., Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), Estrogen and Testosterone in the blood samples of both the groups.
- To assess the effect of intervention with micronutrient supplementation due to exposure to pesticides in the farm children on various biochemical parameters in exposed group subjects.

Methodology

Study Design: Phase 1: Cross-sectional study;
Phase 2: Post-intervention after supplementation with micronutrients (Follow-up study).

Phase 1:Cross-sectional study

Sampling: Children of two age groups (9-12 years, n=66 and 13-15 years, n=63) and their mothers (age group 24-45 years, n=129), who are involved in various agricultural farming activities were selected in the exposed group while the control group consisted of children of the same age groups (9-12 years, n=69 and 13-15 years, n=65), and their mothers (n=134) who were never involved in any farming activities.

Study area: Ranga Reddy District, Telangana.

Inclusion criteria: Subjects in the exposed group were engaged in farming activities (pesticide spraying). While the control group subjects should not be involved in any kind of farming activities.

***Exclusion criteria*:** Subjects who are known to suffer from any kind of chronic disease/illness (viz., Asthma, COPD, Diabetes, Cancer, and HIV/AIDS) or with any physical disability are exempted from the study in both exposed and control subjects. Farm women and control subjects who are pregnant and who have attained menopause are excluded from the study.

Questionnaire: A pre-tested questionnaire was administered to the selected subjects to collect the required information on the demographic particulars of the subjects, types of farming tasks, as well as chemicals, compounds, quantities that are used, handling of pesticides, duration of the pesticide exposure, knowledge about the entry of pesticides, storage and disposal of pesticide containers, any associated morbidity symptoms, etc.

***Blood sample collection and analysis*:** After explaining the details of the study and its importance to the subjects, consent was taken from the subjects for drawing the blood samples (about 8 mL) for analysis of various biochemical parameters to fulfill the objectives of the study. In the case of children, consent was taken from the respective parents before the withdrawal of the blood samples.

The blood samples were processed to estimate the commonly used organophosphorus (OPs) pesticide residues (viz., chlorpyrifos, diazinon, dichlorvos, dimethoate, malathion, monocrotophos, phosalone, phorate, and quinalphos). A baseline data was obtained from the selected/identified children of both the age groups by analyzing the micronutrient status i.e., levels of vitamin A, E, D3, calcium, copper, zinc, magnesium, and manganese. Further, Acetylcholinesterase (AChE), catalase (CAT), superoxide dismutase (SOD) activity, malondialdehyde (MDA), and reduced glutathione and immunological parameters i.e., levels of CD cell markers and hormone levels were also studied.

Phase 2: Post-intervention

Based on the baseline results obtained in the farm children of both age groups (phase 1), supplementation with micronutrients was done to the farm children (9-12 years, n=54 and 13-15 years, n=56) and blood samples were collected from them, 30 days after the supplementation and further subjected to analysis for biochemical parameters viz., pesticide residue levels, micronutrient status, AChE activity, oxidative stress levels, levels of the CD marker and hormone levels to assess the impact of the intervention.

Results

Women

- The analysis for pesticide residues showed four OP residues i.e., Chlorpyrifos (range: 1.11 – 7.82 ng/mL), Diazinon (0.51 – 1.53 ng/mL), Malathion (0.51 – 1.74 ng/mL) and Monocrotophos (0.59 – 1.46 ng/mL) among the farm women (FW). However, no pesticide residues were detected among the women subjects of the control group.
- With regard to the micronutrient status, significantly low levels of vitamins A and magnesium were found in the FW as compared to the control groups ($p< 0.05$). Further, the correlation analysis was found to have shown significance with the residues of monocrotophos and low levels of zinc ($p< 0.05$).
- While the AChE levels were also found to have shown a decrease among the FW as compared

to the control subjects. The oxidative stress parameters viz., CAT, SOD, and MDA levels showed a significant increase while a significant decrease in reduced glutathione levels was observed among the FW as compared to the controls (p<0.01). Further, a significant correlation was also observed between the residues of diazinon and CAT. With regard to the micronutrients, a significant correlation was found between low levels of vitamin D & SOD, and zinc, calcium & CAT in the FW.

- With respect to the CD cell marker levels, significant suppression of the CD4+, CD8+, and CD16+ cells and an increase in the CD19+ was found among the FW as compared to the controls (p< 0.01). Further, the correlation analysis was found to have shown a significant correlation between the residues of chlorpyrifos & CD8+ and monocrotophos & CD19+. Similar significant correlation was noticed with respect to vitamin D levels & CD3+, copper & CD8+ and calcium & CD19+ cell marker (p< 0.05 and p< 0.01).
- A significant decrease was observed in the FSH (follicular phase) and LH (follicular and luteal phase) levels among the FW (p< 0.01). There was also a significant correlation observed between the detected levels of Chlorpyrifos residues and estradiol. In addition, it was also observed that significant correlation was found in the levels of micronutrients viz., vitamin D, & LH, copper & FSH and estradiol, zinc & FSH and LH, and magnesium & testosterone (p< 0.05 and p< 0.01).

Children

- The farm children (FC) of 9-12 years were detected with five OP residues viz., Chlorpyrifos (0.504 – 0.890 ng/mL), Diazinon (0.507 - 0.594 ng/mL), Malathion (0.505 – 1.35 ng/mL), Monocrotophos (0.502 – 0.679 ng/mL) and Phosalone (0.515 – 0.588 ng/mL). The FC of 13-15 years age group were also detected with Chlorpyrifos (0.506 – 1.92 ng/mL), Diazinon (0.511 - 0.924 ng/mL), Malathion (0.503 - 0.623 ng/mL), Monocrotophos (0.504 – 1.63 ng/mL) and Phosalone (0.537 – 1.55 ng/mL). While, the children of control groups (both age groups), were detected with no pesticide residues.
- The micronutrient analysis among the 9-12 years FC showed low levels of vitamin E and copper (p< 0.05), while the 13-15 years showed low levels of vitamin E, copper, manganese, and magnesium in the FC as compared to the controls (p< 0.01). Further, a significant correlation was also found between the residues of chlorpyrifos & calcium, malathion & manganese, and monocrotophos& zinc among 9-12 years of FC. Similarly, a significant correlation was found between the residues of chlorpyrifos, diazinon & low levels of vitamin A, phosalone& vitamin D, and monocrotophos& magnesium levels (p< 0.05) among the 13-15 years FC.
- Interestingly, AChE levels were found to be significantly decreased in both the age groups of FC as compared to the controls (p< 0.05 and p< 0.01). While no such significant difference was found in the oxidative stress parameters in children of both age groups. Significant correlation was also observed between malathion & AChE in 9-12 years and phosalone& CAT in 13-15 years FC (p< 0.05). A significant correlation was observed between low levels of vitamin D & CAT, reduced glutathione levels among 9-12years and phosalone & CAT, and calcium & CAT among the 13-15 years FC (p< 0.05 and p< 0.01).
- With regard to the levels of CD cell markers, a significant decrease in CD4+ levels was observed among the 9-12 years FC while there was a significant decrease in CD8+ among 13-15 years FC as compared to the controls (p< 0.05). It was observed that there was a significant correlation between malathion & monocrotophos residues and CD4+ and, monocrotophos & CD8+ among the 13-15years FC (p< 0.05). Similarly, significant correlation was observed between the levels of copper & CD16+ and zinc & CD19+ among 9-12 years FC; while significant correlation was observed between vitamin A & CD3+, CD16+, and levels of calcium, copper & CD16+ cell markers among the 13-15 years FC (p< 0.05 and p< 0.01).
- However, no significant alterations were observed with respect to the hormone levels among the children of both genders and age groups.

Impact of intervention with micronutrient supplementation among the farm children

The levels of vitamin E, copper, magnesium, and zinc (both age groups) and manganese (13-15 years) were significantly increased among the children supplemented with micronutrients ($p< 0.01$). In addition, a significant improvement in the enzymatic activities like AChE and decrease in lipid peroxidation (both age groups), increase in the CAT levels in 13 - 15 years age group children was also found among the post-supplemented children ($p< 0.01$). Two pesticide residues viz., chlorpyrifos and diazinon were detected among 26 children (out of 110 children) of 9-12 years (chlorpyrifos, n=13, 0.564-0.902 ng/mL and diazinon, n=2, 0.575-0.616 ng/mL) and 13-15 years (chlorpyrifos, n=11, 0.504-0.890 ng/mL and diazinon, n=1, 0.511 ng/mL) age groups after post-supplementation. It was also observed that no statistically significant difference was found in the percentages of CD cell markers among children of both age groups.

Conclusions

- The results of the present study among the FW found to have shown that the residues of OPs detected in the blood samples collected might have led to alterations in the AChE levels, oxidative stress parameters, CD cell markers, and hormone levels in combination with poor micronutrient status.
- Similar observations were made among the FC, with respect to a decrease in the levels of vitamins and minerals, alterations in the levels of AChE activity, and CD markers, except in the oxidative stress parameters and hormone levels.
- Supplementation with micronutrients found to have shown beneficiary effects such as enhancing the metabolism/excretion of residues in the body and also improvement in the biochemical parameters among farm children except among 26 children of both age groups which could have been due to their susceptibility in the retention of the residues in the body either due to poor/less excretion and or low metabolic rate suggesting detailed and more investigations among such susceptible individuals/ children are needed. This study, it shows that nutrition plays a vital role in ameliorating the adverse effects due to pesticide exposure among children.

5. Assessment of chemical contaminants in fresh/ packaged/ bottled tender coconut water

Coconut crops are considered as one of the important oilseed crops and had gained a lot of economic importance for people living in the coastal regions of tropical and sub-tropical countries. On many of occasions, the coconut trees and crops are prone to attacks and diseases due to a variety of pests resulting in low quality and production. Of the several *sps*., infesting the coconut crops, Red Palm weevil, Black-headed caterpillar and *Rhynocerossps*., are very important. The most common practice of controlling the pests are the application of pesticides and the other modern methods of practices are endotherapy of the trees through infusion or systemic injection of pesticides into the trunks of the trees for the better translocation of the pesticides into all the parts of the plant. In agriculture, endotherapy techniques have become popular and predominantly used successful practices for the coconut plants in addition to their use for the several other tree *spp.* like apple, pea, cherry, avocado, palm, and grapevine crops. As India is yet to evolve standards or limits for the tender coconut water; the present preliminary cross-sectional data results will be useful in facilitating the regulatory authority viz., Food Safety Standards Authority of India (FSSAI) in partially arriving at fixing up of the limits.

Objectives

- To administer the questionnaire on the types of the pesticides that are applied on to the coconut crops from Kerala, Tamilnadu, and Andhra Pradesh, India.

- To assess the extent of contamination with pesticide residues and heavy metals in fresh/ packaged/ bottled tender coconut water using GC/LC-MS-MS/AAS.

Methodology

Study Design: The cross-sectional study was carried out in three states, to achieve specific aims and objectives of the proposed study.

Stage 1. Sample collection from the identified locations situated in Kerala, Tamil Nadu, and Andhra Pradesh (A.P.) states.

The pre-tested questionnaire was administered to obtain information from the farmers to obtain the information on the varieties of coconut crops being cultivated, types of chemicals that are applied to the coconut crops, the extent of landholding, types of Good Agricultural Practices that are adopted while during the application of pesticides, duration of the crop, yields, types of infestation and diseases that the crops are prone to and the types and quantity of pesticides used, duration of their application, etc. Simultaneously, the processing units which are processing and packaging the tender coconut water in different kinds of materials (pouches/ tetra packs/ polypropylene containers) for sale/ consumption were also visited to obtain the information on the procurement of tender nuts/ transportation to the respective processing units/ methods of processing, etc.

The total number of samples for the study are as given below:

Fresh Tender Coconut Water:

2 varieties x 3 Locations x 7 Samples each = 42 X 3 identified States = 126 samples

Processed Tender Coconut Water:

2 varieties x 3 Brands x 7 Samples each = 42 X 3 identified States 126 samples

Total no. of samples = 126 + 126 = 252 samples

Stage 2. Sample analyses for the presence of pesticide residues, heavy metals, and minerals.

Both fresh/packaged tender coconut water samples collected were stored using necessary cold storage facilities as per the SOPs to avoid bacterial contamination and enzymatic degradation immediately after their collection. Subsequently, the samples were extracted using SOPs and clean-up procedures for further analyses using LC-MS/MS for the detection of pesticide residues. Similarly, minerals such as Copper (Cu), Zinc (Zn), and the heavy metals viz., Lead (Pb), Chromium (Cr), Arsenic (As), Stannum/Tin (Sn), Cadmium (Cd), Cobalt (Co), Mercury(Hg)analyses were also taken up using the ICP-OES. Analytical methods were standardized in the laboratory in fresh/processed tender coconut water samples before they are analyzed to fulfill the objectives of the project.

Results

Questionnaire data results

- A survey was carried out among 156 coconut crop cultivators in the East Godavari district of A.P. State (60) and Palakkad district of Kerala State(49) and Tanjavur and Polachi of Tamilnadu (47) using a pre-tested questionnaire.
- Information on the educational status of the farmers revealed that, in AP 5 (8.3%), KL 9 (18.4%), and TN 9 (19.1%) respectively, were reported to be illiterates. While, in AP 20 (33.3%), KL 17 (34.7%) and TN 14 (29.8%) completed primary and secondary education, further, in AP 35 (58.9%) KL 23(46.9%), and TN 24(51%) farmers were found to have completed higher education.
- The extent of landholding by the farmers in the cultivation of coconut crops below 10 acres in AP was 50 (83.2%) while they were 10 (16.6%) in case of above 10 acres. with 30 years of average experience. As regards, Kerala, the farmers with 28.3 years of average experience were cultivating the coconut crops below and above 10 acres were 30 (61.2%) and 10 (19%)

respectively. While in TN the farmers with 26.3 years experience in the cultivation of coconut crop were 36 (76.6%) for below 10 acres and 11 (23.4%) for above 10 acres.

- The farmers in all the three States who were engaged in the cultivation of coconut crop were for about a period of 36.5 (AP), 35.3 (KL), and 31.3 (TN) years respectively. While the average harvesting period of the crop was found to be 6 times (60 days), 7-8 times (45-50 days), and 7-12 times (40-50 days) per year for AP, KL, and TN respectively. However, the time interval between the spray and harvest was 40 days in AP and 30-35 days in TN, while there was no such mention by the farmers from KL, as they have reported to be not using any pesticides. The average number of coconut plants planted per acre was found to be 59.6 (AP) 68 (KL) and 70 (TN), respectively.
- The coconut crop varieties that are being cultivated vary from each village/mandal/state. It was found that there are about nine cultivars viz., East Coast Tall (ECT), Ganga Bondam (GB), Godavari Ganga (GG), Philippine Ordinary (PO), Cochin China (CC), Chowghat Orange Dwarf (COD), Malayan Yellow Dwarf (MYD), Yellow Dwarf (YD) and Orange Dwarf (OD) are majorly grown in AP. The majority of the collected samples for the present study belong to MYD,COD, GB, GG, ECT, and OD cultivars which were majorly grown in the respective areas of sample collection. In the KL state, thirteen varieties of tender coconut crops viz., West Coast Tall (WCT), Malayan Yellow Dwarf (MYD), Chowghat Dwarf Orange (COD), Tall X Dwarf (T X D), Ganga Gathram (GGt), Yellow Dwarf (YD), Green Dwarf (GD), WCT small, DJ orange, DJ green, Orange Dwarf (OD), Malayan Orange Dwarf (MOD), Chowghat Dwarf Green (CDG) and Keera Ganga (KG) are being cultivated. These varieties were also found to be popularly grown in the TN and KL state along with West Coast Tall (WCT), Dwarf X Tall (DxT), Ganga Gathram (GGt), Chowghat Dwarf Green (CDG Dwarf X Talk (DxT), and East Cost Tall (ECT).
- It was also found that in AP out of 60 farmers only 8 (13.3%) of farmers were using pesticides, while in the case of TN out of 47 farmers 12 (25.5%) were using pesticides. However, in KL none of the farmers reported having used pesticides.
- The frequency of application in case of their use to control the infestation on to the coconut crop, types of fertilizers used, cropping period, specific period of spraying the pesticides, no. of times of the crop in a year, at which stage (flowering/fruit/nut) of the crop, the application of pesticides is done, the number of sprays in a year, the time interval between the spray and the harvest and the time interval given between the last spray and the harvest, the most popular/common varieties of coconuts cultivated, etc. was obtained. The Application of pesticides on coconut crop was done at both flowering and nut stage by one farmer (1.7%) and 7 (11.7%) were using at the nut stage in AP, while, in TN 4 (8.5%) of the farmers were using at flowering stage, 2 (4.2%) at both flowering and Nut stages, and only 6 (12%) were using at Nut stage.
- The mode of administration in AP was found to be through root by 7 (11.7%) and root+ spraying by 1 (1.7%) farmer/s for about 7 – 8 hours/day, and only 1 – 2 days/per month in a year. In the case of TN, the pesticides through root system were done by 6 (12.8%) farmers, 4 (8.5%) through Spraying, and 2 (4.3%) through both Root + spraying mode for about 5 – 6 hours/day in 2 days/month in 2-6 months/year.
- A total of 12 farmers were using pesticides in Tamilnadu. Of them, 6 were using through the root system. About five farmers were using only Monocrotophos @ 10mL/tree, while one is using Tridemorph @ 2mL/tree.
- Four are using through spraying: One is spraying only Triazophos, @ 2ml/L; oneis using both Triazophos + Nematocide @ 2ml/L each; one is using only Nematocide @ 2ml/L and one farmer is using Profenophos and Imidacloprid (2ml each/L) respectively.
- Two are using both root administration and spraying @10 ml each of Monocrotophos and Nematocide.

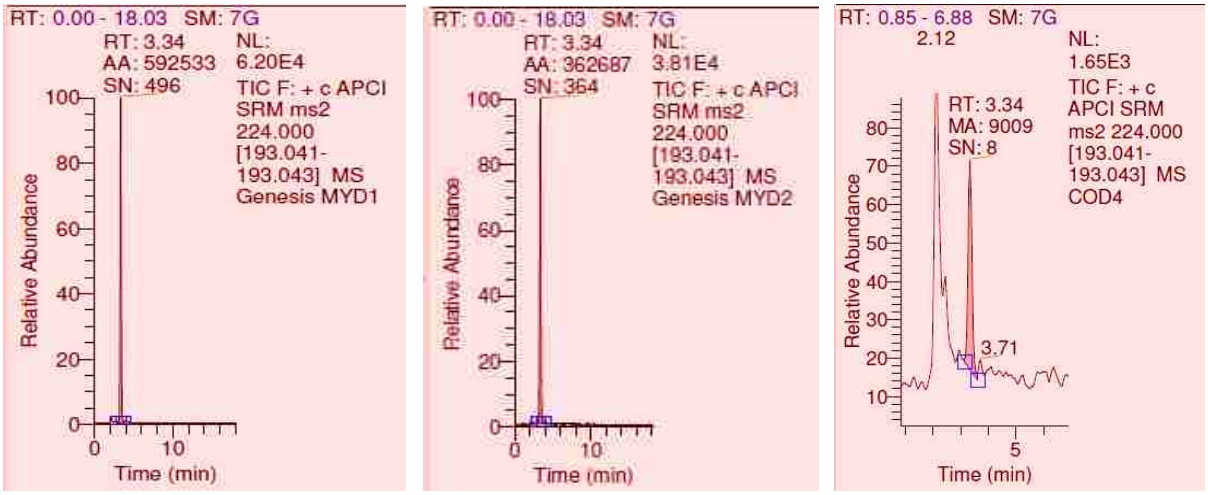
- In A.P., administration of pesticides was done through root system: Out of the 7 farmers, 3 farmers are using only Monocrotophos (@2 mL//tree), one farmer is using Monocrotophos (@2 mL/tree), and Phorate (@ 30g/tree), one is using Monocrotophos (@ 2 mL) , Phorate (@ 30g) and Chlorpyrifos (@ 2 mL) in combination and 2 are using only Phorate (@ 30g).
- Administration through Root+Spraying: Only one farmer is using Monocrotophos (@ 2mL), Phorate (@ 30g) and Chlorpyrifos (@ 2mL) in combination.
- Information on the use of protective devices during pesticides application, if any revealed that all the 8 farmers (7 were using Gloves + Mask + Shoe while one farmer were using (Gloves + Mask + Shoe + Apron) in A.P. Out of 12 farmers in T.N.,11 were using PPEs in different combinations (7 were using Gloves + Mask, 3 were using gloves and one was using mask only), while one was not using any PPEs.
- Details on precautions taken while during/after handling pesticides from the farmers it was found to be 7 (11.7%) were washing hands, whereas one farmer was washing hands, face, mouth and also changing dress after using pesticides in A.P. In T.N. 2 (4.3%) were washing hands, 8 (17%) were washing hands and also changing dress whileone farmer was washing hands, face, mouth and changing dress.

Results of analysis of pesticide residues in fresh/ packaged/ bottled tender coconut water samples:

- Pesticide residues were analyzed in 161 fresh and 126 samples of packaged tender coconut water, collected from A.P., Tamilnadu, and Kerala states.
- Out of the 127 samples of fresh tender coconut water collected from A.P. and Kerala, none of the samples were detected with pesticide residues.
- However, out of the 34 samples collected from Tamilnadu, 4 samples of fresh tender coconut water (one sample of Tall x Dwarf [TxD]; two samples of Malayan Dwarf, and one sample of Chowghat Orange Dwarf [COD]) were detected with the residues of Monocrotophos in the range of 1-51.5 ng/mL, while five samples (three samples of Chowghat Orange Dwarf [COD] and two samples of Tall x Dwarf [TxD]) were detected with Malathion residues in the range of 0.5-0.6ng/mL (Fig 1).
- With respect to the packaged tender coconut water, one sample was detected with Monocrotophos @ a concentration of 0.9ng/mL, while the other four samples (from Tamilnadu were detected with the residues of Malathion at a range of 0.8 to 1.56 ng/mL concentration (Fig 2).

Fig 1. Spectra showing the presence of pesticide residues in fresh coconut water samples collected from Tamil Nadu

Monocrotophos:



Malathion

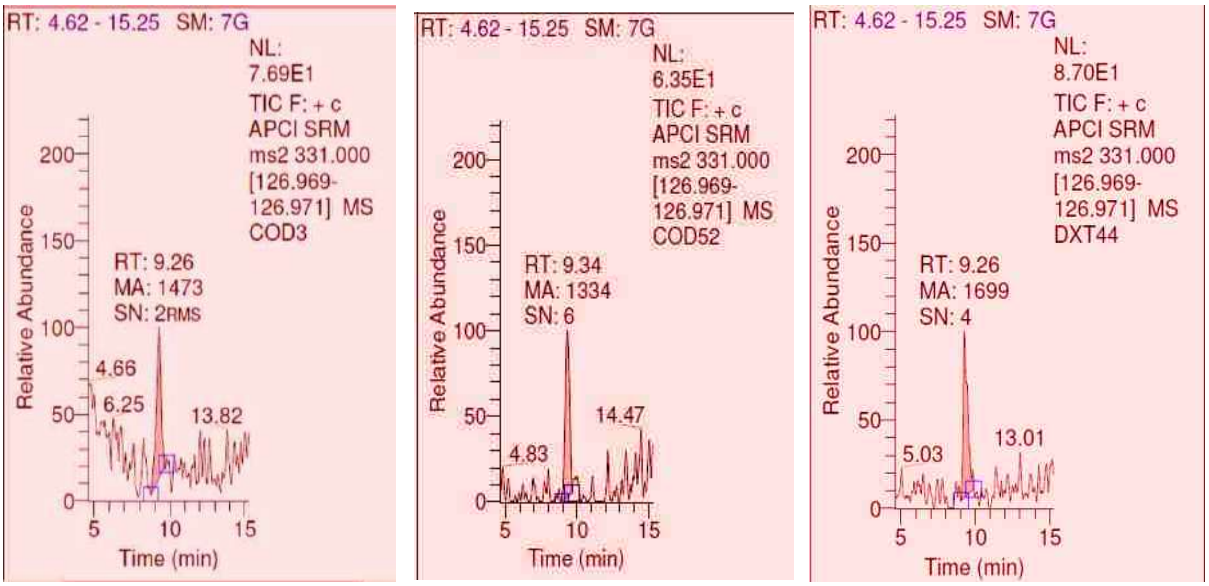
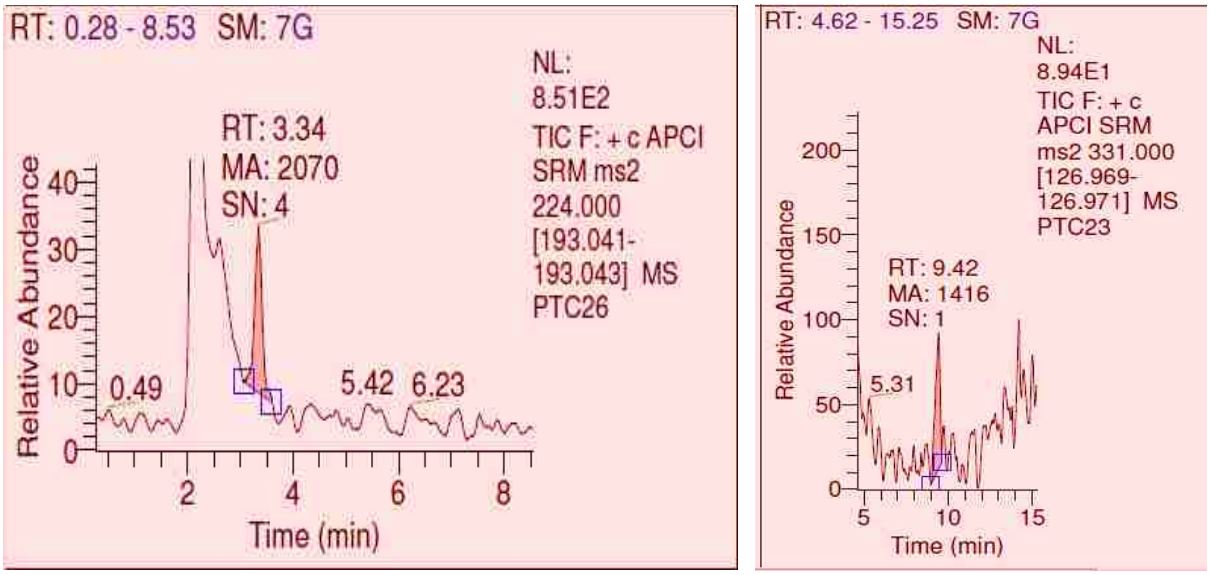


Fig 2. Spectra showing the presence of pesticide residues in packaged coconut water Samples.

Monocrotophos Malathion



Results of analysis of heavy metals/ minerals in fresh/ packaged/ bottled tender coconut water samples

- About 126 samples of fresh and about 126 samples of packaged tender coconut water were analyzed for minerals/ heavy metals viz., Cu, Zn, Pb, Cr, As, Sn, Cd, Co, and Hg.
- The heavy metals such as Cd, Co, Cr, Pb,Sn were detected in six varieties of fresh tender coconut water samples viz., East Cost Tall(ECT), Ganga Bondam, Malayan Yellow Dwarf (MYD), Orange Dwarf, Godawari Ganga, Chowghat Orange Dwarf (COD)collected from A.P., while they were detected in five varieties such as ECT, COD, and Dwarf x Tall (D x T), Chowghat Green Dwarf (CGD) and Malayan Yellow Dwarf (MYD) collected from Tamilnadu respectively. With respect to the samples collected from Kerala were found to have been detected in six varieties viz., West Coast Tall (WCT), COD, MYD, D x T, Ganga Gathramand Godavari Ganga.

- Arsenic was detected in one sample of Godavari Ganga collected from Moolathara village of Kerala State. None other samples of fresh tender coconut water were found to have been contaminated with Hg.
- With respect to the twelve brands of packaged tender coconut water samples collected from A.p., Kerala and Tamilnadu also contained traces of heavy metals viz., Cd, C, Co, and Pb while As and Hg were not detected in any of the packaged/bottled tender coconut water samples.
- The Mean and SD were calculated for both fresh/ packaged tender coconut water samples. The levels of heavy metals were found to be varying in different varieties of fresh tender coconut water samples collected from three different states. It is observed that the average levels of Cd, Cr, Pb, and Sn present in fresh tender coconut water samples were higher in Tamilnadu (TN) as compared to Andhra Pradesh (A.P.) and Kerala (K), while the average levels of Cobalt are higher in AP as compared to K and TN. With respect to minerals, the levels of Zn are higher in K as compared to AP & TN, while the average levels of Cu are higher in AP & TN as compared to K.
- With respect to the heavy metal concentration in fresh tender coconut water in Andhrapradesh, it was found that the average values for cadmium are higher in Ganga Banda, while for Cobalt they were higher in Chowghat Orange Dwarf and for Chromium, lead and stannum they were higher in Godavari Ganga, Orange Dwarf and East Coast Tall respectively.
- In Tamil Nadu, the average values of Cobalt were higher in Malayan Orange Dwarf, and in East, CoastTall Chromium was higher and, the cadmium, lead and stannum were higher in Chowghat Orange Dwarf.
- As regards Kerala the average values of cadmium were higher in Ganga Gathram, Chowghat Orange Dwarf, and Godavari Ganga, whereas Cobalt and Chromium were higher in Dwarf x Tall and Ganga Gathram respectively, and for lead and stannum they were higher in West Coast Tall.
- ANOVA was performed to find out the significant difference between the varieties of fresh tender coconut water samples collected from three different States. The results showed that there is a significant difference in all the heavy metals analyzed (Cd, Co, Cr, Pb, Sn & Zn).
- Based on the ANOVA results (F-value), Post Hoc Test (LSD-Least significant difference) was performed to find the significant difference between the varieties of fresh tender coconut water samples collected from three different states. With regards to Cd concentration, there was found to be a significant difference between samples collected from TN & AP, TN & K. Similarly, with regard to Co concentration, a significant difference was observed between AP & KL, AP & TN. Further, Cr levels were significantly different between KL & TN. With regard to Pb and Sn levels, there was a significant difference between AP and TN, K and TN.
- With respect to the concentration of Zn, there is a significant difference between AP and K, AP and TN. However, there was no statistically significant difference among the three states in the concentration of Cu.
- Furthermore, with respect to the heavy metal concentrations in packaged tender coconut water, they're found to be a significant difference in the levels of Cd, Cr, and Sn among the different varieties of samples collected (Table 2). Zinc levels were also found to be significantly different among the various packaged tender coconut water samples (Table 2). However, no significant difference was found statistically in the levels of heavy metals viz., Co and Pb and mineral.

Table 1. Heavy metals and minerals (ppm) detected in different varieties of FTW samples collected from Andhra Pradesh, Kerala, and Tamil Nadu states

State	Cd		Co		Cr		Pb		Sn		Zn		Cu	
	N	Mean ±SD	N	Mean ±SD	N	Mean ±SD	N	Mean ±SD	N	Mean ±SD	N	Mean ±SD	N	Mean ±SD
AP ($^{\#}$N=42)	42	0.0027 ±0.0009	26	0.0007 ± 0.0004	42	0.0306 ± 0.0196	40	0.0093 ± 0.0052	42	0.0161 ± 0.0077	42	0.2633 ± 0.0757	40	0.0778 ± 0.0560
KL ($^{\#}$N=42)	42	0.0024 ±0.0006	5	0.0002 ± 0.0001	41	0.0253 ± 0.0066	42	0.0114 ± 0.0044	41	0.0173 ± 0.0085	42	0.3504 ± 0.1007	42	0.0733 ± 0.0230
TN ($^{\#}$N=42)	42	0.0034 ± 0.0009	13	0.0002 ± 0.0001	42	0.0356 ± 0.0129	42	0.0148 ± 0.0081	42	0.0504 ± 0.0269	42	0.3217 ± 0.0908	42	0.0778 ± 0.0447
ANOVA (F-value)	15.35 *		9.43 *		5.47 *		8.16 *		55.08 *		10.27 *		0.149	
Comparison between states. (LSD)	AP vs TN * KL vs TN * (Significant)		AP vs KL * AP vs TN * (Significant)		KL vs TN * (Significant)		AP vs TN * KL vs TN * (Significant)		AP vs TN * KL vs TN * (Significant)		AP vs KL * AP vs TN * (Significant)			

ND = Not Detected
$^{\#}$N = Total number of samples analyzed
N = No. of samples detected with heavy metals
*** = (P< 0.05 - Significance)**

Table2: Heavy Metals and minerals (ppm) detected in different varieties of PTW samples

Brand	Cd		Co		Cr		Pb		Sn		Zn		Cu	
	N	Mean ± SD	N	Mean± SD	N	Mean ± SD	N	Mean± SD	N	Mean± SD	N	Mean± SD	N	Mean± SD
Cocoma (#N=15)	15	0.0018 ± 0.0003	0	ND	15	0.0120± 0.0033	6	0.0083± 0.0044	15	0.0087± 0.0030	15	0.0058± 0.0032	13	0.0324± 0.0183
Coconad (#N=12)	12	0.0013 ± 0.0003	3	0.0001± 0.0001	12	0.0041± 0.0019	0	ND	12	0.0040± 0.0018	12	0.3018± 0.3153	0	ND
True coco (#N=10)	10	0.0011 ± 0.0002	0	ND	9	0.0080± 0.0014	1	0.0060	10	0.0500± 0.0188	10	0.0949± 0.0407	5	0.0242± 0.0047
Real active (#N=21)	20	0.0014 ± 0.0003	3	0.0001± 0.0011	21	0.0076± 0.0033	6	0.0024± 0.0018	21	0.0286± 0.0205	21	0.6305± 0.6265	17	0.0094± 0.0043
Treo (#N=15)	14	0.0012 ± 0.0004	1	0.0002	12	0.0168± 0.0030	1	0.0073	15	0.0073± 0.0024	15	0.0985± 0.0420	10	0.0247± 0.0198
Madhurac ocofresh (#N=9)	9	0.0023± 0.0003	0	ND	8	0.0143± 0.0009	3	0.0056± 0.0027	9	0.0275± 0.02519	9	0.1558± 0.0575	7	0.0138± 0.0021
Fresh (#N=11)	10	0.0018 ± 0.0001	0	ND	10	0.0057± 0.0017	3	0.0055± 0.0019	11	0.0041± 0.0004	11	0.3198± 0.1267	11	0.0290± 0.0016
Coco dew (#N=11)	11	0.0016 ± 0.0002	0	ND	8	0.0171± 0.0008	3	0.0048± 0.0009	11	0.0205± 0.0041	11	0.3375± 0.4310	10	0.0174± 0.0009
Cocojal (#N=3)	3	0.0024 ± 0.0003	0	ND	3	0.0168± 0.0021	2	0.0079± 0.0003	3	0.0292± 0.0342	3	0.0863± 0.0028	3	0.0091± 0.0003
Tender coco (#N=3)	3	0.0009 ± 0.00005	0	ND	3	0.0030± 0.0007	0	ND	3	0.0037 ± 0.0002	3	0.8208± 0.1136	0	ND
Siponut (#N=10)	7	0.0025 ± 0.0001	1	0.0005	9	0.0123± 0.0018	1	0.0082	10	0.0194 ± 0.0040	10	0.0200± 0.0009	9	0.0213± 0.0054

Brand	Cd		Co		Cr		Pb		Sn		Zn		Cu	
	N	Mean ± SD	N	Mean± SD	N	Mean ± SD	N	Mean± SD	N	Mean± SD	N	Mean± SD	N	Mean± SD
Mojoco (#N=6)	5	0.0016 ± 0.0001	0	ND	6	0.0131± 0.0023	1	0.0072	6	0.0330 ± 0.0154		0.5448± 0.3153	5	0.0549 ± 0.0204
ANOVA (F- value)	21.17 *		2.38		33.51 *		1.72		11.51*		6.20*		10.13	
Comparsion between states. (LSD)	Cocomavscoconad* CocomavsTruecoco* CocomavsRealactive* Cocomavs Treo* CocomavsMadhurecocofresh* CocomavsCocojal* CocomavsTendercoco* CocomavsSiponut* CoconadvsMadhurecocofresh* Coconadvs Fresh* CoconadvsCocodew* CoconadvsCocojal* CoconadvsTendercoco* CoconadvsSiponut* TruecocovsRealactive* TruecocovsMadhurecocofresh* TruecocovsFresh* TruecocovsCocodew* TruecocovsCocojal* TruecocovsSiponut* TruecocovsMojoco* RealactivevsMadhurecocofresh* Realactivevs Fresh * RealactivevsCocojal* RealactivevsTendercoco* RealactivevsSiponut* Treo vsMadhurecocofresh* Treo vs Fresh* Treo vsCocodew* Treo vsCocojal* Treo vsSiponut* Treo vsMojoco* Madhurecocofreshvs Fresh * MadhurecocofreshvsCocodew*	-	Cocomavscoconad* CocomavsTruecoco* CocomavsRealactive* Cocomavs Treo* CocomavsMadhurecocofresh* Cocomavs Fresh* CocomavsCocodew* CocomavsCocojal* CocomavsTendercoco* CoconadvsTruecoco* CoconadvsRealactive* Coconadvs Treo* CoconadvsMadhurecocofresh* CoconadvsCocodew* CoconadvsCocojal* CoconadvsSiponut* CoconadvsMojoco* Truecocovs Treo* TruecocovsMadhurecocofresh* Truecocovs Fresh* TruecocovsCocodew* TruecocovsCocojal* TruecocovsTendercoco* TruecocovsSiponut* TruecocovsMojoco* Realactivevs Treo* RealactivevsMadhurecocofresh* Realactivevs Fresh * RealactivevsCocodew* RealactivevsCocojal* RealactivevsTendercoco* RealactivevsSiponut* RealactivevsMojoco* Treo vsMadhurecocofresh*	-	CocomavsTruecoco* CocomavsRealactive* CocomavsMadhurecocofresh* CocomavsCocodew* CocomavsCocojal* CocomavsMojoco* CoconadvsTruecoco* CoconadvsRealactive* CoconadvsMadhurecocofresh* CoconadvsCocodew* CoconadvsCocojal* CoconadvsSiponut* CoconadvsMojoco* TruecocovsRealactive* Truecocovs Treo* TruecocovsMadhurecocofresh* Truecocovs Fresh* TruecocovsCocodew* TruecocovsCocojal* TruecocovsTendercoco* TruecocovsSiponut* TruecocovsMojoco* Relativevs Treo* Realactivevs Fresh * RealactivevsTendercoco* Treo vsMadhurecocofresh* Treo vsCocodew* Treo vsCocojal* Treo vsSiponut* Treo vsMojoco* Madhurecocofreshvs Fresh* MadhurecocofreshvsTendercoco* Fresh vsCocodew*	Cocomavscoconad * CocomavsTruecoco* CocomavsRealactive* CocomavsCocodew* CocomavsCocojal * CocomavsSiponut * CoconadvsTruecoco* Coconadvs Fresh* CoconadvsCocojal* CoconadvsSiponut* TruecocovsRealactive* Truecocovs Treo* TruecocovsMadhurecocofresh* Truecocovs Fresh* TruecocovsCocodew* TruecocovsTendercoco* TruecocovsMojoco* RealactivevsMadhurecocofresh* Realactivevs Fresh* RealactivevsCocojal* RealactivevsSiponut* Treo vsCocojal* Treo vsSiponut* MadhurecocofreshvsCocojal* Fresh vsCocodew* Fresh vsCocojal* CocodewvsCocojal* CocodewvsSiponut* CocojalvsTendercoco* CocojalvsMojoco* TendercocovsSiponut* SiponutvsMojoco*	

Brand	Cd		Co		Cr		Pb		Sn		Zn		Cu	
	N	Mean ± SD	N	Mean± SD	N	Mean ± SD	N	Mean± SD	N	Mean± SD	N	Mean± SD	N	Mean± SD
	MadhurecocofreshvsTendercoco* MadhurecocofreshvsMojoco* Fresh vsCocojal* Fresh vsTendercoco* Fresh vsSiponut* CocodewvsCocojal* CocodewvsTendercoco* CocodewvsSiponut* CocodewvsMojoco* CocojalvsTendercoco* CocojalvsMojoco* TendercocovsSiponut* TendercocovsMojoco* SiponutvsMojoco* (Significant)				Treo vs Fresh* Treo vsTendercoco* Treo vsSiponut* Treo vsMojoco* Madhurecocofreshvs Fresh* MadhurecocofreshvsCocodew* MadhurecocofreshvsCocojal* MadhurecocofreshvsTendercoco* Fresh vsCocodew* Fresh vsCocojal* Fresh vsSiponut* Fresh vsMojoco* CocodewvsTendercoco* CocodewvsSiponut* CocodewvsMojoco* CocojalvsTendercoco* CocojalvsSiponut* CocojalvsMojoco* TendercocovsSiponut* TendercocovsMojoco* (Significant)			Fresh vsCocojal* Fresh vsSiponut* Fresh vsMojoco* CocojalvsTendercoco* TendercocovsMojoco* (Significant)		(Significant)			

ND = Not Detected
#N = Total number of samples analyzed
N=No. of samples detected with heavy metals
*** = (P< 0.05 - Significance)**
- = Post hoc test not performed as one group has lesser than two cases.

Conclusion

Pesticide residues viz., Monocrotophos and Malathion were detected in the fresh/ packaged/ bottled tender coconut water samples collected from Tamilnadu State. Heavy metals were detected in both fresh and packaged/ bottled tender coconut water samples. Arsenic was detected in one sample of fresh tender coconut water collected from one of the villages of Kerala State.

VIII. DRUG TOXICOLOGY

1. Evaluation of traditional plants (*cocculushirsutus, cuscutareflexa & tinosporacordifolia)* as immunomodulatory and anti-inflammatory agents

The medicinal values of rarely used plants viz. *i.Cocculushirsutus, ii.Cuscutareflexa, iii.Tinosporacordifolia* being investigated in the current study. As there is a description in traditional literature and one of the plants has a history of use as food. The ancient Ayurvedic literature (Bhavprakashnigantu) has described this plant in rasayana (antioxidant), balya (energy & immune promotion). The combination of *Cuscutareflexa and Tinosporacordifolia* with *Cocculushirsutus* were taken as which is consumed like a green leafy vegetable and evaluated its anti-oxidant and immunity potential.

In the present study, the ethanobotanical and phytochemical profiles are being documented along with *in-vitro,* anti-oxidant, and immune potential in the various extract of the above-mentioned plants.

Results

- ***Ethnobotanical, phytochemical composition:*** The certification of the plant sample confirming the plant species deposited to BSICuscutareflexa (Acc.no.116103), *Cocculushirsutus* (Acc. No.11522), *Tinosporacordifolia* (Acc.no.11524*),* The chromatogram of HPLC, LC-MS have demonstrated the presence of the following in Bergenine, Chlorogenic acid & Ferulic acid in Cuscutareflexa, Protocatechuic acid in Cocculushirsutsu & Tinosporacordifolia
- The free radicals scavenging activity (antioxidant) is evaluated by DPPH assay. The IC50 for antioxidant activity has been established in comparison to Vitamin C. The IC50 values were found to be in a range of 24.5 to 357.55 ug/ml. The potential sequence of inhibition activity is: CR_HA>CR_W>CR_A>CHW>CH_HA>CH_A>TCW>TCHA>TC_A.
- The oxidative stress inhibition activity was found to be in the range of 14.3 to 42.91% in various extracts as demonstrated by LPS stimulated RAW 264.7 macrophage cell line. The potential sequence of inhibition activity is: CR_W (42.91%)>CH_W (40.71%)> CR_HA (33%)>CR_A (31%)>CH_HA=CH_A (25%)>TC_A (22.5%)>TC_W (22%)>TC_HA (14.3%).
- The immunomodulatory activity has demonstrated in different plant extracts CR_W(118%) >CR_HA(89%)>CH_W(87%)>TC_W(85%)>TC_A(82%)>CH_A(74%)>CH_HA= TC_HA(72%) as evaluated by spleenocyte proliferation assay.
- In-vitro Th1/Th2 polarization of all eleven plant extracts showed Th1(increased TNF-alpha & IFN-gamma; decreased IL-4 & IL-10 response) mediated response suggesting immune-stimulating activity.

Conclusion

The water extract of *cuscutareflexa* has shown the potential antioxidant immuno-modulatory activity as compared to other plant extracts.

2. Translation of traditional formulation c*ynodondactylon* (*swarasa of durva)* in menopausal syndrome complication (*rajonivrittijanya-vikritavvastha*) - A reverse pharmacology approach (part - 1)

In the recent past, the incidence of menopausal syndrome (MS) disorder is reported to be increasing and it is estimated to be in 103 million populations by 2026**.** The main symptoms of MS are hot flushes, night sweating, palpitation, insomnia, irritation, hormonal imbalance, etc. Similarly, in classical ayurvedic literature *lakshanas* of *Rajonivrittijanyavikritavastha*are equivalent to described MS complications. The existing intervention strategy includes hormonal therapy (HRT),SERMs (Selective estrogen receptor modulators), Sedative, Hypnotics, bisphosphonates, calcitonin etc. These treatments have limitations therefore use of alternatives like traditional ayurvedic formulations/herbals (Shatawari, *Madhuyasti, Ashwagandha)* can be validated as potential safe effective agents. The current investigation to validate the claims based on the pilot clinical trial with Durva (CynodondactylonLinn.pers.) in *Rajonivrittijanyavikritavastha* (MS). The study objectives include validation of the potential therapeutic activity of Durva juice.

The study has been undertaken with a reverse pharmacology approach to validate the claims of the pilot clinical study. The methodology includes *in-vitro, ex-in-vivo, in-vivo* evaluations after confirming the phytochemical profile of the Durva. The results of the study are as follows:

- ***Ethanobotanical, phytochemical composition:*** The Certification of the plant sample confirming the plant species deposited to BSI (BSI/DRC/17-18/Tech./212, dated 19-06-2017) The chromatogram have demonstrated the presence of 51 Components out of which Ferulic acid, 4-Coumaric acid, Catechin, etc., are abundant ranging around 5%.
- In view of the potential therapeutic benefit identified in the pilot experiment with swarasadurva prepared following traditional and other related concepts. Therefore, the formulation process has been informed in camera to patent as 'ICMR-NIN-MS01/19' (ICMR-PRG recommendations).
- ***In-vitro:*** ICMR-NIN-MS01/ 19 has the potential free radical scavenging activity (62.6%) as compared to Vit C (80%). In the Osteoblast SaOS -2 cells incubated with test compound have shown high proliferation, significant differentiation in osteoblast cells with an increase level of calcium and phosphorus. It was comparable to the standard drug (Sodium alendronate).
- ***Ex-in-vivo:*** ICMR-NIN-MS01/ 19 have reduced the pro-inflammatory cytokine IL-1β, TNFα in concanavalin induces proliferated spleen cells.

Conclusion

The ethanobotanical validations have confirmed the species *'swetadurva'*. The developed formulation coded as 'ICMR-NIN-MS01/19' has demonstrated potential anti-oxidant, anti-osteoporotic, anti-inflammatory (pro-inflammatory marker) activities.

IX. EXTENSION AND TRAINING

1. Edutainment: An approach to inculcate actionable knowledge to promote healthy lifestyle and nutrition

Production of a short film to promote healthy diet and hygiene behavior.

Objectives

- To educate the target audience through entertainment mode (short film) to inculcate actionable knowledge to consume a healthy diet and hygiene behavior.
- To play the short film during extension lecturers and to the visitor's groups of NIN.
- In addition, it is also proposed to telecast in television channels repeatedly.

Work done during the year

Completed the production of the short film and shared the same with major television channels with a request to telecast this short film frequently as their Corporate Social Responsibility (CSR) free of cost**.** Television channels *SAKSHI*, TNN, T-SAT (*Nipuna*), T-SAT (*Vidya*), and *MAA* TV have periodically repeated the telecast of this short film free of cost. In addition, efforts are in progress to screen this short film free of cost in movie theatres before the feature film begins.

2. Celebrities' campaign through mass media to combat anaemia among women and adolescents

Production of a short film to promote healthy dietary habits among adolescent girls and women in general and mother and infants in particular.

Objectives

- To create awareness among adolescent girls, pregnant and lactating women about the importance of nutrition for complete health. To enlighten pregnant and lactating women on the following issues:
- Importance of 1000 days nutrition for complete health, with illustrations. LBW, Iron-rich foods, etc.
- Importance of exclusive breast milk to infants up to 6 months of age. Continuation of breastmilk up to 6 months of age in addition to supplementary foods to the infants from age 6 months to 2 years.
- Promotion of micro-nutrient foods (millets, fruits and vegetables, etc).
- Dos and Don'ts for pregnant and lactating mothers.
- These messages through short films would be endorsed by celebrities.
- This short film is intended to play during the extension lectures of NIN and to telecast on television channels.

Work done during the year

Completed the production of the short film and shared the same with major television channels with a request to telecast this short film frequently as their Corporate Social Responsibility (CSR) free of cost. Television channels *SAKSHI*, TNN, T-SAT (*Nipuna*), T-SAT (*Vidya*), and *MAA* TV haveperiodically repeated the telecast of this short film free of cost. In addition, efforts are in progress to screen this short film free of cost in movie theatres before the feature film begins.

3. Development and validation of a comprehensive index for assessing food safety at household level

Foodborne illnesses are a widespread public health problem globally. Diarrhea is a common symptom of foodborne illnesses. Diarrhoeal diseases cause about 11% of child deaths worldwide. A great proportion of these cases can be attributed to contamination of food and drinking water. Studies show that a significant proportion of foodborne illnesses arises from practices in home kitchens. In order to understand the challenges to food safety at the household (HH) level, it is worthwhile to understand the aspects that typically compromise food safety. In India, ensuring food safety is being increasingly recognized as an important part of public health. However, a majority of foodborne diseases in India go unreported, unrecognized, or uninvestigated. At the household level, various factors that affect food safety may include - knowledge, practices, enabling environment, and awareness. These factors are likely to be different in rural and urban settings as they, in turn, rely on a number of the socio-economic, cultural, and contextual factors. Among these, the location of the domestic kitchen, availability of safe water, refrigeration, and type of cooking fuel also play an important role. Although there are a few research studies on the factors affecting food safety, the incidence of diarrhea, and identifying causative bacterial pathogens for diarrhea among children in India, to date there is no validated comprehensive index to assess food safety at the household level. Such an index, if developed could help in setting goals and taking up suitable strategies to address the gaps in knowledge, practices, and enabling environment and assess the progress in measurable terms. Therefore, the present study attempted to develop, validate and disseminate a comprehensive Household Food Safety Index (HFSI) to assess food safety at the household level.

Objectives

- To review literature for a comprehensive view on assessing the social, cultural, economic, and behavioral factors that affect food safety at the household level.
- To develop and validate a comprehensive index for assessing food safety at the household level.
- To assess differences, if any, among rural and urban households by using the food safety index in food safety-related perception, practices, and reasons thereof.
- To identify critical issues for food safety at the household level and develop key messages for promoting food safety advocacy.

Methodology

This was a cross-sectional study conducted among primary food preparers in both urban (Hyderabad) and rural (Ranga Reddy district) homes of Telangana. For the development of the Household food safety index (HFSI), subjects (N=400) were selected @200 each from rural and urban areas. An 87-item pre-tested questionnaire covering knowledge (43), practices (36), and enabling assets (8) was administered on subjects. Besides, demographic profile, food safety risk

perception, the incidence of food/ water borne diseases also collected. Scores were assigned for responses and the maximum possible score was 205. In addition, at consumption point @400 each stored cooked food, drinking water samples, and hand rinses were collected from all subjects for microbial analysis (USFDA-BAM). Validation of 87 variables' scores was done against the high-risk food borne pathogen (*Salmonella* spp.) risk value (1.55logCFU/g) in homemade foods. Validated HFSI was administered on 200 subjects selected from different areas of rural and urban (@100each) in Telangana. Developed key messages were tested for efficacy on 120 subjects from the slum, urban and rural (@40each) home settings Three types of educational materials were developed - powerpoint presentations, pamphlets, and videos in three languages (Telugu, Hindi, and English) with key messages.

Salient findings

- The major percentage of food borne disease incidence was observed in U5C age among children below 5 years of age.
- Homemade foods and drinking water samples collected at the point of consumption showed a higher percentage of fecal coliforms and other food borne pathogens.
- The majority of households followed poor hand hygiene practices as evidenced by their hand washing practices and higher loads of food borne pathogens on their hands.
- From the 87 item index questionnaire, an 11- item HFSI was developed with specific food safety practices and Enabling assets by correlating with high-risk food borne pathogen (*Salmonella* spp.) contamination loads in homemade foods (Fig 1).
- Rural households have shown significantly poor scores and a higher percentage of microbial contamination in stored cooked foods than urban households.
- Five critical issues were identified related to hand hygiene, cross-contamination, drinking water at the point of contamination, maintenance of the domestic kitchen, and washing of raw fruits and vegetables. To address these issues 5 key messages were developed.
- Educational intervention with the developed material with key messages for promoting household food safety has shown a significant positive change in the HFSI scores (Fig 2) with a more pronounced impact among rural and slum households as well as in subjects who were illiterates and had below secondary education. Proving that the educational intervention was effective and efficacious.

Fig 1. Principal component Analysis - plotted loadings of 11 index parameters (>0.5)

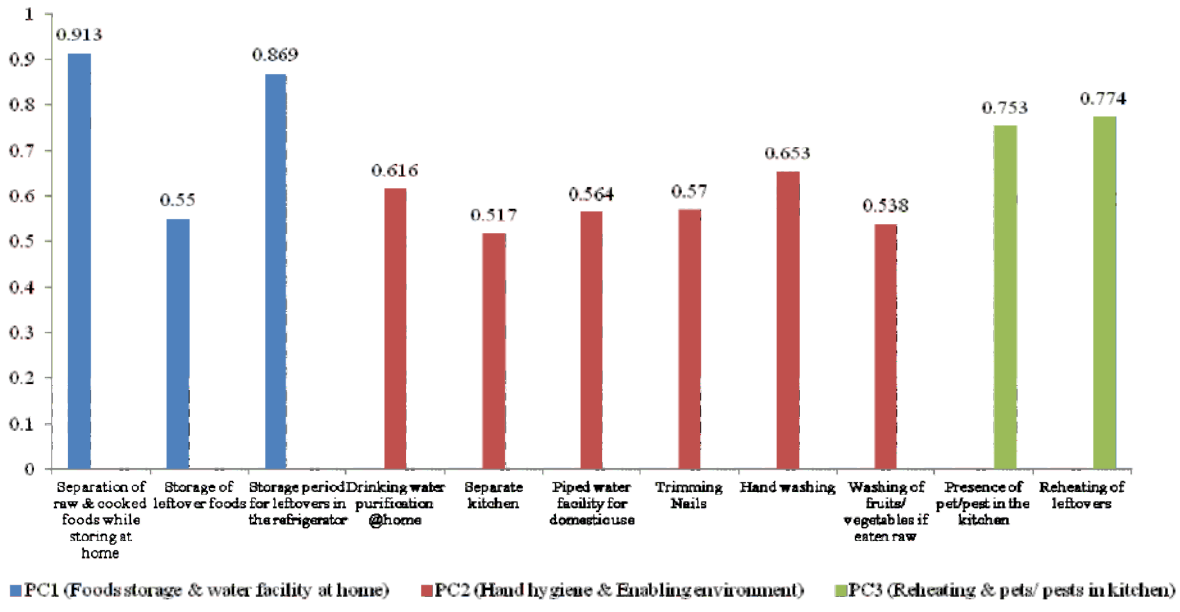


Fig 2. Percentage of participants who followed proper food safety practices based on HHFS Index at baseline and post-intervention (p<0.001)

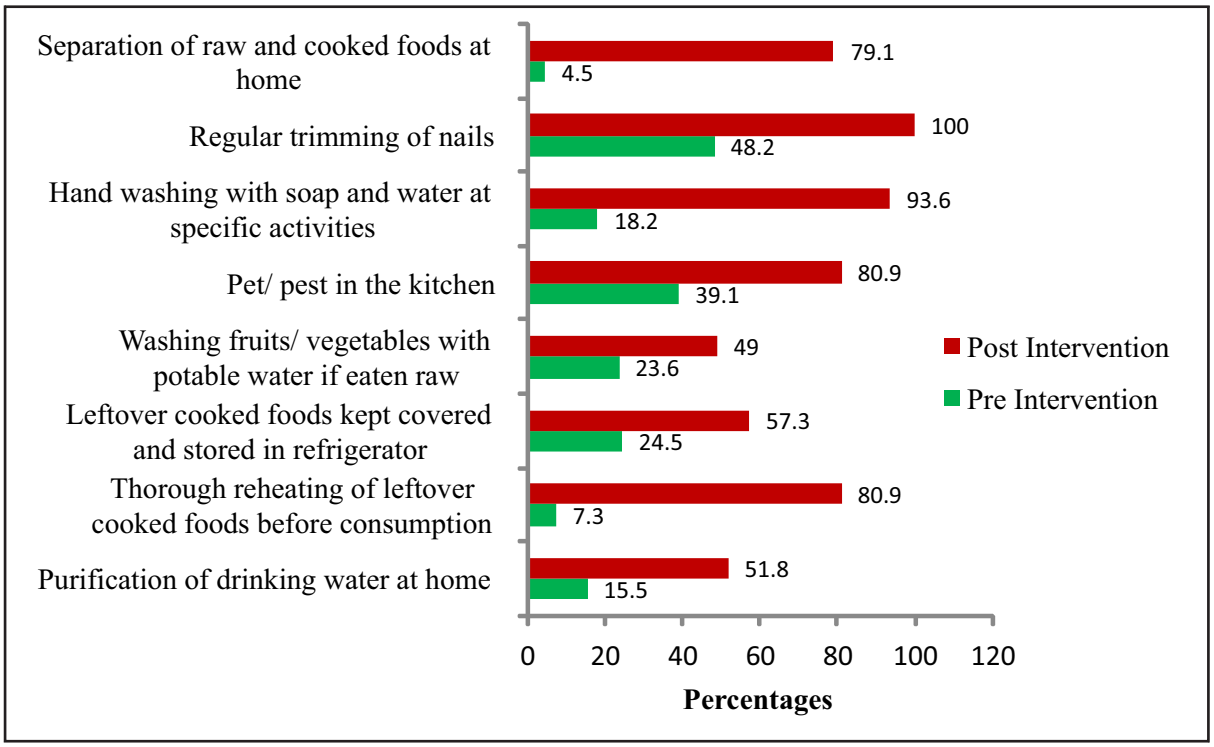


Fig 3. Five keys for food safety - Sample of education material which was brought out in English, Hindi, and Telugu

X. PRE-CLINICAL TOXICOLOGY RESEARCH CENTRE

1. Pre-clinical safety evaluation of oryzanol

Oryzanol is a class of non-saponifiable lipids of rice bran oil (RBO). More specifically, oryzanol is a group of ferulic acid esters of triterpene alcohol and plant sterols. The quality and quantity of dietary fat are known to play a crucial role in plasma lipid concentration. Hence dietary manipulation is known to play an important role in the management of hyperlipidemia-associated obesity and coronary heart disease.

Therefore, this project was proposed to have a systematic study of use of oryzanol as cholesterol-lowering agent by using standard efficacy models and safety studies. As a part of this, the preclinical efficacy profile of the product is completed and the current report is for the Safety investigation.

Methodology

Acute toxicity study in Sprague Dawley (SD) rats and subchronic toxicity study in SD Rats has been conducted. The therapeutic dosage schedule for rat has been calculated based on the intended human dosage – 300mg/70kg man.

Acute toxicity study in SD rats

The study has been conducted in 10 Rats (5 males + 5 females) after 7 days of conditioning period test compound administration was done in High Dose [Oryzanol powder (54mg)] orally through gavage. The animals were observed for mortality and activity for 14 days. The live phase of animals, cage side observation, physical, physiological and neurological parameters were recorded at regular intervals. This is followed by necropsy and the collection of all vital organs.

Subchronic toxicity study in SD rats

The study has been conducted in 40 Sprague Dawley Rats (20M+20F), approximately 6 - 8 weeks old, weighing (150-180gm) after 7 days of conditioning the rats were randomly divided into four groups viz., i. Vehicle Control ii. 1X, iii. 2X and iv. 5X. As a part of a routine examination, all animals were subjected to qualitative urine analysis. This was followed by administration of test material in various concentrations (1X-5.4 mg/1ml, 2X-10.8 mg/1ml, and 5X-27 mg/1ml) by oral gavage daily once for 90 days to respective groups as per SOP. The animals were observed daily for mortality and physical activity. Live phase, cage side, physiological, neurological observations were monitored bi-weekly in all animals till the end of the experiment. The feed intake was quantified and recorded biweekly. Body weights were recorded biweekly. The urine analysis (qualitatively) was monitored pre and post-exposure to the test material followed by the collection of blood samples on the 92nd day of study to evaluate clinical chemistry, hematology, and serum total IgM, IgA, and IgE profile, and all animals were euthanized to conduct gross necropsy and histopathological observations of all vital organs.

Conclusion

Acute toxicity study in SD rats

The Oral administration of Oryzanol powder dissolved in 0.5% CMC at a concentration of 270mg/kg rat which was 10 times higher than the Intended human dose did not show any adverse effect on any of the parameters studied. There was no mortality recorded.

Subchronic toxicity study in SD rats

There was no mortality in any group of animals that received Oryzanol powder at three dosage levels for 90 consecutive days. There was no significant difference in body weights, feed intake, and cage side activities. The clinical chemistry and hematology profile werein the normal range. Serum total IgE, IgM, and IgA levels were not altered in animals and were comparable with control. There were no allergenicity symptoms in the animals. Changes of histological significance were observed in the lungs, liver, and kidneys of both VC and experimental animals. As these changes were observed in the vehicle control group also, it may not be possible to attribute the changes in the experimental group which received the test compound.

2. Preclinical comparative efficacy evaluation of ING010 (Recombinant insulin glargine) with standard drug

M/S Stelis Biopharma Private Limited, Bangalore has developed a product ING010 (long-acting insulin glargine) by recombinant DNA technology. It consists of microcrystals that slowly release insulin, giving a long duration of action of 18 to 24 hours, with a "peakless" profile. The present study was undertaken to evaluate the efficacy of ING010 (recombinant insulin glargine) in comparison with the standard drug (Lantus) in Wistar rats.

Objective

- To compare the efficacy of ING010 (recombinant insulin glargine) with standard drug (Lantus).

Methodology

The study was conducted in Wistar rats (36M+36F) as per the standard schedule Y and OECD guidelines after obtaining IAEC approval (P27F/III-IAEC/NIN/12/2016). The diabetic model was developed by giving STZ. The animals were randomized into 3 different groups and a study was carried out. The ING010 compound has shown significant results which were similar to the standard compound.

Result

- The AUC confirms the bio-similarity between the ING010 and Lantus.

Conclusion

- The test compound has shown the bio-similarity with the standard compound.

3. Preclinical safety evaluation of ADMSCS, ADMSCS pre-conditioned with snap, SDF-1 alpha and SNAP + SDF-1 alpha for therapeutic applications in duchenne muscular dystrophy (DMD)

Duchenne muscular dystrophy (DMD), an X-linked lethal disorder that affects 1 in 3600 male births, is the most prevalent form of muscular dystrophy. DMD is caused by genetic mutations in the dystrophin gene at Xp21, resulting in the absence of this protein in muscle. Dystrophin is a component of the Dystrophin-associated glycoprotein complex and links the muscle fiber cytoskeleton to the extracellular matrix. A promising approach to the treatment of DMD is to restore dystrophin expression by repairing the defective muscle through cell therapy.

Previous studies have suggested that hematopoietic stem cells can contribute to skeletal muscle regeneration. Parent Project Muscular Dystrophy (PPMD) team, isolated mesenchymal stem cells from different donor tissues and found that adipose tissue is the best source of mesenchymal stem cells as they mainly proliferate into muscle cells to form healthy muscle fibers. The present study was conducted to evaluate the tolerability and acute safety of ADMSCs, ADMSCs pre-conditioned with SNAP, ADMSCs pre-conditioned with SDF-1 alpha, and ADMSCs pre-conditioned with SNAP+SDF-1alpha according to the regulatory guidelines approved by Schedule 'Y' of Drugs and Cosmetics Act, 1940 and Rules, 1945 and evaluated by Expert Committee for Stem Cell.

Methodology

Tolerability study in SA Mice and Acute toxicity study in SD Rats and NZW Rabbits has been conducted. The therapeutic dosage schedule for mice, rat, and rabbits has been calculated based on the intended human dose – 250 million cells/person.

Tolerability study in SA Mice

The Mice with normal health reports have been conditioned for 18 days in the experimental room. The conditioned animals were randomized and grouped into five groups viz., i. Vehicle control, ii. *ADMSCs,* iii. *ADMSCs* +SNAP iv. *ADMSCs + SDF-1 alpha and v. ADMSCs + SNAP + SDF-1 alpha*. This was followed by test compound administration to a respective group of animals with the concentration of 0.5 million cells in 0.2ml /mouse as a single dose. The maximum volume of administration is 0.2ml/mice. Animals were observed daily for 14 days after exposure to the test compound. At the end of the experiment, all animals were euthanized and their organs were collected for a gross necropsy. In case of pre-terminal death, an autopsy was conducted to collect the vital organs for histopathological examination.

An acute safety study in SD Rats (IV & IM Routs)

The SD Rats with normal health reports have been conditioned for 9 days in the experimental room. The conditioned animals were randomized and grouped into two groups viz., i. ADMSCs, ii. ADMSCs + SNAP + SDF-1 alpha. This was followed by test compound administration to a respective group of animals with the concentration of 2.8 million cells in 0.5ml /Rat as a single dose. The maximum volume of administration is 0.5ml/rat.Post-exposure, the animals were observed for activity and mortality for 14 days. In addition, bodyweight was recorded biweekly during the live phase. Cage-side observations and neurological activity were also monitored. At the end of the experiment blood samples were collected to evaluate the clinical chemistry and hematology parameters and all animals were euthanized to conduct gross necropsy of the major organs was performed along with weighing of the organs (liver, heart, lungs, testis, spleen, and brain). The autopsy was followed by histopathological evaluation only in the case of the dead animal during the study.

An acute safety study in NZW Rabbits (IV & IM Routs)

The Rabbits with normal health reports have been conditioned for 10 days in the experimental room. The conditioned animals were randomized and grouped into two groups viz., i. ADMSCs, ii. ADMSCs + SNAP + SDF-1 alpha. This was followed by test compound administration to a respective group of animals with the concentration of 11 million cells in 1ml / Rabbit as a single dose. The maximum volume of administration is 1ml/ Rabbit. Post-exposure, the animals were observed for activity and mortality for 14 days. In addition, body weights were recorded biweekly. Cage-side observations and neurological activity were also monitored. At the end of the experiment blood samples were collected to evaluate the clinical chemistry and hematology parameters and all animals were euthanized to conduct gross necropsy of the major organs was performed along with weighing of the organs (liver, heart, lungs, testis, spleen, and brain).

Conclusion

Tolerability study in SA Mice: The Intravenous administration of ADMSCs, ADMSCs + SNAP, ADMSCs + SDF-1 alpha & ADMSCs + SNAP + SDF-1alpha at a concentration of 0.5 million cells/ mouse which was 2.5 times of intended stem cells did not show any adverse effect on any of the parameters studied.

An acute safety study in SD Rats (IV & IM Routs): The Intravenous and Intramuscular administration of ADMSCs, ADMSCs + SNAP + SDF-1alpha at a concentration of 2.8 million cells/ rat which was 2.5 times of intended dose did not show any adverse effect on any of the parameters studied.

An acute safety study in NZW Rabbits (IV & IM Routs): The Intravenous and Intramuscular administration of ADMSCs, ADMSCs + SNAP + SDF-1alpha at a concentration of 11 million cells / rabbit which was 2.5 times of intended dose did not show any adverse effect on any of the parameters studied.

XI. ICMR-NIN ANIMAL FACILITY

The services of the NIN Animal Facility are primarily extended to inter institutional scientific activities in-spite of separating the NARFBR on recommendations of ICMR. Apart from this, the facility is extended to other public sector organizations on a collaborative mode as per the directions and regulations of ICMR. The ICMR-NIN Animal Facility is registered with CPCSEA (154/GO/RBiBt -S/R-L/1999/CPCSEA) and renewed up-to 2023.

1. ***Breeding and Supply of animals:*** During the period, a total 4,047 animals were bred and out of which 2,370 animals were supplied to various outside institutions and 103 animals supplied within the institute. An amount of Rs. 6,37,807/- (Rupees Six lakhs thirty seven thousand eight hundred and seven only) has been generated as sale proceedings (Annexure -I).

2. ***Supply of Animal Feed:*** Department prepared 9,125 kgs of feed (Rat and Mouse feed 7,833 kgs + Guinea pigs and Rabbit feed 1,142kgs; Gerbil feed 150 kgs) during the period. Out of this, a total of 1, 278 kgs of feed was supplied to outside institutions generating an amount of Rs. 5,60,330/-(Rupees Five lakhs sixty thousand and three hundred thirty only). An additional 7,847 kgs of feed (Rat & Mouse feed 6,555 kgs + Guinea Pigs & Rabbit feed 1,142 kgs + 150 kgs Gerbil feed) was also supplied within the institute. In addition, department also prepared 226 kgs of custom made experimental animal feed and supplied to outside institutions (Annexure -II).

3. ***Services extended to inter and intra institutions:*** The Department of Animal Facility has continued to extend the services for seeking the approvals from IAEC, space and related material for conduct of experimental work for inter institutional and intra collaborative projects from public and private sectors, the list of ongoing experiments is enclosed (Annexure -III). The services are also extended to evaluate physiological, pharmacological activities based on state of the art equipments (Annexure -IV).

Human Resource Development: The NIN Animal Facility is the only organization which has a potential to develop the human resources from the lab attendant to mid level supervisory. The syllabus and the training is provided accordingly. In the current year the following activities continued. In the current year a special program was conducted which is supported by UKIERI - Ministry of Skill Development and Entrepreneurship (MSDE), Government of India in collaboration with Drug Safety Division. This program has developed the skills among the middle level supervisory cadre for drug development which is need of the hour. The trainees of this training program are conducting similar type of program in other centre with a limited budget provided by UKIER- MSDE and NIN.

S.No	Title of Training course	Target group	Participants
1	Technicians training course on Experimental animals	10th / 12th classed passed	14
2	Laboratory animals Supervisors Course	Graduation in life sciences	14
3	Ad-Hoc training course on Experimental animals	Masters and Ph.D	20
4	World Laboratory Animal Day- 2019	Animal breeders, suppliers and users	250
5	Developing and sustaining India's capacity for pre-clinical Drug Discovery- Train the Trainer Course for developing human resources under MSDE - UKERI	Middle level research scientists from all over India	40

During this period an Ad-hoc training for 20 candidates belonging different organizations have been trained. The Department organized a one day Seminar on "Strengthening rational use of animals and animal welfare" on the occasion of the World Laboratory Animal Day on 30th April 2019 in association with ICMR, CPCSEA and Humane Society International India. The program is organized mainly to commemorate the sacrifices made by all animals that have improved health, environment and quality of life of every species. In addition, the program included with lectures by eminent personalities on the subjects of animal welfare. There were more than 210 delegates from private and government organizations have participated including CPCSEA nominees of IAEC from various institutions. During these celebrations some of the retired staff of NCLAS and members from local animal welfare organizations has been felicitated. In additions to the above, the department has conducted the first IAEC meeting and approved project proposals.

Table 1. Details of different species and strains of laboratory animals bred and supplied from NIN Animal Facility (January to March-2019)

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)	3405	821	4226	4	834	838	-	800	2588
		C57BL/6J (in bred)	230	99	329	3	31	34	11	-	284
		NIH (S) Nude (in bred)	284	128	412	-	-	-	48	-	364
		NCr. Nude	300	148	448	-	24	24	28	-	396
		FVB/N (in bred)	176	-	176	-	36	36	-	-	140
		Swiss (in bred)	2160	825	2985	3	203	206	20	700	2059
2	Gerbils	…									
3	G.Pig	Dunkin Hartley (white)	162	60	222	-	-	-	-	60	162
		NIH (Colour)	100	20	120	-	-	-	8	65	47
4	Rabbit	New Zealand (white)	54	44	98	20	27	47	8	-	43
Total			6871	2145	9016	30	1155	1185	123	1625	6083

Table 2. Details of different species and strains of laboratory animals bred and supplied from NIN Animal Facility
(January to March 2019)

Sl. No	Species	Strain or Breed	Stock as on	Total number of animals							Balance as on
				Bred during the period	Avail able	Supplied to NIN	Supplied to other institutions	Supplied Total	Died	Disp.	
1	Rat	CFY/NIN (inbred)	69	25	94	--	--	--	26	20	48
		Fischer 344 N (inbred)	180	90	270	--	72	72	--	100	98
		Holtzman (inbred)	83	--	83	--	--	--	18	20	45
		SD (Sprague Dawley)-Outbred	587	295	882	--	268	268	--	40	574
		Wkyoto (inbred)	126	21	147	--	30	30	28	30	59
		WNIN (inbred)	1595	1360	2955	22	789	811	7	165	1972
		WNIN / Gr-Ob	687	--	687	15	--	15	19	40	613
		WNIN / Ob-Ob (inbred)	647	71	718	36	--	36	4	92	586
		SD NIN Nude	133	--	133	--	--	--	40	30	63
2	Hamster	Golden (inbred)	570	40	610	--	56	56	35	160	359
3	Monkey	…									
Total			4677	1902	6579	73	1215	1288	177	697	4417

Table-1 (Total)	6871	2145	9016	30	1155	1185	123	1625	6083
Table-2 (Total)	4677	1902	6579	73	1215	1288	177	697	4417
GRAND TOTAL	11548	4047	15595	103	2370	2473	300	2322	10500

Annexure-III

IAEC Projects 2019: New Proposals

Sl. No.	Title of the project	Name of the Investigator	Funding	Species	Sex & No.		Age	Expected Date of Termination
					Female	Male		
1	Effect of maternal protein restriction (quantity and quality of protein) on protein quality control processes in the brain and muscle of the offspring P1F/IAEC-1/NIN/3/2019/VSR/SD/12F+6M	Dr.V.Sudhakar Reddy, Scientist – B, Biochemistry, ICMR-NIN, Hyderabad	NIN Intramural grant	Rats, SD	12	6	90 days old	4-5 Months
2	Protective effect of hesperidin and chlorogenic acid on fertility changes in obese female rats P6F/IAEC-1/NIN/3/2019/NH/WNIN/GR-Ob/24F	Dr. N. Harishankar Scientist 'E', NIN Animal Facility ICMR-NIN, Hyderabad.	NIN Intramural	WNIN/Gr-Ob	24	-	35 days	12 months
3	Pre clinical Safety evaluation of Formulations (NIN-1, NIN-2, NIN-3) P4F/IAEC-1/NIN/3/2019/BDK/SWISS/78F+78M/SD/78F+78M/SD/156F+156M	Dr. B. Dinesh Kumar Scientist "G" & Head Drug Toxicology Division & NIN Animal Facility ICMR-NIN, Hyderabad	ICMR-NIN	Mice, SA	78	78	4-6 weeks	14 days
				SD Rats	78	78	6-8 weeks	14 days
				SD Rats	156	156	6-8 weeks	43 days
4	Neurotoxicity effects of Lead (pb^{2+}) and amyloid peptide (AP) in altering the mitochondrial function in the Human Brain cells P15F/IAEC-1/NIN/3/2019/NA/SD/	Neelima.A C/o Dr.C.Suresh Scientist 'E', Biochemistry/Stem Cell Research NIN,Hyderabad	NIN-Intramural grant-16BS06	SD Rats	--	--	Weanling stage	02 months
5	Toxicity evaluation and assessment of Domoic Acid (DA) and its metabolites from marines fish in both in vitro and in vivo studies P17F/IAEC-1/NIN/3/2019/JSR/SD/24M	J.Sreenivasa Rao Scientist "D", Food Chemistry Division ICMR-NIN, Hyderabad	NIN-ICMR (project No: 16 FC-03)	SD Rats	---	24	90 days	4 years

Sl. No.	Title of the project	Name of the Investigator	Funding	Species	Sex & No.		Age	Expected Date of Termination
					Female	Male		
6	Efficacy studies on protein hydrolysates from safflower seed and validation of their utility in animal nutrition P7F/IAEC-1/NIN/3/2019/NH/WNIN/60F+60M	Dr. N. Harishankar Scientist 'E', NIN Animal Facility ICMR-NIN, Hyderabad.	NIN Intramural	WNIN wistar	60	60	6-8 weeks	12 months
7	Evaluation and role of isolated compound from Amla fruits on Volproic acid induced Autism Spectrum Disorder (ASD) in experimental Balb/c mice. P5F/IAEC-1/NIN/3/2019/SNN/SD/40F+20M/BALB/c/36F+36M	Dr.Sukesh Narayan Sinha Scientist 'F' Food and Drug Toxicology Research Centre ICMR-NIN, Hyderabad.	3/1/2/62/2014-15(Nut)	Mice and Balb/c	36	36	7 days pups	36 months
8	Maternal exposure of endocrine disrupter Bisphenol A: implication to development programming of glucose homeostasis and insulin resistance in the fetus. Project No: 18 BS 08 P3F/IAEC-1/NIN/3/2019/SB/SD/40F+20M	Dr.SanjayBasak Scientist – E Molecular Biology Division ICMR-NIN, Hyderabad.	DHR	Rats, Sprague Dawley	40	20	3 weeks old	36 Months
9	Role of free Fatty Acid receptors in modulating atherosclerosis in WNIN/Ob rats P8F/IAEC-1/NIN/3/2019/AG/WNIN&WNIN-Ob/36F+36M	Dr. Ajay Godwin P ICMR PDF NIN Animal Facility, Hyderabad	ICMR-PDF	WNIN and WNIN/Ob	36	36	3 months	02 months
10	Effect of zinc supplementation prior to iron on iron absorption, and iron status in deficient rats; in vitro and in vivo studies P14F/IAEC-1/NIN/3/2019/PR/NWZ/1M	Dr.P.Raghu , Scientist-E,Biochemistry, NIN Hyderabad	DBT	New Zealand Rabbits	--	1	3-4 months	15 days

Sl. No.	Title of the project	Name of the Investigator	Funding	Species	Sex & No.		Age	Expected Date of Termination
					Female	Male		
11	Effect of paternal calorie restriction of diet induced obese on metabolism of their offspring	Dr.K. Rajender Rao, Scientist E, NIN, Hyderabad						
12	Compositional Analysis and Pre clinical safety (Regulatory studies) evaluation of Spirulina nutritional Gummy P16F/IAEC-1/NIN/3/2019/BDK/SA/12F+12M/SD/12F+12M/SD/24F+24M	Dr. B. Dinesh Kumar Scientist "G" & Head Drug Toxicology Division& NIN Animal Facility ICMR-NIN, Hyderabad	National Institute of Transforming India (NITI Aayog)	Mice SA	12	12	4-6 weeks	6 months
				SD Rats	12	12	6-8 weeks	
				SD Rats	24	24	6-8 weeks	
13	Developing and sustaining India's capacity for pre-clinical Drug Discovery. P18F/IAEC-1/NIN/3/2019/BDK/SA/30F+30M/SD/30F+30M/NWZ/3F+3M/DH/3F+3M	Dr. B. Dinesh Kumar Scientist "G" & Head Drug Toxicology Division& NIN Animal Facility ICMR-NIN, Hyderabad	Ministry of Skill Development & Entrepreneurship (MSDE)	Mice (SA)	30	30	4-6 weeks	
				SD Rats	30	30	6-8 weeks	
				Rabbits (NWZ)	3	3	3-4 months	
				Guinea Pig (Dunkin-Hartley)	3	3	3-4 months	
14	Evaluation of standardized Aloevera extract for its Simultaneous anti-diabetic and anti-obesity potential in WNIN/Gr-OB Rats P20F/IAEC-1/NIN/3/2019/KVR/WNIN/42F	Dr. Krishnan Venkataraman Director, Centre for BioSeparation Technology (CBST), VIT, Vellore.	CBST, VIT, Vellore	WNIN Rats	42	--	6 months	2 Months
15	Molecular characterization of cancer stem cell in colorectal cancer P9F/IAEC-1/NIN/3/2019/SS/BALB/c/24F	Dr. Sandhya Singh C/o Prof Pallu Reddanna University of Hyderabad.	SB/YS/LS-181/2014	BALB/c Nude mouse	24	-	6-8 weeks	01month

Sl. No.	Title of the project	Name of the Investigator	Funding	Species	Sex & No.		Age	Expected Date of Termination
					Female	Male		
16	Novel nano and micro formulation of PRP for chronic would healing P10F/IAEC-1/NIN/3/2019/JG/SD/	Dr. JyotsnenduGiri Assistant Professor IIT Hyderabad, Kandi, Sangareddy	IIT Hyderabad,	SD Rats			8 weeks	3 years
17	Affordable, Effective Personalized Point of use wound care patches at patient bedside for large asymmetric burn wound P11F/IAEC-1/NIN/3/2019/JG/	Dr. JyotsnenduGiri Assistant Professor IIT Hyderabad, Kandi, Sangareddy	IIT Hyderabad,	Wistar Strain albino rats	96	96	5-8 Weeks	36 months
18	Extraction and characterization of mouse cardiac stem cells for cardiac tissue engineering application P12F/IAEC-1/NIN/3/2019/JG/	Dr. JyotsnenduGiri Assistant Professor IIT Hyderabad, Kandi, Sangareddy		Mouse			23-30 Days	2 months
19	Evolution of anticancer efficacy of drug loaded in Nanostructure Hybrid Lipid Capsules (nHLCs)	Dr. JyotsnenduGiri Assistant Professor IIT Hyderabad, Kandi, Sangareddy		Mice and Wistar Rat			5-8weeks	6 months
20	Design and development of customized 3D printed implants for craniomaxillofacialreconstruction P13F/IAEC-1/NIN/3/2019/FP/WNIN/24M	Dr.B.Sri Sai Ramya Research Sholar, Biomedical Engineering IIT Hyderabad, Kandi, Sangareddy	IIT Hyderabad,	WNIN Rats	--	24		2 months
21	Targeting Triple Negative Breast Cancer Cells using Surface Modified Solid Lipid Nanoparticles of Niclosamide P2F/IAEC-1/NIN/3/2019/PSSSK/C57BL/6J/24F	Mr.Pindiprolu Satya Sesha Sai Kiran Research Scholar Dept. of Pharmacology, JSS College of Pharmacy, Ootacamund	ICMR,Department of Health research	C57BL/6J MICE	24	-	3 weeks	18 days

Annexure-IV

Animal Physiology Lab Equipment Details

Instrument Name	Asset No.	Make
Grip Strength meter	SCH/AR Vol.VII (1)/ P-115/I-07/2008-09	TSE Systems
Total Body Electrical Conductivity (TOBEC) Model SA-3114		SA=3114
Rota Rod 3375-4R	FDTRC/ARV.No. III (2) P-84 /I -4	
Temperature Multiplexer	SCH/AR Vol.VII (1) /P-114/I-6/2008-09	Axiom biotek inc.
Centrifuge Universal 320 R	NCLAS/AR Vol V(2)/ p-101/I-02/11-12	Hettich
Oxy max Test Chamber System		Columbus Instruments
Non-Invasive BP apparatus ((IITC Life science)		IITC LIFE SCIENCE
Leica DMLB Microscope		LEICA
Activity Meter		Columbus Instruments
***In-vivo* imaging system (ivis)**	NIN/AR-VOL IX(2)/ P114/I-35/2011-12	Caliper life sciences
Digital plethysmometer	SCH/AR-VOL VII(1)/P111/I-1/2007-08	Pan lab

PARTICIPATION OF SCIENTISTS IN INTERNATIONAL MEETINGS/ CONFERENCES

SNo	Name of the Scientist	Name of the Meeting/ Conference
1.	Sh.T.Longvah Scientist G	39th Session of the Codex Committee on Methods of Analysis and Sampling held between 6 – 11 May 2018 at Budapest, Hungary as an Indian delegate
		High level expert seminar on Indigenous Food system at FAO Headquarters between 07 – 11 November 2018 at Italy, Rome.
		The IFPRI FAO global event on accelerating the end of hunger and malnutrition held between 28 – 30 November 2018 at Bangkok, Thailand
2.	Dr. Bharathi Kulkarni Scientist F	Meeting related to collaborative research project at London School of Hygiene & Tropical Medicine, London, UK (Sept 27 – 4 October, 2018).
		Collaborative research meeting at London School of Hygiene & Tropical Medicine London, UK (June 27- July 4, 2018).
3.	Dr. GM.Subba Rao Scientist E	Conference of International Association for Media and Communication Research (IAMCR-2019), Oregon, USA, 17-21st July 2018 and presented a paper on "Risk Communication and vendor education for promoting safety of street foods using communication for behavioural impact (COMBI) framework" in the Health Communication and Change Working Group .
4.	Dr.MV.Surekha Scientist D	Conference of International Federation of Placenta Association (IFPA), held at Tokyo, Japan (Sept.21-24, 2018).
5.	Dr.B.Santosh Kumar Scientist C	Codex Alimentarius Commission (CAC): As an Indian Delegation Attended the 40th Session CCNFSDU (Codex Committee On Nutrition And Foods For Special Dietary Uses) from 26-30 November 2018, at Berlin Germany

LIST OF PhD SCHOLARS

S. No	Name/ Fellowship	Guide Name	University	Thesis Title
1	Mr. S. Udaykant, ICMR-JRF	Dr. G.Bhanuprakash Reddy	Osmania University	Studies on vitamin B12 and diabetic neuro degeneration Biochemistry
2	Ms. Paromita Banerjee, ICMR-JRF	Dr. G. Bhanuprakash Reddy	Osmania University	Promoting nutrition and health of corporate employees with workplace interventions A study using communication for behavioral impact (COMBI) approach
3	Mr. HE Harshavardhana, UGC-SRF	Dr. G. Bhanuprakash Reddy	Osmania University	Profibrotic mechanisms in diabetic complications: Role of dietary agents
4	Mr.Ch UdayKumar, ICMR-SRF	Dr. G. Bhanuprakash Reddy	Osmania University	Obesity-induced myocardial pathogenesis; molecular mechanisms and effect of dietary intervention
5	Ms. P. Swathi Chitra, ICMR-SRF	Dr. G. Bhanuprakash Reddy	Osmania University	Micronutrient status of age related cataracts
6	Mr. K. Rajesh Kumar, DBT-SRF	Dr. G. Bhanuprakash Reddy	Osmania University	Role of aurora kinase B in chronic tissue remodeling
7	Ms. T. Shalini, NIN-SRF	Dr. G. Bhanuprakash Reddy	Osmania University	Assessment of nutritional status of geriatric population
8	Ms. Sneha Jakhotia, UGC-Fellow	Dr. G. Bhanuprakash Reddy	Osmania University	Role of small heat shock proteins in diabetic nephropathy
9	Mr. M. Sivaprasad, ICMR-Fellow	Dr. G. Bhanuprakash Reddy	Osmania University	Status of micronutrients and its influence on molecular mechanisms in diabetic nephropathy
10	Ms.J Rishika (DST Inspire) (Biochemistry)	Dr.C.Suresh	Osmania University 2013	Effect of isoflavones isolated from a naturally available cowpea as a source for the treatment of osteoporosis in MG 63 human osteosarcoma cells and to assess its synergetic role with Vitamin D in bone formation.
11	Ms.AS Neelima (DST Inspire)	Dr.C.Suresh	Osmania University (Biochemistry) 2014	Intracellular mechanism of naturally available Neuro protective compounds in mitigating the combined toxicity generated by the Lead(Pb^{2+}) in combination with Amyloid peptides in Human Brain cells.
12	Mr. V .Naresh (ICMR)	Dr.C.Suresh	Osmania University (Biochemistry) 2017	Synergistic Effect of Cowpea Isoflavones, ß -carotene and Vitamin-D on the Osteoblast differentiation

S. No	Name/ Fellowship	Guide Name	University	Thesis Title
13	Ms.B Madhuri (UGC)	Dr.C.Suresh	Osmania University (Biochemistry) 2018	Alteration in the mitochondrial function and energy metabolism in human brain cells by the expose of environment pollutant lead and amyloid peptide combo possible synergistic*in vitro* deleterious intracellular effects
14	Mr. Siva kesavarao kommula	Dr. P. Surya Narayana	Andhra University (Biochemistry)	Effect of longterm pre-diabetes on risk of renal, retinal and lens abnormalities: Biochemical, molecular mechanisms and role of dietary agents
15	Ms. K. Divya Shoshanni SRF	Dr. P. Surya Narayana	Osmania University (Biochemistry)	Assessment of Nutritional and morbidity status and utilization of health care facilities in the elderly population aged 60 years and above
16	Ms.Pallabika Gogoi	Dr. Paras Sharma	To be registered from Osmania University	Tentative Topic/broad area: Bioaccessibilitystudies of Nutrients and phytochemicals from conventional and pigmented cereals after cooking.
17	Ms.Swetha Boddula	Dr M. S. Radhika	Osmania University/29-01-2018	“Etiology of severe anemia and efficacy of treatment in school children”
18	Ms.Srujana Medithi	Dr.J.Padmaja	Osmania University (Registered & Submitted)	Assessment and evaluation of micronutrient status and biochemical parameters in relation to pesticide exposure among farm women and their children.
19	Summaiya Alam Lari	Dr.J.Padmaja	Acharya Nagarjuna University, Guntur, A.P. (Registered)	Assessment of pesticide residues penetration to the skin of farmers and farm women through protective gear in field conditions
20	Mr.Arun Pandiyan P	Dr.J.Padmaja	Osmania University (Registered)	Association between pesticide residue concentration in tissues and with lymphoma,leukaemia and breast cancers
21	Mr.Soumya Ranjan Pradhan	Dr.J.Padmaja	Yet to register	
22	Mr.K.Nagasurya Prasad, ICMR-SRF	Dr.V.Vijayalakshmi	Osmania University	Beneficial effects of mesenchymal stem cells in ameliorating effects of type 2 Diabetes- *in vivo in vitro*
23	Mrs.Pooja Desai DST-WOSA	Dr.V.Vijayalakshmi	Osmania University	Therapeutic Role of BDNF & PUFAS in Type 2 Diabetes

S. No	Name/ Fellowship	Guide Name	University	Thesis Title
24	Ms.Sugeetha Jeyapal	Dr.S.Ahamed Ibrahim	Osmania University	Impact of dietary saturated and trans fatty acids on the progression of non-alcoholic fatty liver disease in fructose induced model of steatosis.
25	Mr.Anil Sakamuri	Dr.S.Ahamed Ibrahim	Osmania University	Modulation of adipose tissue inflammation by dietary n -3 PUFA: Potential role in metabolic syndrome
26	Mr.V.Srinivas	Dr.Sanjay Basak, Scientist-E, Molecular Biology Division	Osmania University	First trimester placental development: Investigating the role of fatty acids and glucose on cellular and molecular mechanisms of placenta related disorders
27	Ms. Kiranmayee Ale	Dr. B. Dinesh Kumar	Osmania University Department of Pharmacy	Effect of vitamin D deficiency on Statin induced myalgia: Genetic Polymorphism
28	Ms. Anita Singh	Dr. B. Dinesh Kumar	Osmania University Biochemistry	Evaluation of traditional plant based formulation(Cocculus hirsutus, Cuscutareflexa, Cichorium intybus,Linum usitatissimum & Dodonea viscosa) for immunomodulatory and anti-inflammatory potential.
29	Dr. Vandana Singh, MD Ayurveda	Dr. B. Dinesh Kumar	NTR Health University, Vijayawada	Translation of traditional formulation Cynodon dactylon (Swarasa of Durva)in Menopausal SyndromeComplication (Rajonivritti janya vikrit avvastha) – A Reverse pharmacology approach
30	Ms. Sindhoora	Dr. B. Dinesh Kumar	Osmania University	Association of candidate gene SNPs with the risk & pathogenesis of PCOS- a hospital based study
31	Mr.Ravindranadh P	Dr.P.Raghu	Osmania University	Purification and characterization of iron absorption enhancers from protein rich foods
32	Mr.Purna Chandra Mshurabad	Dr.P.Raghu	Osmania University	Effect of Dietary Fat on Carotenoid Absorption: *In vitro* and *in vivo* studies

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 has 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 is also accessible.

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	. . . .	127
Reports	. . . .	76
Thesis / Dissertations	. . . .	24
CDROMS	. . . .	2
General CD's	.. . .	2

NEW JOURNALS ADDED

Indian Journals

1) Labour Law Reporter
2) Service Law Reporter

Foreign Journals

1) Appetite
2) Applied Physiology, Nutrition & Metabolism
3) Biofactor
4) European Journal of Public Health
5) Early Human Development
6) Food Security
7) Food Policy
8) Global Food Security (Online only)
9) Hepatology + Liver International (Combined Subs).
10) Health Promotion International
11) Health and Place
12) International Journal of Epidemiology

13) Journal of Bone and Mineral Metabolism
14) Journal of Applied Physiology
15) Journal of Development Studies
16) Journal of Physiology
17) Journal of Science and Medicine in Sports
18) Journal of Sport Sciences
19) Journal of Hepatology
20) Liver International
21) Neuro Toxicology
22) Nutritional Neurosciences (Online only)
23) Public Health
24) Paediatrics and International Child Health
25) Placenta Journal
26) Toxin Reviews (Print + Online)
27) World Mycotoxin Journal

2. Other activities

Journals Bound	. . . .	543
Visitors using the Library	. . . .	2581
Circulation of Books/Journals etc	. . . .	507
No. of E-mails sent outside	. . . .	490
No. of E-mails received	. . . .	3,110
Photocopying (No. of pages)	. . . . .	1,92,462
No. of INTERNET Searches provided	. . . .	150
No. of Reprints sent	. . . .	65

3. Total library collections

Books	. . . .	18,275
E – Books	……	36
Journals (Bound Volumes)	. . . .	41,149
Journals subscribed for 2018	. . . .	312
E – Journals subscribed for 2018	……	68
Journals received (Gratis/Exchange) for 2018	. .	108
Microforms (Microfiche)	. . . .	1,080
Slides	. . . .	280
Reports	. . . .	14,072
Theses & Dissertations	. . . .	453
MEDLINE CDROMS Discs	. . . .	383
Current Contents on Diskettes with abstracts	. . .	664
Proquest (Full Text E-Journals) on CD ROMS	. ..	495
General CD's	. …	331

SCIENTIFIC PUBLICATIONS

A. Papers published in scientific journals

1. Aiswarya R, Sudershan Rao V, Vishnu Vardhana Rao M, Archana K, SubbaRao GM: Perceptions and practices related to consumption of 'energy drinks'. Indian J Nutr Dietet. 55:412-422, 2018.
2. Anantha Krishna V, Vani Acharya, Lakshmi Rajkumar P, Jeyakumar SM, Vajreswari A: Transgenic zero-erucic and high-oleic mustard oil improves glucose clearance rate, erythrocyte membrane docosahexaenoic acid content and reduces osmotic fragility of erythrocytes in male Syrian golden hamsters. J Nutr Intermediary Metab. 12:28-35, 2018.
3. Ananthan R, Subhash K, Longvah T: Capsaicinoids, amino acid and fatty acid profiles in different fruit components of the world hottest Naga king chilli (Capsicum chinense Jacq). Food Chem. 238:51-57, 2018.
4. Anita KP, Anitha Kumari S, Madhusudhanachary P, Turner T, Paul BT: Biochemical and histopathological evaluation of Al_2O_3 nanomaterials in kidney of Wistar rats. Curr Top Biochem Res. 19:1–12, 2018.
5. Anju Sinha, Bharati Kulkarni, Prasanna Mithra, Preetam Mahajan, Ravi Upadhyay, Sree kumar Nair, R. S. Sharma: Research priorities in the field of anaemia in India. Indian J Community Health. 30(Supp):115-118, 2018.
6. Ankita Mondal, Tinku Thomas, Sumathi Swaminathan, Sanjukta Rao, Jithin Sam Varghese, Bharati Kulkarni, Harsh Pal Singh Sachdev, Umesh Kapil, Anura V. Kurpad: Guidelines for iron supplementation for prophylaxis of anemia in a National Programme-A Review. Indian J Community Health. 30(Supp):09-30, 2018.
7. Aruna T, Devindra S: Effect of domestic processing and crude extract of -galactosidase on oligosaccharide content of red gram (Cajanus cajan L.) seeds. Curr Res Nutr Food Sci. 6:852-861, 2018.
8. Aruna T, Devindra S: Role of pigeon pea (Cajanus cajan L.) in human nutrition and health: A review. Asian J. Dairy Food Res. 37:212-220, 2018.
9. Balaji G, Sinha SN: Autism spectrum disorder (ASD): A current review of assessment, risk factors and prevention. Indian J Biochem Biophys. 55:375-383, 2018.
10. Bharathi Kulkarni: Addressing the double burden of malnutrition in developing countries: need for strategies to improve the lean body mass. Food Nutr Bull. 39(2 suppl): S69-S76, 2018.
11. Charles Dorni, Paras Sharma, Gunendra Saikia, Longvah T: Fatty acid profile of edible oils and fats consumed in India. Food Chem. 238:9-15, 2018.
12. Damayanti K, Jeyakumar SM, Sangamitra K, Laxmi Rajkumar P, Vani Acharya, Srinivas E, Stephy Joseph, Vajreswari A: Development of low glycemic index foods and their glucose response in young healthy non-diabetic subjects. Prev Nutr Food Sci. 23:181-188, 2018.
13. Deepika Rani VS, Naveen Kumar R, Sudershan Rao V: A Review on regulatory aspects of food contact materials (FCM'S). Indian J Nutr Dietet. 55: 500-519, 2018.
14. Devindra S, Aruna T, Hemalatha R, Shujauddin Mohd: Hypolipidemic effect of red gram (Cajanus cajan L.) prebiotic oligosaccharides in Wistar NIN Rats. J Diet Suppl. 15:410-418, 2018.

15. Dinesh kumar B, Srinivasa Reddy Y: Nutrition-pollution interaction: An emerging research area. Indian J Med Res. 148:697-704, 2018.

16. Emma Pomeroy, Veena Mushrif-Tripathy, Bharati Kulkarni, Sanjay Kinra, Jay T. Stock, Tim J. Cole, Meghan K. Shirley, Jonathan C. K. Wells: Estimating body mass and composition from proximal femur dimensions using dual energy x-ray absorptiometry. Archaeol Anthropol Sci. 1-13, 2018.

17. Emma Pomeroy, Veena Mushrif-Tripathy, Wells JCK, Bharati Kulkarni, Sanjay Kinra, Stock JT: Stature estimation equations for South Asian skeletons based on DXA scans of contemporary adults. Am J Physiol Anthropol. 167:20-31, 2018.

18. Hemalatha R, Radhakrishna KV, Naveen Kumar B: Undernutrition in children & critical windows of opportunity in Indian context. Indian J Med Res. 148:612-620, 2018.

19. Himadri Singh, Vijayalakshmi Venkatesan: Treatment of 'Diabesity': beyond pharmacotherapy. Curr Drug Targets. 19:1672-1682, 2018.

20. Kankona Dey, SubbaRao GM, Paromita Banerjee, Balakrishna N, Sudershan Rao V: Food scares propagated by media and their impact on consumer perception of food safety and consumption pattern. J Content Commun Communicat. 8:2018.

21. Keren Susan Cherian, Ashok Sainoji, Prem Raj D, Balakrishna N, Venkata Ramana Y: Resting Metabolic Rate of Junior National Weightlifters in India: Development and Validation of Prediction Models. Indian J Nutr Dietet. 55:278-290, 2018.

22. Khandare AL, Hari Kumar R, Meshram II, Arlappa N, Laxmaiah A, Venkaiah K, Amrutha Rao P, Vakdevi V, Toteja GS: Current scenario of consumption of Lathyrus sativus and lathyrism in three districts of chhattisgarh state, India. Toxicon. 150:228-234, 2018.

23. Khandare AL, Vakdevi V, Munikumar M, Bhanuprakash Reddy G, Uday Kumar P, Shankar Rao G, Balakrishna N: Tamarind fruit extract ameliorates fluoride toxicity by upregulating carbonic anhydrase II: a mechanistic study. Fluoride. 51:137–152, 2018.

24. Khandare AL, Vakdevi V, Naveen Kumar B: Fluoride alters serum elemental (Calcium, Magnesium, Copper and Zinc) homeostasis along with erythrocyte carbonic anhydrase activity in fluorosis endemic villages and restores on supply of safe drinking water in school-going children of nalgonda district. Biol Trace Elem Res. 185:289-294, 2018.

25. Khandare AL, Vakdevi V, Shankar Rao G, Gopalan V, Balakrishna N: Dose-dependent effect of fluoride on clinical and subclinical indices of fluorosis in school going children and its mitigation by supply of safe drinking water for 5 years: an Indian study. Environ Monit Assess. 190:110, 2018. doi: 10.1007/s10661-018-6501-1

26. Khandare AL, Vakdevi V, Viswanathan G, Shankar Rao G, Balakrishna N: Role of carbonic anhydrase and triiodothyronine in dental caries affected children in fluorosis endemic areas. Adv Dent Oral Health. 7: 2018.

27. Kranthi Kiran Kishore T, Baba AB, Kowshik J. Bhanu Prakash Reddy G, Nagini S: Gedunin: A neem limonoid in combination with epalrestat inhibits cancer hallmarks by attenuating aldose reductase-driven oncogenic signaling in SCC131 oral cancer cells. Anticancer Agents Med Chem. 18:2042-2052, 2018. doi: 10.2174/1871520618666180731093433.

28. Longvah T, Geissler C, Ananthan R: The 11th International Food Data Conference (IFDC). Food Chem (spl issue). 238:1-2, 2018.

29. Madhavan Nair K, Dripta Roy Choudhury, Archana K: Appropriate doses of iron for treatment of anemia amongst pregnant and lactating mothers; under five children; children in 6-10 years of age; adolescent girls and women in reproductive age groups. Indian j commn health. 30: Supp.39-53, 2018.

30. Mahaling B, Srinivasarao DA, Raghu G, Rajesh Kumar K, Bhanuprakash Reddy G, Dhirendra SK: A non-invasive nanoparticle mediated delivery of triamcinolone acetonide ameliorates diabetic retinopathy in rats. Nanoscale. 10:16485-16498, 2018.
31. Maheshwar M, Narender K, Balakrishna N, Rao DR: Teenagers' understanding and influence of media content on their diet and health-related behaviour. J Clin Nutr Diet. 4: 2018.
32. Manjula T, Srinivasa Reddy Y, Dinesh Kumar B: Evaluation of Rasna panchaka (indigenous drug) as oxidative stress down-regulator using serum-free explant culture system. Indian J Pharmacol. 50:326-331, 2018.
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34. Munikumar M, Vakdevi V, Khandare AL: Reduction of fluoride toxicity by tamarind components: an in silico study. Fluoride. 51:122–136, 2018.
35. Narendra Babu K, Hemalatha R, Satyanarayana U, Shujauddin Mohd, Himaja N, Bhaskarachary K, Dinesh Kumar B: Phytochemicals, polyphenols, prebiotic effect of ocimum sanctum, Zingiber officinale, Piper nigrum extracts. J Herbal Med. 13:42-51, 2018. https://doi.org/10.1016/j.hermed.2018.05.001.
36. Naveen Kumar B, Venkaiah K: Nutritional status of the population in Bundelkhand Region. Indian J Nut Dietet. 55:334-344, 2018.
37. Naveen Kumar B, Vijaya Bhaskara Rao, Venkaiah K: Application of ridit analysis to study the severity of anemia among children, adolescents and pregnant women in Andhra Pradesh. Sri Lankan J Appl Stat. 18:49–68, 2018.
38. Padmavathi AVT, Srinivas PNBS, Raghu P, Bhanuprakash Reddy G, Manohar Rao D: Differential expression of leaf proteins in four cultivars of peanut (Arachis hypogaea L.) under water stress. 3 Biotech. 8 Article:157, 2018.
39. Paromita Banerjee, SubbaRao GM: Wellness programmes in the workplace in India. Lancet Public Health. 3:e515, 2018.
40. Prasad VSS, Hymavathi A, Babu VR, Longvah T: Nutritional composition in relation to glycemic potential of popular Indian rice varieties. Food Chem. 238: 29-34. 2018.
41. Prasanthi PS, Vishnuvardhana Rao M, Bhaskarachary K: Retention of Xanthophylls in Green Foliar Vegetables after Different Food Preparations. Indian J Nutr Dietet. 55:241-256, 2018.
42. Radhika MS, Swetha B, Naveen Kumar B, Bala Krishna N, Laxmaiah A: Dietary and nondietary determinants of nutritional status among adolescent girls and adult women in India. Ann NY Acad Sci. 1416:5–17, 2018.
43. Raja Gopal Reddy M, Mullapudi Venkata S, Uday Kumar P, Jeyakumar SM: Vitamin A deficiency induces endoplasmic reticulum stress and apoptosis in pancreatic islet cells: Implications of stearoyl-CoA desaturase 1-mediated oleic acid synthesis. Exp Cell Res. 364:104-112, 2018.
44. Sai Santhosh V, Laxmi Rajkumar P, Arlappa N, Balakrishna N, Divya Shoshanni K, Prasad U, Bhoja Raju B, Sivakesava Rao K, Ravinder P, Suryanarayana P: Prevalence of nutritional anemia and hyperhomocysteinemia in urban elderly. Indian J Clin Biochem.1–6, 2018.
45. Sangita Mukhopadhyay, Sudip Ghosh: Mycobacterium tuberculosis: What is the role of PPE2 during infection? Future Microbiol. 12:457-460, 2018.

46. Sanjay Basak, Arnab Sarkar, Santosh M, Duttaroy AK: Cellular growth and tube formation of HTR8/SVneo trophoblast: effects of exogenously added fatty acid-binding protein-4 and its inhibitor. Mol Cell Biochem. 437:55–64, 2018.

47. Sanjay Basak, Vilasagaram Srinivas V, Duttaroy AK: Bisphenol-A impairs cellular function and alters DNA methylation of stress pathway genes in first trimester trophoblast cells. Reprod Toxicol. 82:72–79, 2018.

48. Sanjukta Rao, Thomas T, Sumathi Swaminathan, Varghese JS, Kurpad AV, Ankita Mondal, Bharati Kulkarni, Sachdev HPS, Umesh Kapil: Current practice of iron doses for treatment of iron deficiency anaemia-a review. Indian J Commun Health. 30:31-38, 2018.

49. Sarin S Jose, Shalini T, Balakrishna N, Radhika MS, Brahmam GNV, Bhanuprakash Reddy G: A raw food based quantitative food frequency questionnaire to assess long-term dietary-intake among urban adults of South India: Relative validity and reproducibility. Indian J Nutr Dietet. 55(1):1-17, 2018.

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51. Shalini T, Sivaprasad M, Balakrishna N, Madhavi G, Radhika MS, Naveen Kumar B, Raghu P, Bhanuprakash Reddy G: Micronutrient intakes and status assessed by probability approach among the urban adult population of Hyderabad city in South India. Eur J Nutr. https://doi.org/10.1007/s00394-018-1859-y, 2018.

52. Shruthi K, Sreenivasa Reddy S, Swathi Chitra P, Bhanuprakash Reddy G: Ubiquitin-proteasome system and ER stress in the brain of diabetic rats. J Cell Biochem. 2018. doi: 10.1002/jcb.27884.

53. Singh P, Nagender Rao R, Sudip Ghosh, Sangita Mukhopadhyay: Cell envelope lipids in the pathophysiology of Mycobacterium tuberculosis. Future Microbiol. 13 000:000 doi: 10.2217/fmb-2017-0135, 2018.

54. Sinha SN, Venkat Reddy B: Correlation of pesticide exposure from dietary intake and bio-monitoring: The different sex and socio-economic study of children Ecotoxicol Environ. 162:170-177, 2018.

55. Sneha Jakhotia, Sivaprasad M, Shalini T, Yadagiri Reddy P, Viswanath K, Khubchand Jakhotia, Rakesh Sahay, Manisha Sahay, Bhanuprakash Reddy G: Circulating levels of Hsp27 in microvascular complications of diabetes: Prospects as a biomarker of diabetic nephropathy. J Diabetes Complicat. 32:221-225, 2018.

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