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Studies on systemic inflammatory marker, TNF- α and oxidative stress in Zn induced obese wistar rats

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Abstract
The role of Zn in the induction of systemic inflammation in obesity and obesity related diseases has been documented. Excessive Zn in diet was found to increase the subcutaneous fats resulting higher gain in body weights resulting obesity. The present study revealed that Zn- induced obesity leads to systemic inflammation. TNF- α is directly related to concentration of Zn in tissues, insulin and correlating leptin levels causes insulin and leptin resistance which have been linked to the fall in antioxidant enzyme activities including superoxide dismutase and catalase due to Cu-deficiency in the tissues leading to rise in liver lipid peroxidation reactions pertaining to increased outcome of oxidative stress.

Keywords: Zn, Cu, TNF- α, hyperleptinemia, hyperinsulinaemia, Oxidative stress

1. Introduction
Zn is known as an essential micronutrient for normal growth and development [1, 2]. The previous epidemiological studies suggested that excessive Zn in diet causes accumulation of body fat both in rodents [3] as well as humans [4,5] and results in etiology of obesity, hyperinsulinaemia, hypercortisolemia [3] and leptin resistance [6]. Zn is also acting as the important component of numerous enzymes. Its alteration cause implication in various pathophysiological diseases via decreasing the activities of antioxidant enzymes such as superoxide dismutase and catalase [7, 8] resulting in oxidative stress [9, 10]. Moreover, it has been found that TNF- α, a proinflammatory cytokine, release from the adipose tissue also plays a role in oxidative stress [11]. As TNF- α is regulated by Zn, affects the energy balance, fat accumulation, fatty liver disease, glucose homeostasis and insulin resistance [12-16] and leptin secretion [17, 18] resulting in systemic inflammation. Thus, the growing list of protein signals and factors released from adipose tissue are interlinked and suggests that Zn may playing a wide ranging role in metabolic regulation and physiological homeostasis far beyond simply acting as obesity inducer. Therefore, in the present studies it was investigated that excessive Zn in diet, stimulates systemic inflammation and oxidative stress in male Wistar rats.

2. Material and methods
For the present investigations, 18 male Wistar rats weighing 80-85gm were used as an experimental animal model procured from Central Animal House, Panjab University, Chandigarh and maintained in a separate plastic cages with stainless steel top grills at room temperature 25-28 °C with 12:12 hrs L: D cycles and 70-80% relative humidity. They were used in this study after taking approval of the protocol from Institutional Ethics Committee. Semi-synthetic basal diet (Orgebin-crist *et al*. modified by Taneja *et al*.) [19, 3] was employed with increasing Zn concentration to induce hyperleptinemia

2.1 Basal Diet Composition
The semi-synthetic basal diet contained (g/100 of diet) : Casein, 30; Agar, 2.0; Corn oil, 5; Cellulose, 8; Sucrose,51; Vitamin mixture 0.5 [Vitamin mixture (mg/Kg): Ascorbic acid, 500; Biotin, 4; Calcium pantothenate, 320; Choline chloride, 2500; Folic acid,10; Inositol,1000; Nicotinic acid, 300; Pyridoxine HCl, 180; Riboflavin, 120; Thiamin HCl 200; α-Tocopherol acetate (E), 60; Cyanocobalamin,0.40; Retinol, 0.30; Ergocalciferol, 0.0031] and mineral mixture, 3.5[Mineral mixture (gm/Kg) : CaH_2P0_4, 25.30; $COCl_3$, 0.04; $CuCl_2$, 0.10; $FeS0_4.7\ H_20$, 0.60; $MnS0_4.H_20$, 0.30; $MgS0_4$. H_20, 4.05; KCl, 43; KI, 0.004; Na_2C0_3, 1.15; NaF,0.008; $ZnS0_4.7\ H_20$, 0.088g]. The basal diet was divided into 3 parts and modified as (i) Basal diet containing 20 mg Zn/Kg diet for control group-I, (ii) Zn-induced-hyperleptinemic-diet-1 (Zn-HL-diet-I) containing 40 mg Zn/kg diet and (iii) Zn induced hyperleptinemic-diet II (Zn-HL-diet-II) containing 80 mg Zn/Kg diet by accordingly increasing $ZnS0_4.7\ H_20$ in the basal diet. For the preparation of these diets, the ingredients were weighed. The water soluble vitamins were thoroughly grounded and mixed along with sucrose, while the fat soluble vitamins were dissolved in corn oil. Agar, used as a binding agent was dissolved in 100 ml of double distilled deionized water with constant stirring on water bath maintained at 60 °C. On its cooling to 40 °C, the contents of each diet were thoroughly mixed in separate containers. The dough so formed was put in petri dishes and solidified in refrigerator for 3 hours to harden the wet fresh diets. The solidified diets were then cut into small pieces of 2 cm× 2 cm× 2 cm size and stored in the containers at < −4 °C.

2.2. Experimental design and feeding of rats.
The male Wistar rats (n = 18) were divided into three groups, group-I, group-II and group-III i.e. 6 rats per group. Each group was fed on the semi-synthetic basal diet containing Zn as 20mg, 40mg and 80mg/kg diet for a period of 4 months.

2.3. Record of Data

The data of food intake and body weight were recorded on monthly basis during dietary treatment.

Urine samples of rats were subjected to Benedict's test for estimation of the presence of glucose every week. At the end, they were sacrificed using diethyl ether as anesthesia at the end of the experiment.

2.4. Hormones and mineral analysis

The blood was collected into centrifuge tube by puncturing the heart of rats in each group and serum was prepared by centrifuging blood at 2400-2600 rpm for 10-15 minutes. The serum was analyzed for glucose by using commercially available diagnostic CHOD-POD kit (Reckon Diagnostics PVT, LTD, Baroda, India), serum cholesterol by CHOD-POD kit method (Reckon Diagnostics PVT, LTD, Baroda, India) and serum triglyceride by GPO kit method (Reckon Diagnostics PVT, LTD, Baroda, India). Hormonal analysis included insulin by a solid phase two site enzyme immunoassay (DRG diagnostics, USA), cortisol by enzyme immunoassay (Microtiter strip kit supplied by Immune-Biological Laboratories, Hamburg), leptin by solid phase two site enzyme-immunoassay (Mediagnost Aspenhaustr, D-72770 Reutlingen/Germany) and TNF-α by using solid phase sandwich enzyme linked immunosorbent assay (ELISA Kit).

Other two parameters Lipid peroxidation and antioxidant enzymes superoxide dismutase and catalase activity evaluate in the liver. Lipid peroxidation [20] was analyzed by measuring thiobarbituric acid-reactive substances (TBARs) in homogenates. The samples were mixed with 10% trichloroacetic acid and 0.375% thiobarbituric acid. Then the samples were heated in a boiling water bath for 15 min. After centrifugation TBARs were determined from the absorbance at 532 nm.

Superoxide dismutase (SOD) [21] activity was measured at 560 nm on a spectrophotometer on supernatant. The reduction of nitro blue tetrazolium (NBT) with NADH mediated by phenazine methosulfate (PMS) under aerobic conditions initiate by addition of SOD. $NADH_2$ then is reduced to PMS, which on reoxidation with O_2 produces superoxide anion O_2^-. This O_2^- anion reduces NBT from blue formazan that can be measured at 560 nm.

Catalase activity (CAT) [22] was measured at 240 nm on a spectrophotometer by the decomposition of H_2O_2. Previously, homogenate of liver or kidney be centrifuged. Then 2 ml of phosphate buffer added to 20µl of post nuclear fraction of liver or kidney. The reaction was started by the addition of 1ml of 30mM H_2O_2. Initial absorbance should be 0.500 (approx.). The values for change in absorbance at 240nm/ 10 sec should not be greater than 0.100 and not smaller than 0. 020.

Protein content [23] is assessed by taking O.D. at 750nm. 50µl of liver homogenate/PMS was taken and volume was made to 1ml with DDW. For blank 1ml of distilled water was taken. To all these tubes was added 3ml Lowry's reagent. Vortexed and allowed to stand for 10 min at room temperature. 0.3ml Folin's reagent was added to each tube, vortexed and allowed to stand for 30 minutes. The O.D. was taken at 750nm. A standard curve was also made by taking different concentration of BSA ranging from 10 to 100ug/ml.

The minerals including Cu and Zn were estimated in serum and liver. The tissues were digested in nitric acid and perchloric acid mixture (3:1 v/v) on a sand bath until a white ash was formed. The ash was dissolved in 6 ml of 10 mM $HN0_3$ and filtered through ash free filter paper before analysis. Minerals Cu, Zn and Mg were then estimated on atomic absorption spectrophotometer (Electronic Corporation of India Limited Hyderabad- AAS 4139) using hollow cathode lamps (213.9 and 324.8 2 nm for Zn and Cu respectively). Standards (sigma) for these metals were prepared by dilution in triple distilled deionized water.

The data were subjected to statistical analysis applying one way ANOVA and Tukey post-hoc test to determine the differences between groups. A level of $p<0.05$ was selected to indicate statistical significance. Data were expressed as mean ± SD.

3. Results and Discussion

In the present investigations, Zn induced systemic inflammation and oxidative stress was reported in group-II containing 40mg Zn/kg diet and group-III containing 80mg Zn /kg per diet in male Wistar rats elaborating its role in the etiology of obesity and obesity induced systemic inflammation, a cause of hyperleptinemia (leptin resistance) and hyperinsulinaemia/insulin resistance. The induction of obesity as evident from the increased body weights in group-II and III (Figure 1) was due to excessive intake of dietary Zn resulted from increased food consumption in them (Figure 2). As Zn is known as the essential component of numerous enzymes and transcription factors [24] it increases the absorption of nutrients [3] by activating the ovine metallothionein-ovine growth hormone (OMT-1a-OGH) and induces the proliferation of adipocytes by filling with triglycerides as observed in present study (Table 1).

Further, urine analysis of rats for glucose shows that it reacts negatively with Benedict's test in control group-I while positively in group-II and group-III rats after 8th week and 10th week and thereafter, suggesting the onset of obesity in the present study (Table 1). As it had already suggested that excessive Zn supplementation increases the absorptive surface area in intestine and enhance the uptake of nutrients [6].The antagonistic effect of Zn-Cu at intestinal absorption level leads to increase in Zn concentration and reduction in Cu concentration in liver of group-II and group-III rats as compare to control group-I (Table 3) which further impaired the glucose tolerance secondary to copper deficiency [25]. Moreover, it also alters the glycosylation and hemoglobin A_{1C} (HbA_{1c}) activity which is a reliable quantitative indicator of long-term hyperglycemia as observed in group-II and III rats in the present studies (Table 1). Also higher serum Zn concentrations observed in group-II and group-III (Table 3) resulted in blocking of peripheral insulin receptors [26] leading to decreased glucose tolerance [27] and consequent higher insulin levels in group-II and group-III rats in the present study (Table 1).

The effect of Zn supplementation on systemic inflammation was estimated by evaluating the obesity induced inflammatory marker, TNF- α which is higher in group-II and group-III rats (Table 1). This led to aggravated inflammation and higher mortality [28] and impaired glucose homeostasis due to decrease in insulin sensitivity in the present investigation [16,29,30].The molecular mechanism of TNF- α induced insulin resistance primarily involves the destruction of insulin receptor mediators [31] of lipoprotein lipase with elevated plasma triglyceride concentration and down regulation of glucose transporter 4 [32].

As already suggested, Zn plays an important role in appetite regulation [33] and metabolism by influencing the leptin system [34]. In the present study, excessive Zn –supplementation has

been reported to result in increase appetite as evident from food intake/body weight and cause hyperleptinemia in group-II and group-III (Table 1). The present increase in serum concentrations of leptin is also positively correlated with insulin [35,36] cellular Zn, food intake and body mass which were higher in group-II and group-III rats stimulate the glucose uptake into adipose tissue to increase leptin production [37].

Several studies also show that circulating TNF- α levels are increased in obese patients [38,39] and were positively correlated with serum leptin concentration which were higher in the present investigations (Table 1). The presence of hyperleptinemia in group-II and group-III suggests that the appearance of leptin resistance in them. The present increase in serum concentration of leptin reflects the excessive Zn in diet increases the nutrient levels [6] which resulted in obesity induced systemic inflammation evident by higher TNF- α levels in group-II and group-III rats than control group-I (Table 1).

Higher levels of cortisol evaluated in group-II and group-III rats [40] support the concept that the increased synthesis of corticotrophin-releasing hormone is a sequence of higher leptin levels, as observed, interfere further with leptin's interaction with its receptor and induction of central leptin resistance in them [41-43].

Previously, the involvement of TNF- α in oxidative stress and its relation with antioxidant enzymes was reported [11] which is observed in the present study that the higher levels of TNF-α in group-II and group-III stimulates the generation of obesity induced oxidative stress by reduction in the activities of antioxidant enzymes superoxide dismutase and catalase (Table 2). Along with this, high serum Cu levels in group-II and group-III rats in the present study (Table 3) attributed to hyperglycemia that may stimulate release of more Cu ions that accelerates the oxidative stress [44]. Furthermore, fall in tissue Cu / Zn ratio also adversely affected the cytosolic superoxide dismutase and catalase resulting in liver lipid peroxidation in the present study (Table 2). Also, the reduction of selenoglutathione peroxidase and increased synthesis of cholesterol promotes lipoprotein oxidation under Cu deficiency [45, 46].

Thus, the data suggests that Zn influences the levels of cytokine leptin concentration, a product of ob (obesity) gene and systemic inflammatory marker TNF- α in group-II and group-III rats.

4. Tables and Figures

Table 1: Mean serum glucose, triglycerides, cholesterol and hormones including serum insulin, cortisol, leptin and inflammatory marker TNF-α in male Wistar rats fed on basal diet in control group-I containing 20mg Zn/kg diet, group-II containing 40mg Zn/kg diet and group-III containing 80mg Zn/kg diet after the four months of dietary treatment. n=6, value are mean ± S.E.M.

Parameters	Group-I (Control)	Group-II	Group-III
Glucose*	89.6 ± 1.29	190.3 ± 3.72 ^{a}	245.6 ± 4.81^a
Triglycerides*	61 ± 6.12	98 ± 5.0 ^{a}	139 ± 6.12^a
Cholesterol*	106 ± 4.65	159.6 ± 4.42 ^{a}	180.1 ± 64^a
Insulin $^+$	18 ± 1.29	73 ±0.85 ^{a}	106.2 ± 1.12^a
Cortisol$^€$	58.3 ± 4.40	99.3 ± 3.98 ^{a}	104.8 ± 65^a
Leptin$^\&$	655 ± 16.48	1269 ±70.70^a	1530 ± 659 ^{a}
TNF-α$^\&$	6.0 ± 0.96	14 ± 0.46 ^{a}	24.3 ± 0.78 ^{a}

Units:*mg/dl; $^+$pmole/L; $^€$ (ng/ml); $^\&$ (pg/ml); *p*-value: ^{a}<0.001 (values of group-II and group-III were compared with control group-I).

Table 2: Mean liver lipid peroxidation and antioxidant enzyme superoxide dismutase and catalase activities in male Wistar rats of control group -1 fed on 20mg Zn/kg diet, group-II fed on 40mg Zn/kg diet and group-III fed on 80mg Zn/kg diet after four months of dietary treatment. n=6, values are mean ±, mean S.E.M.

Parameters	Group-I (Control)	Group-II	Group –III
Lipid peroxidation*	0.9 ± 0.04	1.5 ± 0.39 ^{a}	2.1 ± 0.04^a
SOD^β	14.2 ± 0.14	10 ± 0.17 ^{a}	8.0 ± 0.28^a
Catalase$^\&$	48.2 ± 0.25	40.40 ± 0.64 ^{a}	35.7 ± 0.94^a

Units: *nmol MDA produced/hr/mg protein; ^{β}unit /mg protein; $^\&$µ mol H_2O_2 decomposed /min/mg Protein; *p*-value: ^{a}<0.001 (values of group-II and group-III were compared with control group-I).

Table 3: Mean Zn and Cu concentration in male Wistar rats fed on basal diet in control group-I containing 20mg Zn/kg diet, group-II containing 40mg Zn/kg diet and group-III containing 80mg Zn/kg diet after the four months of dietary treatment. n=6, value are mean ± S.E.M.

Parameters	Group-I (Control)	Group-II	Group-III
Serum Zn*	0.6 ± 0.04	1.5 ±0.01^a	1.8 ± 0.05^a
Serum Cu*	0.8 ± 0.02	2.5 ±0.03 ^{a}	3 ± 0.17^a
Liver Zn$^\&$	35 ± 0.66	42.9 ± 0.48 ^{a}	60 ± 0.63^a
Liver Cu$^\&$	63 ± 0.90	40.2 ± 0.24 ^{a}	32.6 ± 0.44 ^{a}

Units: *mg/dl; $^\&$µg/g; *p*-value: ^{a}<0.001 (values of group-II and group-III were compared with control group-I).

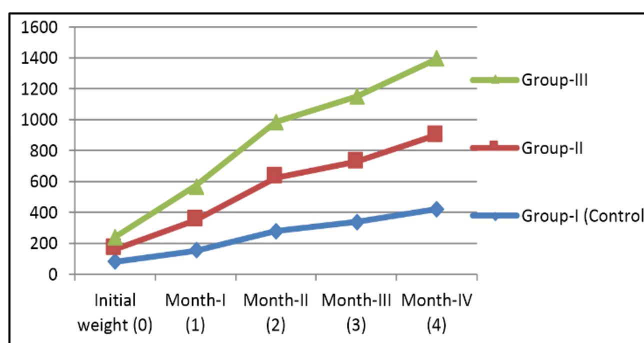

Fig 1: Line graph showing mean month wise body weight of Wistar rats in groups control group-I, group-II and group III during 4 months of dietary treatment. [Values are mean ± SEM of n= 6].

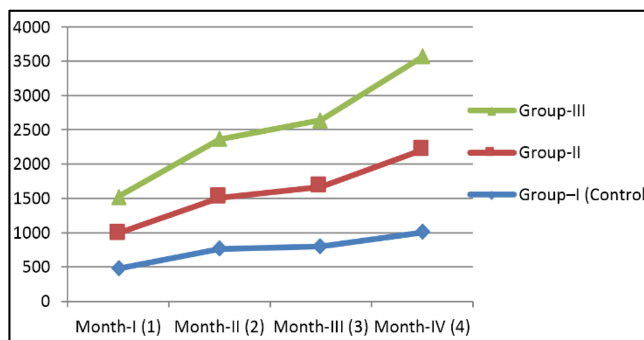

Fig 2: Line graph showing mean month wise food intake of Wistar rats in groups control group-I, group-II and group III during the 4 months of dietary treatment. [Values are mean ± SEM of n= 6].

5. Conclusion

Therefore, Zn –induced obesity leads to systemic inflammation. TNF- α is directly related to concentration of Zn in tissues, insulin in blood serum and correlating leptin levels causes insulin and leptin resistance which have been linked to the fall

in antioxidant enzyme activities including superoxide dismutase and catalase due to Cu-deficiency in the tissues leading to rise in liver lipid peroxidation reactions pertaining to increased outcome of oxidative stress. The present findings are very significant in term of excessive Zn being consumed all over the world including the northern Indian population. This excessive Zn intake through food chain is due to its indiscriminate use in agriculture and animal husbandry practices, Zn fortified baby foods in U.S.A. and other Zn rich dietary supplements which makes the children, adults and other age groups more vulnerable to obesity induced systemic inflammation, the cause of all obesity and obesity related disease and therefore requires to develop a method (strategies) to reduce the Zn burden on the body so as to contain the increasing prevalence of obesity and obesity induced systemic inflammation and oxidative stress.

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