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Article

Assessment of the impact of high zinc intake on leptin receptor gene expression in wistar rats

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Abstract: In recent years, zinc (Zn) has been extensively employed in agricultural and livestock practices, as well as in baby foods and multivitamin supplements, due to its perceived non-toxic nature and its ability to promote linear growth and body weight in consumers and consequently, its usage is increasing steadily. This study investigates the impact of prolonged excessive zinc intake on the expression levels of the leptin gene in adult Wistar rats without a genetic predisposition to obesity. Three groups of rats were fed basal diets containing 20 mg Zn per kg diet (control group, Group-C), 50 mg Zn per kg diet (Group-T1), and 80 mg Zn per kg diet (Group-T2) for 180 days. Following the dietary treatment, gene expression studies were conducted using adipose tissue from the experimental rats. The findings indicate that dietary zinc supplementation significantly increased leptin receptor gene expression in adipose tissue in a dose- dependent manner. Compared to the control group (Group C), leptin receptor mRNA levels were 3.19-fold (± 0.54) higher in Group T1 receiving 50 mg Zn/kg diet and 4.70-fold (± 0.59) higher in the group receiving 80 mg Zn/kg diet. Our findings indicate that excessive zinc intake can resulted in the upregulation of the leptin gene expression which may lead to leptin resistance and ultimately may contribute to obesity.

Keywords: trace element; leptin, gene expression; adipose tissue; obesity; animal models

1. Introduction

Zinc is one of the essential micronutrients which plays a crucial role in several biological processes such as enzymatic functions, immune response, gene expression and cellular metabolism [1,2]. It is also responsible for regulating several hormonal pathways, which in turn impact metabolic health. Among all those hormones, Leptin is a fundamental hormone that regulates satiety, body weight and energy balance through its interaction with receptors in hypothalamic neurons [3,4]. Leptin, described by Zhang et al. in 1994 [5] is a 167-amino-acid hormonal protein product of the obesity gene. Various tissues such as white adipose tissue, brown adipose tissue, placenta, ovaries, and skeletal muscles among others produce it.

Leptin operates through leptin receptors, which are crucial for mediating its physiological functions. Leptin signaling is important for maintaining energy balance and any disturbances in this pathway can result in obesity and metabolic disorders.

Long-term studies have uncovered that a deficiency in Zn can result in a variety of metabolic disorders, such as non-alcoholic fatty liver disease, insulin resistance,

inflammation in adipose tissue, and high blood sugar levels [6–8]. However, recent research has revealed potential negative effects of zinc supplements at relatively low levels or doses in certain situations. Moreover, studies have begun to reveal the potential risks associated with excessive consumption across various groups, including pregnant women and children, emphasizing the importance of careful consideration when supplementing with zinc [9]. Although zinc is an essential micronutrient, excessive intake has been linked to several disorders, including obesity, diabetes, dyslipidemia, and hypertension in experimental animals [10–13].

Recent studies have highlighted the complex relationship between zinc and leptin regulation, suggesting that zinc plays a role in both the secretion and function of leptin [14,15]. While zinc deficiency has been linked to lower leptin levels, excessive zinc intake may result in elevated leptin concentrations, potentially leading to leptin resistance—a condition where high leptin levels fail to suppress appetite effectively [16]. This effect is of particular interest in the context of obesity, where leptin resistance contributes to weight gain and metabolic dysregulation [17]. Dysregulation of leptin signaling, including impaired leptin receptor function, has been associated with obesity and other metabolic disorders.

Given the increasing use of zinc in dietary supplements and its widespread inclusion in multivitamins, it is essential to explore the effects of prolonged zinc supplementation on leptin regulation, particularly in non-obese models. Previous studies have demonstrated conflicting findings on the relationship between zinc levels, leptin secretion, and body weight, which highlights the need for further investigation [18–20]. Despite the well-known significance of zinc and leptin, the precise impact of high zinc intake on leptin receptor gene expression remains unclear. Therefore, it is crucial to conduct a thorough assessment of the impact of high zinc intake on leptin receptor gene expression in wistar rats to better understand the potential implications of zinc supplementation on obesity and related metabolic disorders. This study aims to explore the influence of excessive zinc supplementation on the gene expression of the leptin receptor in Wistar rats. By examining varying zinc concentrations in the diet, we seek to elucidate the dose-dependent effects of zinc on leptin receptor expression in adipose tissue and the potential implications for metabolic regulation and obesity.

2. Materials and methods

2.1. Experimental design and experimental diet

In the present study, the experimental models used were Wistar rats. 24 adult male rats (aged around 2 months), weighing between 80–100 g, were obtained from Chakraborty Enterprise, a CCSEA (Committee Control and supervision of Experiments on Animals, Government of India) registered breeder located in Kolkata, India. They were housed in accordance with the guidelines of the Institutional Animals Ethics Committee, Sikkim University, Gangtok and in compliance with internationally recognized standards for laboratory animal care. The housing conditions provided a room temperature of 25–28 ℃ , with a 10:14 h light-dark cycle and a relative humidity of 70%–80%. Ethical approval for all animal-related procedures was obtained from the Institutional Animal Ethical Committee of

Sikkim University, which is fully accredited by CCSEA; under the reference number IAEC/SU/Ph.D./2019/01, issued on 29 April 2019.

At the outset, the newly acquired rats were provided with a standard pellet rat diet for 1 week to facilitate their acclimation to the new environment. The study utilized a semi-synthetic diet [21] , which was specifically formulated to be rich in fat and refined sucrose, to ensure consistency in the composition of key nutrients and to eliminate potential interactions between zinc and dietary fibers or phytates[22,23]. The composition of the diet is mentioned in **Table 1.** To prepare the diets, firstly the mineral and water-soluble vitamins were mixed with sucrose, while the fat-soluble vitamins were dissolved in corn oil. Agar, used as a binder, was dissolved in 25 mL of warm water and then cooled to 40 ℃. The components of each diet were thoroughly mixed in separate containers, resulting in a dough-like consistency. The dough was then placed in petri dishes. The prepared diets were then solidified and stored for use in the study. Over a period of 180 days, each group of rats was fed their respective diets and provided with double distilled water. The weights of the rats were recorded at the beginning of the feeding regimen and then on a weekly basis thereafter. Additionally, their food intake was monitored throughout the experiment.

The rats were randomly assigned to three groups: Group C (Control), Group T1 (Test group-1), and Group T2 (Test group-2), with the basal diet containing 20 mg Zn/kg (control diet) for Group C, 50 mg Zn/kg (Zn supplemented feed 1) for Group T1, and 80 mg Zn/kg (Zn supplemented feed 2) for Group T2. The increase in dietary zinc content was achieved by adding $ZnSO_4.7H_2O$ to the basal diet.

Table 1. Composition of basal diet [21].

Total Diet	**(g/100g)**	**Vitamin Mixture (mg/kg)**	**Mineral Mixture(g/kg)**
Casein	30	Ascorbic acid–500	CaH_2PO_4–25.3
Agar	1.5	Biotin–4	$COCl_3$–0.04
Corn oil	5	Calcium Pentothenate–320	$CuCl_2$–0.10
Cellulose	8	Choline Chloride–2500	$FeSO_4.7H_2O$–0.60
Sucrose	51.5	Folic acid–10	$MnSO_4.H_2O$–0.31
		Inositol–1000	$MgSO_4.H_2O$–4.05
Vitamin Mixture	0.5	Nicotinic acid–300	KCl–3.43
		Pyridoxine HCl–80	KI–0.004 Na_2CO_3–1.15
MineralMixture	3.5	Riboflavin–120 Thiamin HCl–200 α-tocopherolacetate (E)–60 Cyanocobalamin–0.40 Retinol–0.3 Ergacalciferol–0.0031	NaF–0.008 $ZnSO_4.7H_2O$–0.088
Total Diet	100		

The selection of zinc doses (20 mg/kg, 50 mg/kg, and 80 mg/kg) was based on a tiered approach to evaluate the dose-dependent effects of zinc on leptin receptor gene expression. These doses were strategically designed to represent a gradient, allowing the investigation of how gradual increases in dietary zinc intake influence the

expression of the leptin receptor in adipose tissue. The lowest dose, 20 mg/kg, reflects the basal dietary requirement for zinc, while the higher doses (50 mg/kg and 80 mg/kg) were chosen to simulate moderate and excessive zinc supplementation, respectively. This approach is consistent with previous research where varying zinc concentrations have been used to explore its broader metabolic impacts in animal models [16].

2.2. Collection of tissues

After concluding their respective dietary treatments, the experimental rats in each group were anesthetized using thiopental sodium and subsequently euthanized. White adipose tissues were harvested for subsequent analyses. These tissues were cleaned, blotted dry using filter paper, and rinsed with saline solution prior to weighing. A portion of the adipose tissue was sectioned and immediately frozen in liquid nitrogen for gene expression analysis, while the remaining tissues were stored at −70℃ for future examination.

2.3. Leptin gene expression of Wistar rats

Total RNA extraction from adipose tissue was performed using the RNeasy Lipid Tissue Mini kit (Qiagen) according to the manufacturer's instructions. The purity and concentration of the extracted RNA were assessed with a NanoDrop QIAxpert. To eliminate any DNA contamination, the RNA was treated with an RNase-free DNase kit (Qiagen). Subsequently, the RNA was reverse-transcribed into first-strand cDNA using the Quantinova™ Reverse Transcription Kit, with 1 μg of total RNA in a 20 μl reaction mixture. *β*-actin served as the internal control for leptin. Primer sequences for the leptin receptor and the housekeeping gene β-actin are listed in **Table 2**. Real-time RT-PCR amplification and analysis were conducted using the Quantitect SYBR Green PCR Kit on a Rotor-Gene Q, following the manufacturer's protocol. The PCR conditions utilized were an initial denaturation at 95℃ ℃ for 10 min, followed by 40 cycles of 95℃ for 15 s, 60℃ for 1 min, and 72℃ for 10 s, with a final extension at 72℃ for 10 min. Gene expression levels were calculated using the 2-ΔΔCt method.

Table 2. Primers used for the amplification of cDNA [24]

cDNA	Forward primer	Reverse primer
β-actin	5′- GGCTGTATTCCCCTCCATCG-3'	5′- CCAGTTGGTAACAATGCCATGT-3'
Leptin	5′- GAGACCCCTGTGTCGGTTC-3'	5′- CTGCGTGTGTGAAATGTCATTG-3'

2.4. Statistical analysis

The experimental results were expressed as the mean value ± standard deviation (SD). Statistical analysis of the data was conducted using the SPSS software package (Chicago, IL, USA). To identify significant differences between the treatment groups and the control group, a One-way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison test was performed. A significance threshold of $p < 0.05$ was applied to determine statistically significant differences in the data.

3. Results

3.1. Effect of zinc supplementation on body weight

During the experiment, the body weight of the rats was recorded monthly. A significant increase in body weight was observed with the increase in dietary Zn concentration. Specifically, Groups T1 and T2 exhibited a significant rise in monthly body weights ($P < 0.05$) for the first 150 days of nutritional treatment. However, in the subsequent 30 days, the body weights of these groups decreased compared to the control group (Group C) **(Figure 1).**

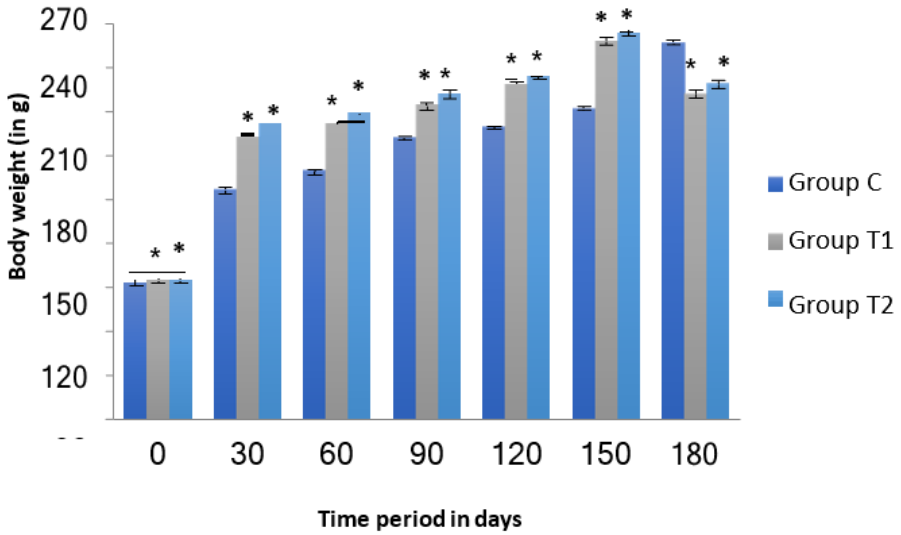

Figure 1. Body weight of the experimental group of rats after 180 days of dietary treatment. (Values aremean ± SEM).

3.2. Effect of Zn supplementation on food intake

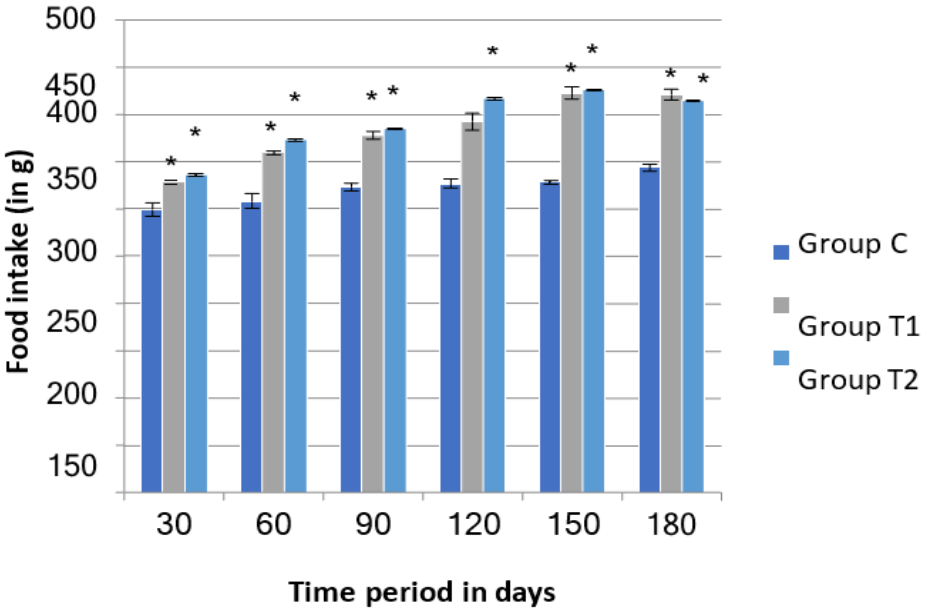

Figure 2. Food intake of the three group of rats during 180 days of dietary treatment. (Values are mean ± SEM).

Similar trend was observed while studying the effect of Zn supplementation on food intake too. Food intake among the rats increased over time across all three groups (Group C, Group T1, and Group T2). Additionally, Groups T1 and T2 demonstrated a significantly greater increase in food consumption compared to the Control group (**Figure 2**).

3.3. Effect of Zn supplementation on the gene expression of leptin in Wistar rats

The findings indicate that dietary zinc supplementation significantly increased leptin receptor gene expression in adipose tissue in a dose-dependent manner. Compared to the control group (Group C), leptin receptor mRNA levels were 3.19-fold (± 0.54) higher in Group T1 receiving 50 mg Zn/kg diet and 4.70-fold (± 0.59) higher in the group receiving 80 mg Zn/kg diet ($p < 0.05$). The expression of the leptin receptor gene in adipose tissue of the three groups of Wistar rats subjected to different dietary zinc treatments is presented in **Figure 3**.

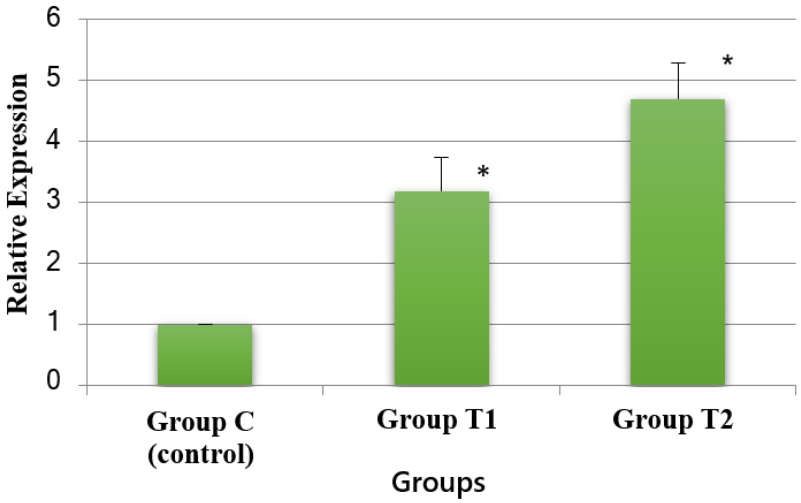

Figure 3. Effect of Zn on the expression of Leptin of rats of Group C, i.e, the Control group [(fed on a basaldiet (Control)],Group-T1 (fed on Zn supplemented feed 1) and Group-T2 (fed on Zn supplemented feed 2) after 180 days of dietary treatment.
*$p < 0.05$ versus the control group using the Dunnett test.

4. Discussion

This study aimed to investigate the effects of prolonged zinc supplementation on the expression of leptin receptor genes in Wistar rats, with particular attention to the implications for obesity and metabolic health. The significant increase in body weight observed in the zinc-supplemented groups during the first 150 days of treatment aligns with prior studies that have demonstrated zinc's involvement in weight gain and fat storage [25–27]. This can be explained by zinc's involvement in multiple metabolic processes, including the regulation of appetite and fat storage. However, the subsequent decline in body weight observed in the final 30 days of the study suggests that prolonged exposure to excessive zinc may disrupt these metabolic processes, potentially leading to metabolic dysregulation. As observed, elevated dietary zinc levels were associated with increased body weight and food

consumption, aligning with previous research that highlights the role of zinc in growth and metabolism [1]. In addition to it, another study demonstrated that both zinc deficiency and excess zinc intake influenced body weight and food intake in growing rats. Studies have shown that zinc deficiency can lead to impaired growth, anorexia and weight loss [28]. Excess zinc led to increased body weight, thereby, highlighting its impact on growth and metabolism [29]. Thus, research consistently demonstrates that increased zinc intake can result in higher body weight and food consumption. These effects have been observed across diverse species and experimental settings, highlighting the important role of Zn in the regulation of appetite and metabolism. As fat mass escalates, there is an elevation in leptin concentrations, leading to a reduction in appetite and a boost in energy usage [30]. Also, other reported research studies indicate that leptin is crucial in regulating both food intake and the accumulation of body fat [3,31] It has also been observed that an overly high intake of Zn may disrupt these regulatory mechanisms by elevating leptin concentrations, which might then foster a state of leptin resistance [4]. Such findings highlight the intricate interactions among dietary Zn, leptin, and factors associated with obesity.

Our study highlights the dose-dependent effect of Zn on leptin receptor expression, where higher Zn doses led to a significant increase in leptin receptor mRNA levels compared to the control group. This aligns with earlier research showing that Zn plays a role in modulating leptin production and signaling [14,15,17]. This result suggests a complex interaction between Zn intake and leptin regulation, highlighting Zn's potential role in promoting leptin resistance when consumed in excess. The upregulation of the leptin receptor gene observed in our study raises the possibility that excessive Zn intake could contribute to the development of leptin resistance, a condition in which elevated leptin levels fail to produce their expected physiological effects on appetite suppression and energy expenditure [14,17].

The dose-dependent upregulation of leptin receptor gene expression in Zn-supplemented groups (T1 and T2) suggests that Zn may significantly affect leptin signaling pathways. This supports findings by Ott and Shay (2001), which demonstrated that both Zn deficiency and excess Zn intake alter leptin dynamics, affecting its role in energy homeostasis. The biphasic response observed, where weight gain increased during the early stages but decreased with prolonged Zn exposure, indicates a complex relationship between Zn and long-term metabolic regulation. This response may be due to Zn-induced alterations in nutrient absorption, leading to hyperleptinemia and potential leptin resistance, as suggested by previous studies [15]. Zn has been shown to regulate serum leptin concentration in humans [32]. Studies suggest that elevated levels of Zn-induced glucocorticoid cortisol may lead to hyperleptinemia, potentially resulting in leptin resistance. Additionally, Zn deficiency has been shown to decrease leptin gene expression and secretion in rat adipocytes [17]. The decrease in leptin gene expression may be attributed to a decline in transcription, as Zn is involved in the structure and function of RNA polymerase, and Zn deficiency can specifically alter the composition of mRNA synthesis in cells [33]. Excessive dietary zinc intake has been linked to the development of leptin resistance in Wistar rats, potentially due to enhanced nutrient absorption at the

intestinal level. This suggests a complex relationship between zinc and leptin, where both deficiency and excess can affect leptin levels and functionality [16,17]. The elevated leptin receptor expression observed in this study indicates that excessive zinc may disrupt leptin's physiological roles, fostering a state of leptin resistance. Leptin resistance has been linked to increased adiposity and metabolic disturbances, and our results align with studies showing that excessive zinc intake can impair leptin's ability to regulate appetite and energy expenditure [17]. This disruption in leptin signaling could explain the initial weight gain followed by reduced weight, as prolonged leptin resistance may diminish its anorexic effects.

White adipose tissue (WAT) is used in this study of leptin gene expression and its receptors because of its crucial role in leptin synthesis, metabolic regulation, and maintaining energy homeostasis. As the main site of leptin production, WAT provides a direct means to investigate the gene expression of leptin and the pathways that regulate it, making it essential for understanding leptin's role in energy balance. Furthermore, WAT exhibits high levels of leptin receptors (Ob-R), which are critical for mediating leptin's effects on appetite control and metabolism. In the context of obesity, WAT often expands and becomes dysregulated, contributing to leptin resistance, which further supports its relevance in studies exploring the molecular mechanisms of this condition. The practical accessibility and abundance of WAT, compared to tissues like the brown adipose tissue, also make it a suitable choice for large-scale research into leptin signaling and metabolic regulation [34,17].

Additionally, insufficient Zn levels might contribute to leptin resistance through direct mechanisms or as a component of oxidative stress in obese and diabetic individuals [35]. Research indicates that excessive Zn can increase the intestinal mucosal epithelial surface area, thereby augmenting nutrient absorption. This nutrient surplus prompts insulin production to transport the extra nutrients to adipose tissue, promoting adipocyte growth and increasing leptin synthesis and secretion. These findings have significant implications for metabolic regulation and energy homeostasis [16]. In this study, Wistar rats were administered diets with varying Zn concentrations, revealing significant outcomes. Higher dietary Zn levels resulted in increased food intake, body weight gain, and elevated blood levels of leptin, glucose, insulin, and cortisol. These findings suggest that high Zn concentration supplementation may influence leptin gene expression, highlighting the complex relationship between Zn and leptin regulation. The results corroborate previous studies by Mantzoros et al. [32] and Mangian et al. [20], which demonstrated that Zn deficiency reduces leptin levels in both humans and rodents. Conversely, it has been found that serum leptin levels increased during Zn depletion. Zn deficiency in osteoblastic cells resulted in an upregulation of leptin receptor expression, thereby activating the JAK2/STAT3 signaling pathway, which is essential for leptin's cellular effects. This study highlights the importance of Zn in regulating leptin receptor expression and its associated signaling pathways [36]. Thus, researches confirm that Zn deficiency affects leptin levels and gene expression in rodents, particularly rats, reinforcing the significance of our findings that indicate a correlation between Zn concentration and leptin levels. Additionally, Ueda et al. [19] showed that inadequate or excessive dietary Zn intake affects the leptin system in pregnant mice by regulating plasma leptin levels through the expression of soluble

Ob-R mRNA in the placenta and leptin mRNA in visceral fat. This supports our results, which demonstrate an increase in leptin levels with higher Zn concentrations. Studies by Taneja et al. [37,38] also indicated that elevated Zn levels can lead to hyperleptinemia, potentially resulting in leptin resistance due to Zn's role in leptin synthesis and function regulation. Excessive Zn intake, along with elevated CRP levels observed in our previous research [39], may contribute to leptin resistance, likely due to the association between increased CRP and leptin resistance, which inhibits leptin binding to its receptors and disrupts its signaling [40]. Consequently, this can attenuate leptin's physiological effects. Elevated CRP is associated with inflammation, which can interfere with leptin signaling, inhibiting leptin's ability to regulate appetite and energy balance. CRP directly binds to leptin in the bloodstream, inhibiting leptin's ability to bind to its receptors and thus blocking its physiological actions. This mechanism likely contributes to leptin resistance in the presence of elevated CRP levels [40]. In addition to it, CRP also impedes leptin's entry into the central nervous system, further contributing to leptin resistance [41], prolonged consumption of Zn in amounts exceeding the body's requirements has been shown to disrupt the absorption and utilization of other essential trace elements. Research revealed a significant decrease in copper and manganese levels, along with a substantial increase in Zn levels, in the tissues of Wistar rats fed a high-Zn diet [39]. This observation is consistent with our previous findings. Excessive Zn intake may lead to diminished copper levels in soft tissues, resulting in oxidative stress. This oxidative stress can induce inflammation, which in turn raises cortisol levels and contributes to leptin resistance and elevated insulin levels. Therefore, it can be suggested that leptin resistance induced by excessive Zn can be mitigated by balancing the levels of Zn, copper, and magnesium ions in the diet, along with the inclusion of antioxidants to reduce oxidative stress.

The concentration-dependent effect of Zn on leptin expression suggests a dose-response relationship, indicating that higher levels of Zn supplementation may lead to more pronounced changes in leptin gene expression. Furthermore, it is essential to consider the specific mechanisms through which Zn may influence leptin receptor gene expression. One possible mechanism may be the regulation of signalling pathways involved in leptin signalling, such as the JAK-STAT pathway. It has been shown that Zn can modulate the activity of this pathway, potentially affecting the expression of leptin receptor genes [36]. Additionally, Zn may also play a role in epigenetic modifications that can affect gene expression [42]. Understanding these mechanisms is crucial in assessing the impact of high Zn intake on leptin receptor gene expression in Wistar rats. Additionally, the use of a semi-synthetic diet in this study enabled the controlled manipulation of Zn content without the confounding effects of dietary fibers or phytate interactions. This approach enhanced the internal validity of the study findings and provided valuable insights into the specific impact of Zn on leptin gene expression.

5. Conclusion

In conclusion, this study demonstrates that excessive Zn supplementation leads to a dose-dependent increase in leptin receptor gene expression in white adipose

tissue, which may contribute to the development of leptin resistance. While Zn is an essential micronutrient, its prolonged excessive intake can disrupt normal leptin signaling and energy balance, ultimately promoting metabolic disturbances such as hyperleptinemia and obesity. These findings highlight the need for careful consideration of Zn intake levels, particularly in the context of dietary supplements, and underscore the importance of further research into the mechanisms underlying Zn's effects on leptin regulation and metabolic health.

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Ethical approval: The Institutional Animals Ethics Committee, of Sikkim University, Gangtok, India approved the animal study protocol. This committee is fully accredited by CCSEA, and the approval was granted under the reference number IAEC/SU/Ph.D./2019/01, issued on 29 April 2019. The study was conducted in accordance with the Declaration of Helsinki.

Conflict of interest: The authors declare no conflict of interest.

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