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Modulatory Role of NTSR1 Antagonist SR48692 in HFD-Induced Thyroid Impairment in Mice: An *In Vivo* Study

Himanshu Pal*, Pradeep Verma and Banalata Mohanty

Department of Zoology, University of Allahabad, Prayagraj - 211002, Uttar Pradesh, India; himanshu.pal.315860@gmail.com

Abstract

Thyroid hormones are one of the crucial regulators of metabolism, especially lipid, and is linked with lipid metabolism dysregulation. High-Fat Diet (HFD) induces the secretion of Neurotensin (NTS), which enhances lipid absorption, and deposition, proportionately increases leptin. Increased leptin is associated with central leptin resistance and affects thyroid regulation which may lead to hypothyroidism. We hypothesized that HFD-induced increased NTS may cause dyslipidemia leading to thyroid alterations, which could be antagonized by the NTS receptor-1 (NTSR1) antagonist SR48692. Swiss albino mice (n = 24) were divided into two sets and fed with Standard Diet (SD) and HFD for 4 weeks. After that each set of mice were further divided into two groups and administered with 0.9% saline and SR48692 (100 µg/kg bw) intraperitoneally for 4 weeks and maintained as Group-I: (Control/SD), Group-II: (HFD), Group-IV: (HFD+SR48692), and Group-IV: (SD+SR48692). HFD-fed mice showed significantly decreased T3 and T4 with increased TSH and leptin, suggesting leptin-induced hypothyroidism, also reflected in the histological alterations observed in thyroid and adipose tissue. Co-treatment with SR48692 (HFD+SR48692) showed amelioration in the thyroid, as levels of T3, T4, TSH, and resensitization of leptin, were evident in histopathological analysis compared to HFD suggesting the therapeutic efficacy of SR48692 on thyroid impairment in dyslipidemia. Only SR48692-treated group showed an exacerbating effect.

Keywords: High-Fat Diet, Leptin, Neurotensin, SR48692, Thyroid Dysfunction

Abbreviations: GIT- Gastrointestinal Tract; HFD- High-Fat Diet; HPT- Hypothalamic-Pituitary-Thyroid; NTS- Neurotensin; SD- Standard Diet; T3- Triiodothyronine; T4- Thyroxine; THs- Thyroid Hormones; TRH- Thyrotropin Releasing Hormone; TSH- Thyroid Stimulating Hormone.

1. Introduction

Thyroid Hormones (THs), the peripheral end products of the Hypothalamic-Pituitary-Thyroid (HPT) axis are one of the most significant regulators of energy expenditure[1]. Thyroid illness is frequently connected with dyslipidemia which affects all aspects of lipid metabolism, including synthesis, mobilization, and breakdown[2]. High-Fat Diet (HFD) intake increases the secretion of Neurotensin (NTS), a Gastrointestinal Tract (GIT) peptide secreted by N cells, which is a metabolically active neuropeptide[3]. NTS acts as an anorexigenic peptide and plays a crucial role in the regulation of energy balance, and also participates in lipid absorption and accumulation

**Author for correspondence*

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in adipose tissue as well as other peripheral organs and leads to the development of obesity[4]. Studies over the past few decades have concentrated on the role of NTS in intestinal function, particularly in relation to fat absorption and reduced food intake through direct infusion or HFD-induced NTS secretion[5-7]. HFD-induced NTS release enhances the absorption of lipids via NTS Receptors (NTSRs), NTSR1, NTSR2, and NTSR3 and increases the lipid content in plasma. It has been recently shown that there is a positive link between NTS and leptin levels, indicating that NTS influences peripheral leptin concentration[8]. Obese individuals have elevated levels of leptin expression in adipose tissue as well as in plasma[9,10]. Functional aspects and mass of adipose tissue are independently regulated by THs and Thyroid Stimulating Hormone (TSH); though, adipokines, such as leptin, also influence the HPT axis[11]. Hypophysiotropic Thyrotropin-Releasing Hormone (TRH) is released to increase pituitary thyrotropin (Thyroid Stimulating Hormone; TSH) could be the consequence of HPT axis stimulation by leptin, which increases the production and surge of THs[12,13]. Triiodothyronine (T3) affects appetite regulation via the central nervous system, primarily the hypothalamus, and affects lipid deposition in adipocytes[1]. Decreased THs along with increased TSH level have commonly been observed in obese individuals in primary subclinical hypothyroidism[11,14]. Hypothyroidism is one of the indicators of leptin resistance, characterized by the diminished response of leptin at the central level, which may be caused by different mechanisms, including impaired leptin transport through the blood-brain barrier, inflammation in the hypothalamus, and disturbance in leptin signalling of hypothalamic neurons[15,16]. It affects major metabolic pathways in the body, including those linked to obesity and metabolic syndrome, and plays an important role in the de novo production of fatty acids and the breakdown of lipids[1]. In this respect, studies indicated that thyroid dysfunction could serve as an obesity risk factor and vice versa[14]. Collectively, these findings imply that endocrine variables, such as plasma leptin imbalance in extreme obesity, are significant causative factors disrupting the HPT axis in obese conditions. However, there is no study available to corroborate the regulation of peripheral NTS in the aspect of the HPT axis. Due to the minimal stability of NTS in plasma, targeting its receptor through its antagonist might be one of the effective operative methods to detect the involvement of NTS in the pathophysiology of the thyroid. SR48692 is a specific antagonist of NTSR1, and it has several biological and physiological functions through central and peripheral regulation by crossing the blood-brain barrier[3,17]. SR48692 effectively counteracts many of the exogenous NTS-induced effects, such as hypertension in guinea pigs, and behavioral modification in rodents, and has been reported to have an inhibitory effect on NTS neurotransmission by systemic administration[18,19]. In the current study, the role of NTSR1 antagonist SR48692 in the regulation of HPT axis has been investigated by utilizing the well-established HFD-induced obese mice model. We hypothesized in this study that HFD-induced alterations in NTS level may cause lipid metabolic disturbances leading to thyroid alterations, which could be antagonized by the NTSR1 antagonist SR48692.

2. Materials and Methods

Swiss albino mice (male, age; 8 weeks) were purchased from IITR Lucknow, India and housed in polyvinyl chloride cages in standard laboratory conditions with temperature (23 ± 2 °C), light (12-hour light-dark cycle), and relative humidity (55 ± 5 %). Mice (n = 24) were acclimatized for one week, and after that divided into two groups, half fed with commercially available rodent Standard Diet (SD) (3.1 kcal/gm) contained 10% energy from fat however other half fed with freshly prepared HFD pellets (5.58 kcal/gm) contained 60% energy from fat. The HFD was prepared in the laboratory (lard as the major source of fat)[20]. The dietary composition (macronutrients) is represented in the table. After 4-week feeding of SD and HFD, both SD and HFD-fed mice were received intraperitoneally (i.p) 0.9% saline as vehicle and SR48692 (Sigma Aldrich, Bengaluru, India) (SR48692 100 µg/kg bw) for 4 weeks[21]. Thus 4 groups containing 6 mice each were made as follows: SD (vehicle control group), SD+SR48692, HFD (vehicle control group), and HFD+SR48692. After the end of experiment mice were anaesthetized using pentobarbital (100 mg/kg) and sacrificed. The mice handling was done following the Committee for the Purpose of Control and Supervision of Experimental Animals guidelines, Ministry of Environment and Forest, Government of India. The protocols were approved by the University of Allahabad, Institutional Animal Ethical Committee (Approval No: IAEC/AU/2019(1)/02).

Table 1. Macronutrient composition of standard rodent chow and high-fat diet.

	Diets	
	Standard diet	**High-fat diet**
Carbohydrate, % kcal	76	26
Protein, % kcal	14	14
Fat, % kcal	10	60
Energy, Kcal/g	3.1	5.58

2.1 Hormonal Assays

Mice were anaesthetized and blood was immediately collected from cardiac puncture and centrifuged at 3000 rpm for 20 min, plasma was separated and pooled for further analysis. Assays for T3, thyroxine (T4), TSH, and leptin were conducted using commercially available ELISA kits following the manufacturer's protocol (T3, T4, TSH and leptin: Krishgen Biosystems, Ltd, Mumbai, India). The samples were run in duplicate and optical density was measured by iMark microplate reader (Bio-Rad Laboratories, CA, USA) at 590 nm.

2.2 Histopathological Analysis of Thyroid and Adipose Tissue

After anaesthetizing mice, the thyroid and adipose tissue were quickly dissected out, and fixed in freshly prepared 4% paraformaldehyde for 24 hours. After proper washing and dehydration, tissue was embedded in paraffin. The sections were cut at 5-6 µm using a rotary microtome, stretched on standard sterilized glass slides, and stained with haematoxylin-eosin (H and E) following the standardized protocol of our laboratory[22], and microphotographs were produced using Leica DM 2500 (Germany) light microscope.

The Nucleus-to-Cytoplasmic (N/C) ratio of thyroid follicular epithelial cells and the number of adipocytes were carried out using Image 1.52r (Image analysis software package, NIH, Bethesda, MD). In brief, total cytoplasmic area (Acn), nuclei area (An), and cytoplasmic area excluding nuclei (Ac) were calculated in every selected cell. The N/C ratio of epithelial cells was determined at magnification 100× in each section by the equation: $\frac{N}{C} = \frac{An}{Acn - An}$. Each of the histology measures for the thyroid gland was conducted in replicates and 20 replicates were conducted per animal[23]. To estimate the number of adipocytes, a distinct counting frame (200 × 200 µm^2) was used for 10 randomly selected fields of H and E-stained adipose sections per sample for each mouse and were measured at ×40.

2.3 Statistical Analysis

Graph Pad Prism 9 (Graph Software Inc., USA) statistical software was used for all analyses. Data with normal distribution and homogeneity of variance were analyzed using one-way analyses of variance (one-way ANOVA), represented as mean ± SEM, followed by Tukey's posthoc test for multiple comparisons. The results with a p-value of less than 0.05 are considered significant.

3. Results

3.1 Plasma Level of Thyroid (T3 and T4) and Pituitary (TSH) Hormones

Plasma level of T3 and T4 significantly decreased in HFD-fed mice ($P < 0.001$) compared to SD. In the antagonist co-treated group (HFD+SR48692) both T3 and T4 significantly increased ($P < 0.001$) compared to HFD but remained significantly decreased ($P < 0.001$) compared to SD. T4 decreased significantly ($P < 0.01$) in only antagonist treated group but T3 remained equivalent to SD (Figures 1(a) and (b)). In the HFD-fed mice plasma TSH was significantly increased ($P < 0.001$) compared to the SD. In the antagonist co-treated group (HFD+SR48692) significantly decreased ($P < 0.01$) compared to HFD but remained significantly increased ($P < 0.01$) compared to SD. Only antagonist-treated group remained equivalent to SD (Figure 1(c)).

3.2 Plasma Leptin Assay

The plasma leptin level was significantly increased ($P < 0.001$) in HFD-fed mice. The antagonist co-treated group (HFD+SR48692) showed a significant decrease ($P < 0.001$) compared to HFD but remained significantly increased ($P < 0.001$) compared to SD. Only antagonist-treated group remained equivalent to SD (Figure 1(d)).

3.3 Histopathological Analysis of Thyroid

The thyroid gland in mice of the SD group had a characteristic lobular structure and consisted of round and oval follicles. The wall of the follicles was formed by cuboidal epithelial cells. The thyroid of HFD-fed mice showed disorganization of follicular cells, with hypertrophy, and hyperplasia

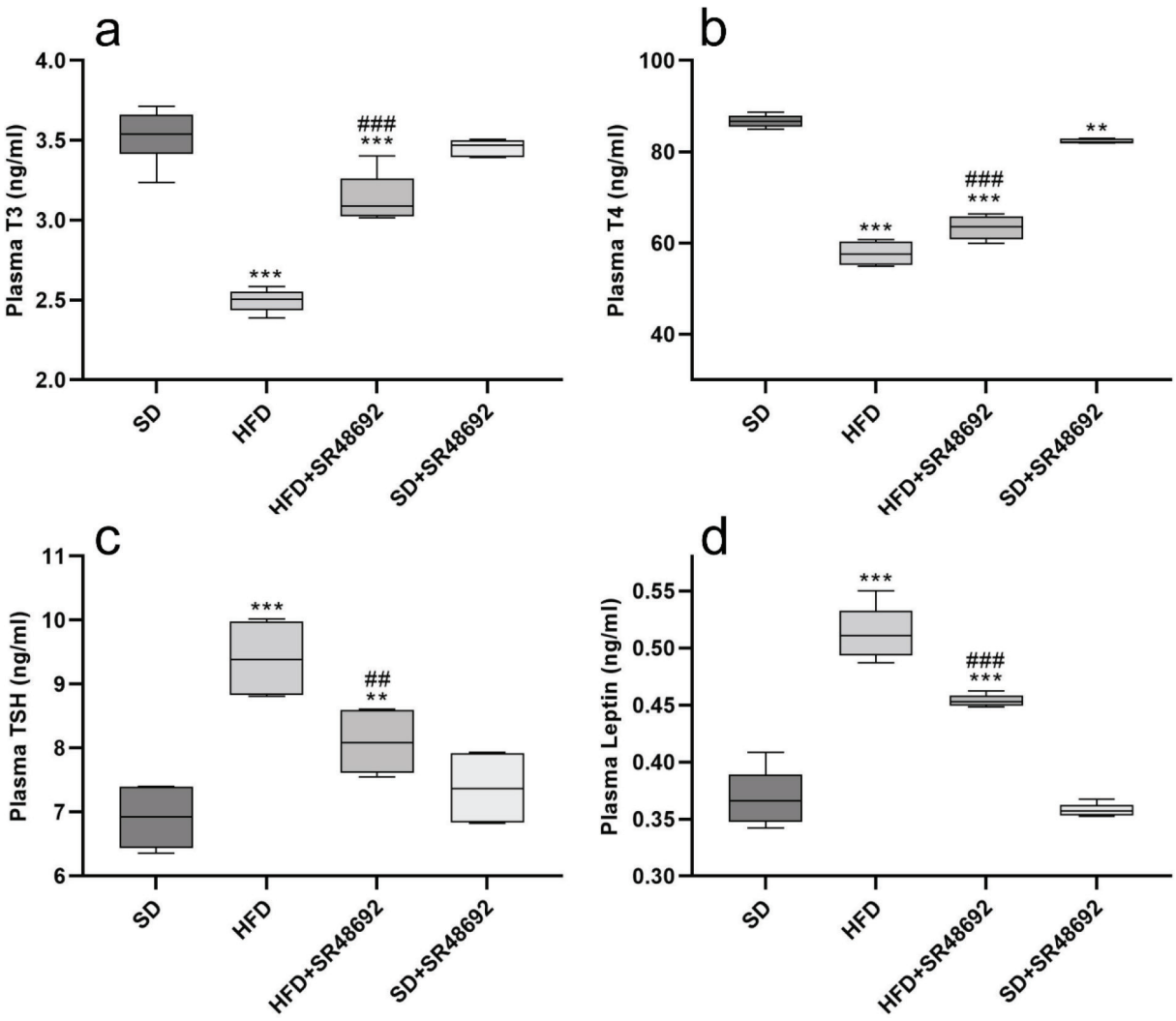


Figure 1. Graph showing plasma T3 **(a)**, plasma T4 **(b)**, plasma TSH **(c)**, and plasma leptin **(d)** in experimental groups as well as vehicle controls. The values were given as the Mean ± SEM. Statistical significance was assigned as SD Vehicle control vs Experimental groups **P < 0.01 and ***P < 0.001; HFD Vehicle control vs Experimental groups ##P < 0.01 and ###P < 0.001.

additionally the cytoplasm was vacuolated in some follicular cells, and the basement membrane was disrupted which involves the loose connective tissue adjacent to the thyroid follicle. With the co-treatment of SR48692 (HFD+SR48692), the ameliorating effects were observed compared to HFD, as no empty follicles, and a reduction in the hypertrophied follicles, interstitial space between follicles with increase in compactness (Figure 2(a)). N/C ratio significantly decreased in HFD-fed mice (P < 0.001) compared to SD-fed mice. In the antagonist co-treated group (HFD+SR48692) significantly increased (P < 0.001), compared to HFD but significantly decreased (P < 0.01) compared to SD. Only antagonist treated group significantly decreased (P < 0.05) compared to the SD (Figure 2(b)).

3.4 Histopathological Analysis of Adipose Tissue

The number of adipocytes per unit area of adipose tissue in HFD-fed mice, showed a significant decrease (P < 0.001), reflecting more lipid deposition compared to SD. With the antagonist co-treated group (HFD+SR48692), lipid deposition was considerably less, and the number of adipocytes significantly increased (P < 0.001) per unit area than that of HFD-fed mice but remained significantly decreased (P < 0.001) compared to SD. Only antagonist treatment group significantly increased (P < 0.05) than that of SD-fed mice (Figure 2(c)).

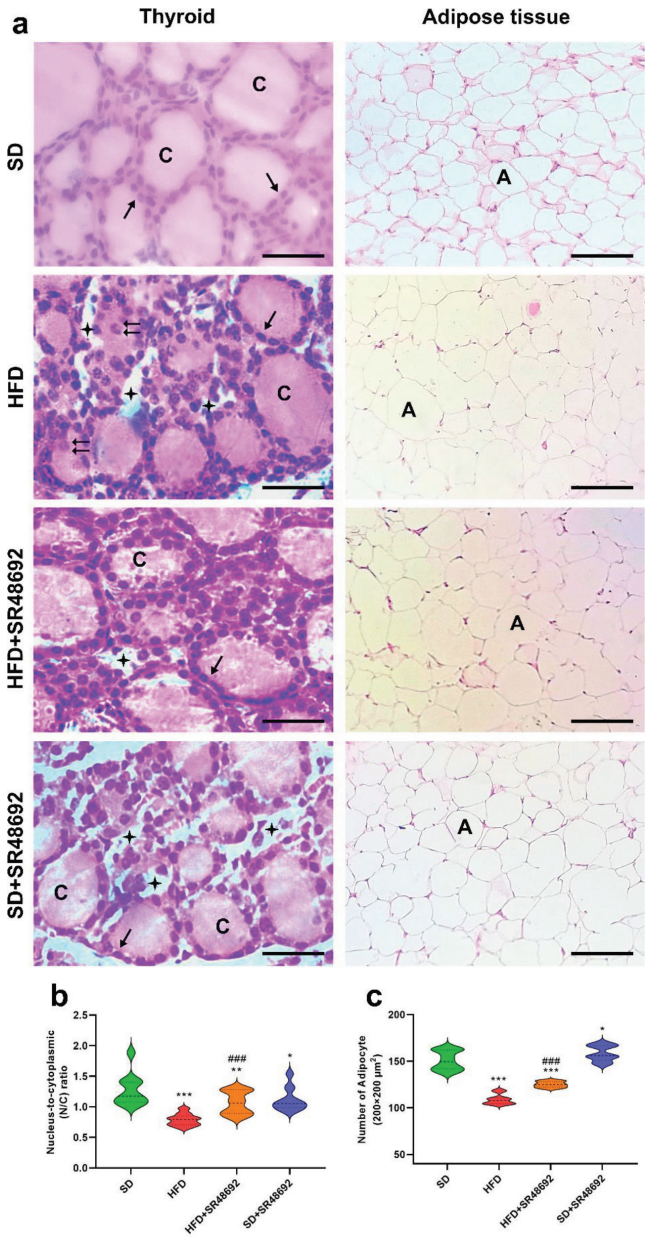


Figure 2. H&E stained sections showing thyroid and adipose tissue of vehicle control and experimental groups **(a)**; colloid filled follicle (**C**), follicular epithelium (➞), shrinkage (✦), exfoliation of epithelial cells into colloids (⇆) and adipocyte (**A**). Graph showing nucleus to cytoplasmic (N/C) ratio of follicular epithelium of thyroid **(b)**, and number of adipocytes in a given area **(c)** in experimental groups as well as vehicle controls. The values were given as the Mean ± SEM. Statistical significance was assigned as SD Vehicle control vs Experimental groups *P < 0.05, **P < 0.01 and ***P < 0.001; HFD Vehicle control vs Experimental groups ###P < 0.001. Magnification ×40, Scale bar 50μm.

4. Discussion

The present study focused on the ameliorative effects of NTSR1 antagonist SR48692 in HFD-induced thyroid dysfunction. The diet and duration of HFD have significant impact on THs level is reflected in this study. Studies suggested that TSH level in plasma are a good indicator for the existence of thyroid dysfunction. Thyroid produces T4 in response to TSH, and D2 deiodinases locally convert this to the physiologically active form T3. TSH secretion is regulated by several hormones, including leptin[12]. Leptin is directly correlated with hypothalamus which participates in food intake regulation and aids in the regulation of the satiety centre[24].

Studies reported that consumption of HFD enhances the NTS secretion, which may involve in prolonged stomach emptying,[25] resulting increased lipid absorption leads to adipocytes as well as ectopic fat accumulation, and increased energy expenditure[6]. HFD has been reported for long-lasting increase in circulating leptin, which is a potential marker of the body's lipid levels[26]. Leptin increases the production of the TRH peptide in the paraventricular nuclei to regulate the HPT axis by both direct and indirect pathways[12]. Pre-treatment of fasting rats with a melanocortin antagonist completely inhibits the leptin-induced activation of the HPT axis, thereby establishing the necessity of the indirect pathway for leptin-mediated HPT activity[13]. It is well established that chronic HFD-induced plasma leptin leads to leptin resistance[16], due to the disruption of the indirect pathway of leptin functioning, though the direct pathway remains active, corroborated to previous report[27]. HFD-fed mice showed a decrease in T3 and T4 along with an increase in TSH level due to leptin resistance, characterised hypothyroidism and reported that a lower level of T3 in hypothyroidism is related to obesity[28]. In this study, the thyroid axis alterations in mice given an HFD resembled the thyroid abnormalities in animals with hypothyroidism as earlier reported[29]. Interestingly, we noticed flattened epithelial cells and expanded follicles in the thyroids of HFD-fed mice, also reported by Shao and co-workers[30], concurrently with considerably lower plasma THs levels. In this study, HFD-induced histopathological alterations were distinct in the thyroid, such as decreased N/C ratio, as earlier reported[29]. Similar histomorphological changes were reported to have a "damaging effect on the thyroid gland" by Pandey and co-workers[23]. These results imply that thyroid dysfunction is positively correlated with

altered thyroid histomorphological structure. Collectively, suggesting the disruption of thyroid pathophysiology in response to the increased plasma level of leptin due to HFD feeding.

The protective effect of SR48692 co-treatment with HFD was observed via leptin, T3, T4, and TSH modulation under HFD-fed conditions in the present study. It may be partly attributed to the fact that TSH, which was correlated with adiposity, was higher in HFD as compared to co-treated (HFD+SR48692) mice. Earlier studies reported that SR48692 treatment diminishes peripheral NTS-induced bile acid cycling and is subject to the regulation of intestinal bile acid[31]. The decreased level of bile acid surge could be responsible for less absorption of lipid content, and adiposity and ultimately lowering the leptin level. The leptin sensitivity restoration could become a useful approach to treating obesity maintaining weight loss and reducing the rate of weight regain[32]. In the current study, the SR48692 co-treated group showed less lipid deposition in adipose tissue, associated with a decreased leptin level, which increases the THs level, which collectively showed the protective role of SR48692 in the thyroid pathophysiology by restoring leptin resistance. Previously reported that, after leptin resensitization, leptin-mediated normal thyroid regulation becomes activated, and the level of thyroid hormone returns to normal[32]. Several studies have reported the restoration of leptin resistance by various dietary factors that could decrease body adiposity and hyperleptinaemia[16,32,33]. Moreover, the chronic administration of SR48692 to SD-fed mice showed noteworthy adverse effects. Contrary to acute exposure, chronic exposure to SR48692 may impair several parameters, most likely by preventing endogenous NTS from binding to its receptors, as suggested by Karinch *et al*[34]. The exacerbating effects of only SR48692 treatment causing less lipid in the body could be due to fast gastric emptying[35] and disruption of normal NTS signalling due to SR48692. Supported by the fact that SR4892 pre-treatment before olive oil consumption decreased intestinal fatty acid absorption, as reported by Li and co-workers[6]. Previously, a study reported that SR48692 administration created systemic toxicity and caused greater body weight loss. As reported before, SR48692 treatment caused greater body weight loss, which might be due to increased systemic toxicity[36]. The selected dose of antagonist mitigates the detrimental effects of HFD-induced thyroid impairments, indicating a potential therapeutic use of SR48692 in thyroid impairments.

5. Summary and Conclusion

The current study shows that the NTSR1 antagonist SR48692 has some efficacy in modulating HFD-induced thyroid impairment. Resensitization of the central leptin signalling reflects that SR48692 actively participates in the central leptin signalling pathway through peripheral regulation, suggesting its therapeutic role in thyroid impairment. While it is widely known that NTSR1 plays a major role in lipid absorption, it is equally important to investigate the roles played by other NTS receptors in this regard. Furthermore, more studies are to be conducted in a dose-dependent, regulated manner to explore the potency of the receptor analogues.

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