Original Article

Regulation of Neuropeptide Y Receptor Gene Expression and Hormone Level in Obese Male Rats Receiving 6-Gingerol and L-Arginine Supplementation

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ABSTRACT

Obesity and its associated disorders, such as hyperlipidemia, have become a global issue following the consumption of unhealthy, high-fat, and high-carbohydrate foods, which burdens the economies and the health systems of human societies worldwide. This study aimed to evaluate the effect of oral consumption of 6-gingerol and L-arginine supplements on obesity factors. Thirty rats in five groups were fed a diet specific to each group for 12 weeks and then treated with the oral administration of L-arginine (200 mg/day) and 6-gingerol (100 mg/day) for 12 weeks. The food and water intake and weight change, were then measured. In addition, plasma glucose, triglyceride, cholesterol, high-density lipoprotein (HDL), very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and serum hormone levels, including corticosterone, testosterone, and insulin, were measured, and NPY, Y1, and Y5 receptor gene expression were recorded using real-time PCR. Administration of 6-gingerol and L-arginine decreased food intake, weight gain, glucose levels, insulin levels, and homeostasis model assessment-insulin resistance (HOMA-IR) index compared to the HCD control group. In addition, corticosterone and testosterone levels in the study groups showed a significant decrease (P<0.05) and increase (P<0.01) compared to the control groups, respectively. Triglyceride, total cholesterol, HDL, and VLDL levels in the groups treated with L-arginine and gingerol alone or combined significantly decreased compared to the control group (P<0.01). This study confirms that 6-gingerol and L-arginine supplements prevent HCD-induced hyperlipidemia by controlling hormones and neurotransmitters involved in the general metabolism.

Keywords: Carbohydrate-Rich Diet, Corticosterone, Ginger, Insulin, Nitric Oxide

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1. Introduction

Today, obesity, being overweight, and its associated problems are reaching epidemic proportions and attracting increasing press and official attention. Statistics from the World Health Organization show that in 2016, more than 1.9 billion grown-ups (18 years and older) were overweight (1), of which more than 650 million were obese, and 39 million children aged 0–5 were overweight or obese in 2020 (2). Obesity enhances the risk of many diseases, including type-2 diabetes, musculoskeletal disorders, hyperlipidemia, and cardiovascular disease (3). Therefore, the prevention and treatment of obesity are appropriate for promoting health. Ginger is a spice from the roots of *Zingiber officinale* plants. Ginger contains active ingredients, such as Shogaol, Gingerol, and flavonoids, whose antimicrobial, antioxidant, and anti-inflammatory effects have been proven in various studies (4, 5). In addition, 6-gingerol is one of the essential components of ginger, whose anti-inflammatory, anti-tumor, and anti-obesity effects have been proven (6). On the other hand, L-arginine is an essential amino acid in the human body that has been proven to have anti-hypertensive and anti-inflammatory effects, and there is evidence of its positive impact on cardiovascular disease (7). The L-arginine is the immediate precursor of Nitric Oxide (NO), which has numerous effects on performance and health and regulates mammalian central food intake (8). Additionally, arginine stimulates the pancreas to inhibit the release of insulin. The neurohypophysial hormone arginine vasopressin is produced in the hypothalamus and helps release growth hormones. Several studies have shown that L-arginine supplementation affects appetite and food intake. A positive relationship between L-arginine intake and serum nitrate + nitrite (NO_x) was noteworthy in overweight, obesity, and chronic diseases but not in normal-weight cases (9). Clinical and animal studies have demonstrated the anti-obesity, vasodilating, anti-infertility, and anti-inflammatory effects of L-arginine supplementation (10). In addition, numerous studies have investigated the role of brain neurotransmitters (11, 12), such as NPY and their precursor amino acids, in controlling mammalian nutritional behaviors and mood (13). The hypothalamus NPY regulates energy expenditure and is a potent appetite stimulant. This neurotransmitter is released in the brain and the autonomic nervous system (14). Several studies have reported evidence of the positive effect of gingerol and L-arginine on fat profile (15-17). The present study explored the effects of gingerol and L-arginine supplementation on the expression of the NPY receptors gene and their relationship with insulin levels, lipids profile, corticosterone, and testosterone levels in obese male rats.

2. Materials and Methods

2.1. Animals and study design

Thirty male Wistar rats were provided by the Pasteur Institute of Iran, 10 weeks old and weighing 220-280 g body weight. The animals were kept until the end of the experiment in highly controlled temperature (22-24°C), relative humidity (55.5%), and 12 h light/12 h dark cycles. Then, they were randomly divided into five groups. This study was approved by the National Committee on Ethics in Biomedical Research (ethics.research.ac.ir) of the Islamic Azad University, Science and Research Branch and approved with the ethics ID IR.IAU.SRB.REC.1396.189, and all animal tests followed the guidelines for the care and use of research laboratory animals of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. During the 14-day adaptation period before starting the test, a basal diet (containing 23% protein, 3.5% fat, 4.5% fiber, 10% ash, 0.1% calcium, 0.7% phosphorus, 0.5% salt, 3% Amino acid, and 10% moisture) were constantly available to the rats' *ad libitum*. Each unit of high-carbohydrate food consisted of 33% standard food, 33% condensed milk, 7% crystallized sugar, and 8.6% water. The HCD consisted of 16% fat, 68% carbohydrates, and 16% protein, containing 428 kcal per 100 g. At the end of the adaptation period, the animals were weighed and randomly segregated into five groups of six animals each. Group 1 was fed a standard diet, other groups were fed HCD for 12 weeks to induce obesity, and then groups 3, 4, and 5 were treated with oral administration of L-arginine or 6-gingerol alone or simultaneously for six weeks.

The experimental groups were as follows:

1: Control group: Normal Diet: (ND)

2: Carbohydrate diet control group: High Carbohydrate Diet (HC)

3: Carbohydrate-rich diet + 200 mg/kg L-arginine: (HCA)

4: Carbohydrate-rich diet + 100 mg/kg 6-gingerol: (HCG) (18)

5: Carbohydrate-rich diet + 200 mg/kg L-arginine + 100 mg/kg 6-gingerol: (HCAG)

This experiment recorded food and water intake as behavioral factors. Animals' weight was measured on the first day and at the end of the experiment (day 84) by the Dual-X ray absorptiometry (DXA) system. The following formula calculates the Body Mass Index (BMI). BMI = body weight (g)/length2 (cm2), where "length" corresponds to the "nose-to-anus" length.

2.2. Biochemical analysis

At the end of the experiment, overnight-fast animals were anesthetized under CO_2. Blood samples were collected, and serum was separated following centrifugation of the blood after coagulation at 3000 rpm for 10 min. The HDL, LDL, VLDL, Total cholesterol, and triglycerides were measured using a special diagnostic kit in a spectrophotometer (UV-Vis 2100, China) in three independent replications at 550 nm. Blood glucose was measured at the end of the last day of treatment using a glucose enzymatic kit (Megazyme, Ireland).

2.3. Serum hormone levels

According to the manufacturer's instructions, serum insulin, corticosterone, and testosterone concentrations were determined using a rat-specific enzyme immunoassay kit (Qiagen, USA). Fifty microliters of serum samples from each rat were added to 96-well plate wells. Hormone concentrations were measured by absorption at 405 nm using an ELISA reader. Each piece was analyzed in three different experiments. Furthermore, the amount of insulin resistance was determined based on the homeostasis model using the formula: HOMA-IR=fasting glucose in nmol/L × fasting insulin in μU/ml/22.5 (19).

2.4. Gene expression

The rats were then euthanized by CO_2, and the brain was carefully dissected and examined for NPY gene expression (20). Total RNA was extracted from rat hypothalamic tissue with a special RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The Nanodrop device confirmed the quality of extracted RNA and cDNA synthesized using the Revert Aid First Strand cDNA Synthesis Kit (Thermofisher). Quantitative RT-PCR was performed with an initial SYBR Premix Ex Taq II Kit (CinnaGen, Iran) on a thermocycler system (StepOneplus, Thermo Fisher Scientific, Germany) incubation at 95°C for 30 sec, followed by 38 cycles of 15 s at 94°C and 1 min at 62°C. The relative expression of the target genes was obtained using the GAPDH housekeeping gene. The detection system software confirmed the threshold cycle one number, and data were analyzed using the $2^{\Delta\Delta CT}$ method (21). Each reaction was carried out in triplicate, and statistical analysis was done via SPSS (version 25). The primers used for qRT-PCR are as follows: Forward 5′-GCT AGG TAA CAA ACG AAT GGG G-3´ and reverse 5′-CAC ATG GAA GGG TCT TCA AGC-3´ for NPY gene, forward CTCAGGGACTGTCACGTGTT and reverse primers were CCAGGACTGTGCTTCATCCA for NPY1R gene, forward 5-TTCTTACCGATCTATGCATG-3' and reverse 5´-CCTTACATGCGTTAAAACCA-3´ for *NPY5R* gene.

2.5. Statistical analysis

Data analysis was performed using SPSS software (version 25). Smirnov-Kolmogorov test was performed on the normality of data distribution and homogeneity of variance of groups. In addition, a One-Way Analysis of Variance was done to compare the effects of different drug doses and compare it with the control. A *P-value* of < 0.05 was considered statistically significant.

3. Results

It was additionally shown that the food and water intake in the study group, in which gingerol and L-arginine were prescribed, were lower than the HC group ($P<0.05$; Figure. 1). The results showed that by receiving the 6-gingerol or L-arginine supplementation, the body weight was significantly reduced compared to the HC control group ($P< 0.01$) but did not vary from each other (Figure. 1). In addition, the results revealed that the food and water intake of the animals in the study group for which gingerol and L-arginine were prescribed together was lower than the HC group ($P<0.05$; Figure. 1).

3.1. Biochemical test results

The data showed that oral administration of 100 mg/kg gingerol and 200 mg/kg L-arginine alone ($P<0.02$) and in combination ($P<0.05$) significantly reduced serum insulin and glucose levels and HOMA-IR. In addition, serum corticosterone and testosterone levels in the study group compared to the control group showed a significant decrease and increase, respectively (Table 1). The effect of oral administration of 100 mg/kg 6-gingerol and 200 mg/kg L-arginine alone and in combination on the level of blood lipids in the experimental groups compared to the control group is shown in Table 2. The results showed that triglycerides, total cholesterol, HDL, and VLDL levels in

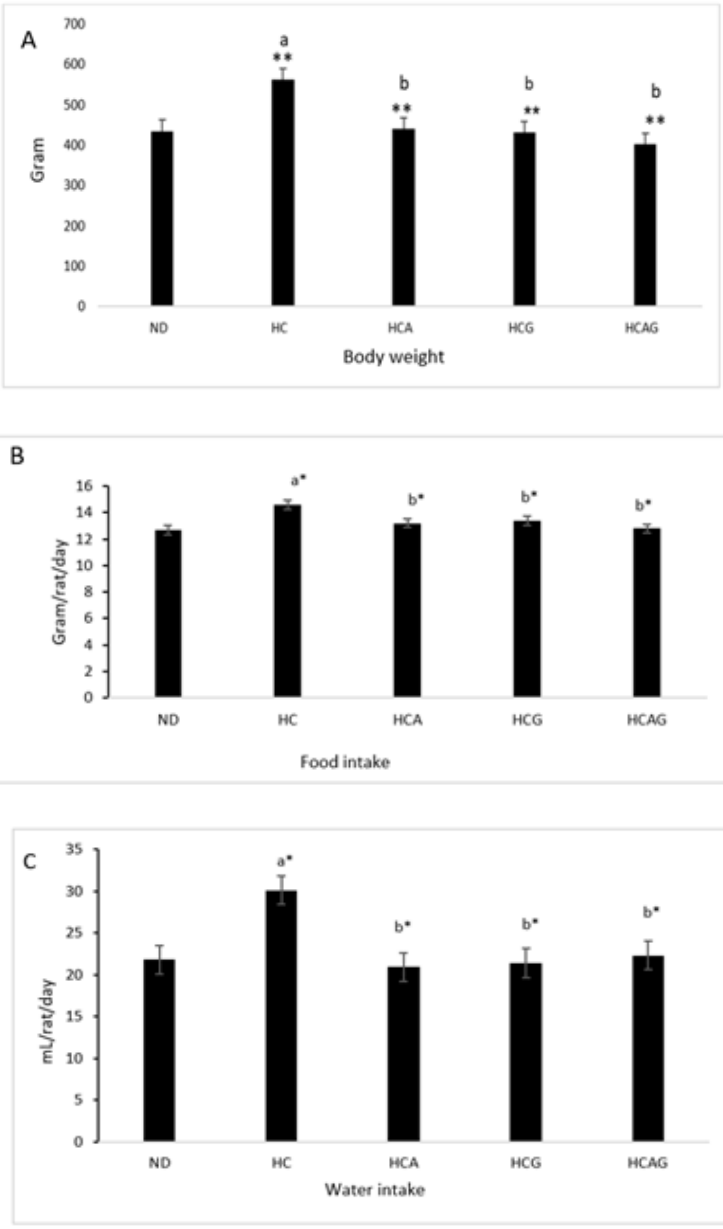

Figure. 1. Effect of 6-gingerol and L-arginine on animals' weight (A), food (B), and water intake (C) in the standard and experimental obese rats. Values at the level of ** p <0.01 are significant. a: significant difference with a standard control, b: significant difference with carbohydrate receptor (HC) control group.

Table 1. Effect of 6-gingerol and L-arginine treatment on serum glucose, insulin and HOMA-IR index, testosterone, and corticosterone in normal and HCD-induced obese rats

Groups	Insulin (ng mL^{-1})	Glucose (ng mL^{-1})	HOMA-IR	Testosterone (ng mL^{-1})	Corticosterone (pg mL^{-1})
ND	2.1±0.4	93.6±1.6	9.81±0.213	0.69±0.01	101.84±3.38
HC	3.1±0.9^{a*}	96.2±2.1 ^{a*}	16.14±1.1^{a**}	0.58±0.02^{a*}	104.27±2.3 ^{a*}
HCA	2.3±0.02^{b*}	94.1±0.9	10.32±0.29^{b*}	0.71±0.12^{b*}	94.29±1.96 ^{b*}
HCG	2.6±0.7	94.9±1.7	10.01±0.36^{b*}	0.73±0.04*	92.76±0.84 ^{b*}
HCAG	2.7±0.4	93.9±2.5	9.19±0.31 ^{b**}	0.76±0.03^{b**}	93.55±1.16 ^{b*}

*** Mean ± SD with different letters superscripts (a, b) in the same column using the one-way ANOVA test. n=6; Values are significant at ****P*** < 0.05, *****P*** < 0.01.**

the groups treated with L-arginine and gingerol alone or combined significantly decreased compared to the control group (*P*<0.01).

3.1. Gene expression profile

Figure 2 shows the expression level of NPY mRNA and two Y1 and Y5 receptor genes. The NPY gene expression in the experimental group was significantly reduced compared to the control group by oral administration of 100 mg/kg gingerol or 200 mg/kg L-arginine compared to the HC group (*P*<0.01). In addition, the combination of 6-gingerol and L-arginine treatment significantly reduced NPY gene expression (*P*=0.001). In addition, the expression level of the Y1R gene in the HCAG group by receiving 6-gingerol and L-arginine was higher than in the control groups.

4. Discussion

Statistics demonstrated that the consumption of simple carbohydrates, especially fructose and sucrose, is increasing worldwide (22). A diet rich in carbohydrates or fats, inactivity, and genetic background are the most critical factors in obesity and related diseases such as hyperlipidemia and diabetes (23). Obesity directly affects the incidence of cardiovascular disease as the leading cause of death worldwide (24). Studies have shown that rats exposed to high-fat, high-carbohydrate diets have similar characteristics to humans (25). It has been reported that rodents exposed to a high-carbohydrate, high-fat diet had symptoms such as hyperglycemia, insulin resistance, hyperlipidemia, and decreased bone mineral density (26). In this study, a high-carbohydrate diet resulted in weight gain, increased blood glucose, and insulin resistance in the experimental group, which is consistent with previous studies (27). These features can be attributed to a high-carbohydrate diet that leads to adipose tissue hypertrophy (3). Studies have shown that insulin increases serum glucose and fat levels by preventing lipolysis and inducing lipogenesis (28). Previous findings demonstrated that hyperinsulinemia and increased insulin resistance are essential to obesity in Wistar rats (29). In the present study, administering L-arginine and 6-gingerol significantly reduced serum glucose, serum insulin levels, and HOMA-IR index in experimental groups compared with the HC group. The L-arginine and gingerol appear to increase insulin sensitivity and reduce insulin resistance by reducing free fatty acids or increasing cellular metabolism, leading to hypoglycemia, weight gain, and food intake in experimental animals. In other studies, lowering the level of leptin hormone has been proven as another anti-obesity mechanism of 6-gingerol and L-arginine (30). Ghlissi et al. suggested that dietary ginger for 30 days decreased blood glucose and oxidative stress factor, improved reproductive organ weights and testosterone levels, enhanced semen quantity and motility, and ameliorated antioxidant activities of male diabetic rats (31). Serum testosterone levels in the L-arginine and the 6-gingerol-treated group were significantly increased compared to the HC control group, similar to other studies (32-34). Increasing testosterone levels is a fundamental goal of researchers, not only because of its effect on enhancing lipolysis and its anti-obesity effects but also because of stimulating the fertility process (32, 33). The present study, in agreement with a large group of studies, showed that oral administration of L-arginine and 6-gingerol significantly augmented serum testosterone levels in the study group compared to the HC group. Therefore, the effect of these two agents in increasing testosterone levels and anti-obesity impact can be further studied in treating infertility disorders. The association

Table 2. Effect of 6-gingerol and L-arginine treatment serum lipids profile in normal and experimental obese rats

Groups	Triglyceride (mg dL^{-1})	Total cholesterol (mg dL^{-1})	HDL (mg dL^{-1})	LDL (mg dL^{-1})	VLDL (mg dL^{-1})
ND	63.84±8.06	98.9±2.41	36.44±2.36	50.19±1.94	12.37±0.40
HC	177.12±10.02^{a***}	119.5±4.75^{a**}	28.32±1.9 ^{a**}	56.47±2.07^{a*}	46.12±3.64^{a***}
HCA	93.22±5.67^{b*}	96.31±3.96 ^{b*}	35.6±2.09 ^{b**}	47.19±1.38^{ab*}	23.19±2.31^{b**}
HCG	101.41±8.62^{b**}	93.0±3.68 ^{b*}	34.11±1.32^{b**}	45.58±2.74^{ab*}	30.56±1.7 ^{b*}
HCAG	90.87±6.36^{b**}	94.06±4.38 ^{b*}	34.29±1.26^{b**}	45.60±0.88^{ab*}	19.94±1.01 ^{b**}

*** a: Significant difference from normal control (ND), b: Significant difference from HC. Values are Mean ± SD, n=6; Values are significant at * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001.**

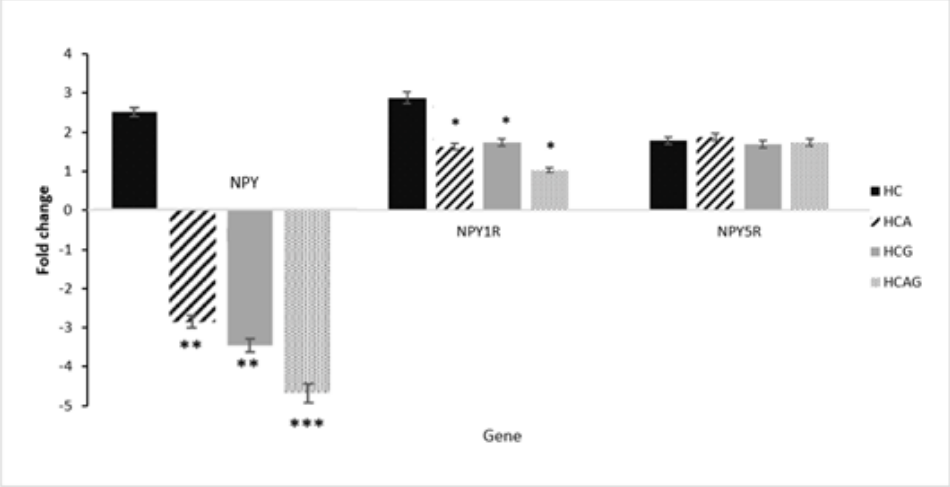

Figure. 2. Effect of 6-gingerol and L-arginine on Y1R, Y5R, and NPY gene expression. Values are statistically significant at *p < 0.05, **0.01, ***0.001. a: significant difference from normal control (ND) and b from HC group. Data are expressed as mean ± standard deviation.

between dietary intakes of L-arginine and serum NOx levels, independent of BMI, was diminished in hypertensive patients. However, NO overproduction in overweight and obese people has been attributed to augmented inducible nitric-oxide synthase (iNOS) activity in response to insulin and pro-inflammatory cytokines (9). The hypolipidemic effects of L-arginine and gingerol (35) reported in the present study agreed with previous studies for L-arginine (30, 33) and gingerol. Previous studies have concluded that both factors can lower triglycerides, total cholesterol, and LDL in humans and rats. The fat-lowering effects of gingerol have been attributed to high levels of flavonoids, polyphenols, and phenols (32) and its inhibitory effect on intestinal cholesterol uptake and hypocholesterolemic activity (30, 36). *In vitro* studies have shown that ginger supplementation can affect glucose transport and insulin resistance. In addition, Isa *et al*. showed that ginger's 6-gingerol and 6-shoall content increased the adiponectin gene expression (37). Adipocytes maintain fat homeostasis by modulating lipogenesis and lipolysis. Various factors such as enzymes, nutrients, hormones, and neurotransmitters control the process of fat synthesis and lipolysis. Studies have shown that amplified lipogenesis is strongly associated with insulin resistance, obesity, and type-2 diabetes. The NPY is a 36-amino acid neurotransmitter protein that is a potent stimulator of appetite and a regulator of energy expenditure, and its mRNA expression level increases under food deprivation (21). Our study revealed that oral administration of 100 mg/kg gingerol and 200 mg/kg L-arginine alone and, in combination, significantly reduced the expression of the NPY gene. These results suggest that 6-gingerol and L-arginine reduce this neurotransmitter production by reducing its mRNA expression level, leading to decreased appetite and reduced fatty acid synthesis. In this regard, food intake in the -gingerol and L-arginine-treated groups was lower than in the HC control group. In addition, previous research has shown that in people with polycystic ovary syndrome (PCOs), the amount of NPY is increased, which is involved in regulating the secretion of sex hormones by controlling GnRH secretion (38). Therefore, our study showed that L-arginine and 6-gingerol reduce the expression level of the NPY gene and can be considered a field of study in treating PCOs and enhancing lipolysis. The orexigenic effects of ghrelin (a peripheral gut hormone) regulated food consumption by releasing the hyperphagic peptides involving NPY and agouti-related protein (AgRP)-expressing neurons and enhancing NPY gene expression (39). The NPY receptors, including Y1R, Y2R, and Y5R antagonists, inhibited the stimulatory effects of ghrelin to control energy homeostasis. Caloric restriction augments the hypothalamic NPY and plasma ghrelin (40). Expression of the ghrelin gene was down-regulated in all groups fed 3% ginger (41). Ginger has regulated the lipid profile by decreasing total cholesterol and enhancing HDL. In addition, ginger can affect some of the peptides involved in appetite stimulation, such as the increase in ghrelin (42). Diet-induced obesity with hypothalamic leptin resistance interferes with the homeostatic control of body weight and appetite. Although Proanthocyanidins (PACs), the phenolic compounds, modulate adiposeness and food consumption by preventing hypothalamic inflammation, they do not reverse hyperleptinemia and body weight gain (43). Flaxseed polysaccharides interfere with body weight and abdominal fat by eliminating leptin resistance and recovering satiety through the downregulation of NPY and the upregulation of glucagon-like peptide 1. Adiponectin activates the AMPK signaling pathway to improve lipid metabolism (44). Furthermore, it has been shown that both 6-gingerol and L-arginine boost NO production, which plays a crucial role in mammalian food intake (45). Therefore, the synergistic effect of these agents could be attributed to triggering the NO cycle. In this line, as revealed in our research, several studies demonstrated that L-arginine (33) and 6-gingerols enhance testosterone levels (35). In conclusion, previous studies confirmed that 6-gingerol and L-arginine showed promising anti-obesity, lipid-lowering, and anti-diabetic effects. High-carbohydrate diet for 12 weeks induced overweight and obesity in rats. Daily oral administration of L-arginine and 6-gingerol maintained the hormone level and lipid profile for six weeks led to obesity development. The impact of these factors on reducing the expression of the NPY gene in the hypothalamus of Wistar rats was proven, which can be considered a new mechanism in the field of anti-obesity that affects these factors. This study provides scientific evidence on the traditional use of ginger and L-arginine supplementation in treating obesity and diabetes.

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Authors' Contribution

All authors contributed to the study's conception and design. Meysam Karbasian and Negar panahi performed material preparation and data analysis. Meysam Karbasian wrote the first draft of the manuscript. Reza Badalzadeh, Delavar Shahbazzade, and Seyed Hamed Shirazi-

Beheshtiha performed the writing review, editing, and data analysis.

Ethics

This study was approved by the National Committee on Ethics in Biomedical Research (ethics.research.ac.ir) of the Islamic Azad University, Science and Research Branch and approved with the ethics ID IR.IAU.SRB.REC.1396.189. Moreover, according to Helsinki's declaration, it was conducted with appropriate caution to respect the animals' welfare in this research.

Conflict of Interest

The authors declare no conflict of interest.

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