Single Head-Neck Irradiation Intervention Increases Thyroid Hormone Level and Enhances Energy Metabolism in High-Fat Diet Mice

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Abstract: Radiotherapy, an established treatment of malignant diseases of the head and neck, increases the risk of chronic metabolic disorders. However, the molecular mechanisms responsible for metabolic dysfunction after irradiation remain unknown. We aimed to determine whether single head-neck irradiation intervention changes the levels of thyroid hormones and affects energy metabolism in high-fat diet mice and in chow diet mice. C57BL/6 mice were treated with a single dose of 6 Gy X-ray head-neck irradiation and were fed a high-fat diet. Body weight, accumulated food intake, fasting blood glucose and glucose tolerance were measured during the study. Plasma, brown adipose tissue, thyroid, liver and white adipose tissue were collected for histological analysis. We found that head-neck irradiation significantly increased food intake and decreased body weight in high-fat diet mice. However, there were no obvious changes in chow diet mice. Further studies showed that head-neck irradiation significantly increased levels of 3,3',5'-triiodothyronine and thyroid-stimulating hormone, as well as expression of uncoupling protein 1 in brown adipose tissue and glucose transporter 2 in liver in high-fat diet mice. Our results suggest that single head-neck irradiation intervention increases thyroid hormones levels and enhances energy metabolism in high-fat diet mice.

Keywords: head-neck, irradiation, HFD, TH, energy metabolism, Ucp-1, GLUT2

1. Introduction

In recent years, the incidence of head and neck tumours has increased significantly. Annually, an estimated 650,000 new cases of head and neck squamous cell carcinoma are diagnosed worldwide [1]. Radiotherapy is an established treatment of malignant diseases of the head and neck [2]. The role of irradiation therapy to ensure tumour control and to avoid cancer recurrence has been well established since the 1900s and has been continuously increasing owing to several benefits, including time efficiency, adequate tolerance and cost-effectiveness [3]. However, patients with cancer treated with irradiation therapy have increased risk for developing metabolic disorders such as type 2 diabetes, hyperinsulinemia, and components of the metabolic syndrome [4-7]. Nevertheless, the molecular mechanisms underlying the changes in metabolic function after irradiation are not well understood.

Thyroid hormone (TH) controls key metabolic processes including carbohydrate and lipid metabolism as well as energy metabolism [8]. TH is an important determinant of energy expenditure that contributes to appetite regulation. Secretory products from adipose tissue act on the central nervous system (CNS) to provide information about the quantity of energy stores, and this may have an impact on the activity of the hypothalamus–pituitary–thyroid axis [9-11]. TH production is controlled by thyroid-stimulating hormone (TSH) that is secreted by the anterior pituitary gland [12]. The hormone 3,3',5'-triiodothyronine (T3), derived from the prohormone levothyroxine (T4), is the predominant bioactive form of TH [13]. In the context of hyperthyroidism, especially elevation of T3 levels, body weight
increased while the appetite increased due to the promoted lipid turnover in white adipocyte and the increased heat production in brown adipocyte, associated with augmentation of total energy expenditure [12]. White adipose tissue (WAT) is the main depot where metabolic energy is stored in the form of triglycerides, and brown adipose tissue (BAT) is the main site of non-shivering thermogenesis in mammals. Brown adipocytes contain many highly oxidizing mitochondria and have uncoupling protein-1 (Ucp-1) in the inner membrane [14]. Ucp-1, which is expressed only in brown adipocytes, uncoupled the respiratory chain from oxidative phosphorylation, producing a high oxidation rate and enabling cells to use metabolic energy to provide heat [15]. BAT produces heat to adapt the organism to the cold environment and prevents obesity by promoting energy expenditure in rat and mouse models [16]. Therefore, we hypothesized that the effect of head-neck irradiation on energy metabolism might be mediated by changing levels of TH and affecting the metabolism of adipose tissue.

TH not only has a profound effect on body weight, thermogenesis and lipolysis, but also has a profound effect on carbohydrate metabolism. TH, especially T3, affects the surrounding glucose metabolism through its effects on many organs, especially the liver [17]. Obvious hyperthyroidism is related to increased endogenous glucose production in the liver and increased lipolysis and proteolysis, thereby providing the necessary substrates for the subsequent increase in energy expenditure [18]. The increase in endogenous glucose production is promoted by the increased activity of liver gluconeogenic enzymes (including phosphoenolpyruvate carboxykinase and pyruvate carboxylase), and the liver expression of glucose transporter 2 (GLUT2) also increases [19]. GLUT2 is the main glucose transporter in the liver, which promotes the bidirectional transport of glucose through the cell membrane [20]. Liver GLUT2, as a glucose sensor, plays a vital role in regulating systemic glucose homeostasis [21]. Therefore, we suspected that the effects of irradiation on metabolism were also related to the liver GLUT2.

A recent study demonstrated that ionizing radiation potentiated high-fat diet (HFD) to induce an insulin resistance state in skeletal muscle and adipose progenitor cells taken from C57BL/6 mice after irradiation at a single dose ranging from 3 to 6 Gy [22]. It is thought that nutritional rapport is crucial in the context of irradiation therapy or exposure. Consequently, in this study, we aimed to determine whether single head-neck irradiation intervention changes the levels of TH and affects energy metabolism in HFD mice and in chow diet (CD) mice.

2. Results
2.1 Effects of Single Head-Neck Irradiation Intervention on Body Weight and Accumulated Food Intake

To determine how head-neck irradiation affects metabolism, we recorded changes in body weight and accumulated food intake before the mice were sacrificed. HFD mice exhibited increases in body weight compared with CD mice. In response to irradiation, body weight decreased significantly in mice subjected to irradiation and HFD compared with non-irradiated mice fed a HFD from 7th week after head-neck irradiation (P < 0.05; Figure 1a and 1b). There was no obvious change in CD mice. The trend of body weight in irradiated mice fed a HFD was similar to that of CD mice.

An analysis of feeding behaviour showed that single head-neck irradiation intervention significantly increased accumulated food intake compared with sham-treated counterparts in HFD mice. At 9 weeks post-irradiation, the weight of accumulated food intake in the mice subjected to irradiation and HFD was heavier than the mice subjected to non-irradiation and HFD, and the weight of accumulated food intake in the mice subjected to irradiation and HFD was also heavier than the mice subjected to irradiation and CD in the first two hours (P < 0.05; Figure 1c). Moreover, our statistic on the calorie of accumulated food intake showed that it was heavier in the mice subjected to irradiation and HFD compared with the mice.
subjected to non-irradiation and HFD \( (P < 0.05; \text{Figure 1d}) \) as well as the mice subjected to irradiation and CD \( (P < 0.01; \text{Figure 1d}) \). However, there was no significant difference in CD mice.

\[ \text{Figure 1} \]

2.2 Effects of Single Head-Neck Irradiation Intervention on Thyroid Function

To investigate whether head-neck irradiation affects thyroid function, fresh thyroid samples were received (Figure 2a) and thyroid follicles showed abnormal changes by H&E staining. In CD mice, thyroid follicles showed an irregular structure and were devoid of colloid in irradiated mice compared with non-irradiated mice. In HFD mice, thyroid follicles showed an irregular structure and were devoid of colloid in non-irradiated mice compared with irradiated mice (Figure 2b).

We further studied levels of thyroid hormones. T3 levels were lower in non-irradiated mice fed a HFD compared with non-irradiated mice fed a CD \( (P < 0.05; \text{Figure 2c}) \). T3 levels were higher in irradiated mice fed a HFD compared with non-irradiated mice fed a HFD \( (P < 0.05; \text{Figure 2c}) \). T3 levels were not altered in CD mice. The changes of TSH levels was consistent with T3 levels: TSH levels were lower in non-irradiated mice fed a HFD compared with non-irradiated mice fed a CD \( (P < 0.05; \text{Figure 2d}) \); TSH levels were higher in irradiated mice fed a HFD compared with non-irradiated mice fed a HFD \( (P < 0.05; \text{Figure 2d}) \); TSH levels were not altered in CD mice.

\[ \text{Figure 2} \]
2.3 Effects of Single Head-Neck Irradiation Intervention on The Expression of Ucp-1 in BAT

We next studied BAT, the vital organ of energy metabolism. Fresh interscapular BAT gross samples were received, and we found that BAT samples appeared shallower in non-irradiated mice fed a HFD; however, irradiated mice fed a HFD exhibited darker BAT samples than those of the mice subjected to non-irradiation and HFD (Figure 3a). We found no changes in the weight of BAT between the four groups ($P > 0.05$; Figure 3b).

Brown adipose cells were larger in the mice subjected to irradiation and CD and in the mice subjected to non-irradiation and HFD than in control-treated mice according to H&E staining, and the volume of brown adipose cells in the mice subjected to irradiation and HFD were smaller than those of mice subjected to non-irradiation and HFD, which appeared similar to those of control-treated mice (Figure 3c). Furthermore, the number of brown adipose cells per unit area decreased in the mice subjected to non-irradiation and HFD compared with control-treated mice, but increased in the mice subjected to irradiation and HFD compared with the mice subjected to non-irradiation and HFD ($P < 0.05$; Figure 3d).

The expression of Ucp-1 in BAT was significantly greater in irradiated mice fed a HFD compared with non-irradiated mice fed a HFD and irradiated mice fed a CD ($P < 0.05$; Figure 3e). But there was no significant change in CD mice.
2.4 Effects of Single Head-Neck Irradiation Intervention on the Weight and Morphology of WAT

To determine whether head-neck irradiation affects WAT, subcutaneous fat such as inguinal fat and visceral fat such as epididymal fat were both weighed. HFD increased the weight of inguinal fat and epididymal fat compared with CD mice, while irradiation decreased the effect of HFD \((P < 0.05; \text{Figure 4a and 4b})\).

White adipose cells were larger in the mice subjected to non-irradiation and HFD than in control-treated mice according to H&E staining, and the volume of white adipose cells in the mice subjected to irradiation and HFD was smaller than the mice subjected to non-irradiation and HFD, which appeared similar to control-treated mice (Figure 4c). Furthermore, the number of white adipose cells per unit area decreased in the mice subjected to non-irradiation and HFD compared with control-treated mice \((P < 0.01; \text{Figure 4d})\), but increased in the mice subjected to irradiation and HFD compared with the mice subjected to non-irradiation and HFD \((P < 0.05; \text{Figure 4d})\).
2.5 Effects of Single Head-Neck Irradiation Intervention on Glucose Tolerance and Liver GLUT2

To determine whether head-neck irradiation also influences glucose metabolism, we further measured fasting blood glucose and glucose tolerance before mice were sacrificed. Fasting blood glucose levels were not significantly different among four groups (Figure 5a). Next, we performed IPGTT at 5 and 9 weeks post-irradiation. Glucose tolerance was significantly impaired in HFD mice, however, irradiation improved the effect of HFD ($P < 0.05$; Figure 5b and 5c). Nevertheless, in CD mice, the result of glucose tolerance showed no obvious change between two groups.

Next, we examined the possible molecular mechanisms of glucose-regulating effects of head-neck irradiation. The expression of GLUT2 level in liver were measured, and results showed significantly greater expression of liver GLUT2 level in irradiated mice fed an HFD than the mice subjected to non-irradiation and HFD and the mice subjected to non-irradiation and HFD ($P < 0.05$; Figure 5d). However, there were no change in CD mice.

![Figure 5](image_url)

3. Discussion

As previous studies have shown, the development of advanced radiotherapy technologies such as stereotactic body radiotherapy, which provides high-dose radiotherapy in a single or small fraction, has made radiotherapy increasingly used for cancer treatment [23-25]. However, some patients receiving radiation therapy exhibit a series of side effects that may cause treatment interruption or radiation dose limitation. In fact, irradiating normal tissues will cause a series of chain reactions including oxidative stress, and ultimately lead to changes in biological functions [26]. Exposure to ionizing radiation also increases the risk of chronic metabolic disorders such as insulin resistance and type 2 diabetes. Nylander et al. [22] demonstrated that mice subjected to a single dose 6 Gy total body irradiation showed alterations in glucose metabolism. However, the effects of local radiation interventions such as head and neck radiation on metabolism remain unclear. In this study, normal C57BL/6...
mice were treated with a single dose of 6 Gy X-ray head-neck irradiation. Moreover, high fat calorie intake has been significantly increasing in modern societies and it is known that HFD is the basis of many complicated health problems such as obesity, type 2 diabetes, cardiovascular diseases and metabolic syndromes in model animals [27-29]. Therefore, mice received either CD or HFD in this study to determine whether the effects of head and neck irradiation intervention on metabolism were different. Body weights in mice fed a HFD without irradiation were significant greater than those of other groups. Interestingly, irradiation may diminish the effect of HFD. We found that body weights in irradiated mice fed a HFD were less than those of non-irradiated mice fed an HFD, but were similar to those of CD mice with or without irradiation. By contrast, in HFD mice, head-neck irradiation increased accumulated food intake compared with that of irradiated mice. However, there was no obvious change in CD mice. It is well known that increased food intake but decreased body weight are typical characteristics of enhanced energy metabolism. Apparently, single head-neck irradiation intervention did not influence the energy metabolism of CD mice, but significantly increased the energy metabolism of HFD mice. We further investigated the molecular mechanism by which single head-neck irradiation intervention may affect energy metabolism.

The thyroid gland is usually located in the front and lower neck and plays a key role in regulating metabolic functions, including cardiac rate and output, lipid catabolism, bone growth, and oxygen and heat production [30]. For decades, TH has been considered a hormone that has a profound effect on energy expenditure and the ability to control weight [13]. TH increases the compulsory thermogenesis, which is due to the stimulation of many metabolic pathways, including development, remodeling, and energy delivery to tissues. In addition, TH may particularly stimulate the selected heat generation mechanism during the constant temperature evolution. TH also plays an important role in facultative thermogenesis that interacts with the sympathetic nervous system (SNS) at different levels. At the periphery, TH enhances the role of SNS at the level of adrenergic receptor and adenosine cyclase complex, and the distal role from this point [31]. Santini et al. [12] have reported that despite increased appetite, hyperthyroidism is usually associated with a variable decrease in body weight, due to a decline in both lean and fat mass caused by strengthening the influences of the SNS, associated with an increase in total energy expenditure. Our results showed that single head-neck irradiation intervention destroyed the structure of thyroid follicles; however, the levels of T3 and TSH were unaltered in CD mice. When mice were fed with a HFD for 10 weeks, thyroid follicles showed irregular structures and were devoid of colloid; however, they returned to normal health state in mice subjected to irradiation and HFD. It is possible that single head-neck irradiation intervention may damage the structure of thyroid follicles without affect the levels of TH in CD mice, but activate the damaged thyroid follicles caused by HFD. The results of TH levels suggested that single head-neck irradiation intervention increased T3 and TSH levels and increased energy expenditure in HFD mice, but did not alter T3 and TSH levels or energy metabolism in CD mice. Nevertheless, in this study, we only detected the levels of T3 and TSH, the most critical TH. Other TH such as TRH, T4 need to be measured in future studies to investigate the effects of single head-neck irradiation intervention on TH levels.

The classical view is that the metabolism of T3 is mediated through its effects on peripheral organs such as liver, muscle and BAT. In these organs, T4 is converted to T3, activating a series of tissue-specific T3-dependent genes, thereby regulating metabolism and energy consumption [32]. Compared with WAT, which mainly provides energy storage and release based on system requirements, BAT is a special organization that consumes energy through heat generation. The unique functional role maintained by brown and white adipose tissue is also obvious in structure-BAT has dense mitochondria content, innervated by sympathetic nerve fibers, and is highly vascularized, while WAT has few mitochondria and is much less
innervated and vascularised. Mitochondrial uncoupling in BAT is mediated by Ucp-1, a 32-kDa protein expressed in the inner membrane of the mitochondria; this activity dissipates the proton gradient of the inner mitochondrial membrane without adenosine diphosphate (ADP) phosphorylation. Since BAT can consume energy by producing calories, it is a promising target for potential obesity treatment [33, 34]. Thyroid hormone signals substantially affect energy balance and accelerate energy expenditure, partly by activating BAT through TH activation mediated by local D2 (type II deiodinase). As shown in animal and cell models, d2-dependent T3 is necessary for the tissue properties and functions of BAT, and for brown adipogenesis [33]. In this study, we showed that, although single head-neck irradiation intervention enlarged the volume of brown adipose cells in mice fed a CD, the weights of BAT and WAT, the expression of Ucp-1 and the volumes of adipose cells did not show significant changes in CD mice with or without irradiation. This could be due to the insignificant effect of single head-neck irradiation intervention on mice fed a CD. However, increased expression of BAT Ucp-1 levels, decreased weight of white fat, and changes in morphology of adipose cells in irradiated mice fed a HFD suggested that single head-neck irradiation intervention changes TH and affects lipid metabolism in HFD mice.

Glucose metabolism, like lipid metabolism, is also an important form of energy expenditure. We next assessed fasting blood glucose and glucose tolerance to determine whether single head-neck irradiation intervention influences carbohydrate metabolism. Glucose homeostasis is strictly regulated to meet the energy needs of vital organs and maintain health. The liver plays an important role in controlling glucose homeostasis by controlling various pathways of glucose metabolism, including glycogenesis, glycolysis, and gluconeogenesis [35]. Previous studies have shown that the liver of mammals is a classic organ for studying the effects of TH. TH regulates liver lipid and carbohydrate metabolism in a multi-layered complex manner. T3-bound thyroid receptors directly upregulate target genes and key gluconeogenic enzymes in the liver, such as phosphoenolpyruvate carboxykinase 1 and glucose-6-phosphatase. The increase in deacetylation and activation of Forhead box O1, a major gluconeogenic transcription factor caused by SirT1, is also regulated by T3 to increase gluconeogenesis. In addition to regulating the transcription of gluconeogenesis genes, increased alanine transport and inhibition of insulin signaling may also lead to TH-induced liver glucose production [36]. GLUT2 is the main glucose transporter in rodent and human liver cells [37, 38]. The generally accepted role of this transporter is to absorb glucose during the absorption phase and release glucose in the blood during fasting. In hepatocytes, glucose can be stored as glycogen, degraded through glycolysis or converted into fatty acids through lipogenesis. The release of glucose in the circulation follows the degradation of glycogen or gluconeogenesis [20]. Our results indicated that HFD mice impaired glucose tolerance; however, there was no significant difference in glucose tolerance between the mice subjected to irradiation and HFD and control-treated mice. We proposed that single head-neck irradiation intervention restored damaged glucose tolerance to normal levels in mice fed a HFD. In this study, we found elevated levels of GLUT2 in liver in irradiated mice fed a HFD compared with non-irradiated mice fed a HFD. This might suggest that liver GLUT2 is involved in the effects of single head-neck irradiation intervention on glucose metabolism. We consider that when blood glucose levels rose, single head-neck irradiation intervention enhanced the role of GLUT2 in transporting glucose from the blood to hepatocytes, where it was converted by gluconeogenesis into glycogen for storage in the liver. However, interestingly, single head-neck irradiation intervention did not alter fasting blood glucose.

As is shown in Figure 6, we first observed that single head-neck irradiation intervention changed levels of TH, affected lipid metabolism and glucose metabolism as well as energy metabolism, and these effects varied in response to different diets.
In conclusion, our results provide insight into mechanisms by which single head-neck irradiation intervention affects energy metabolism in different nutritional states. Results demonstrate that single head-neck irradiation intervention altered the structure of thyroid follicles and the morphology of adipose tissue, decreased weights of adipose tissue, increased levels of T3, TSH, the expression of Ucp-1 in BAT and the expression of GLUT2 in liver, and that it maintained energy homeostasis in HFD mice. These results shed some light on the impact of the irradiation exposure on TH and energy metabolism, and this effect is closely related to nutritional behavior.

4. Materials and Methods

4.1 Animals and Treatments

Male C57BL/6 mice were purchased from Qingdao Institute of Drug Control when they were 8 weeks old and weighed between 20 g and 22 g. They were kept in a 12-h light/dark cycle (illumination from 8:00 to 20:00) and were housed in standard housing conditions (23–25°C and 56 ± 20% humidity). Food and water were provided ad libitum. All the procedures used in this study were approved by Qingdao University Animal Care and Use Committee and were in accordance with the National Institutes of Health guidelines. The study was carried out in compliance with the ARRIVE guidelines.

After 2 weeks of acclimatization, 10-week-old mice were irradiated in the head and neck with a single dose of 6 Gy X-ray (n = 15) at 9 am (Figure 7a). Non-irradiated mice (n = 15) were sham-treated. One day later, irradiated mice and non-irradiated mice were randomly assigned into CD and HFD (15% lard, 3% soybean oil, 5% egg yolk, 18% sugar, and 59% chow diet) for 10 weeks. All mice were randomly divided into four groups: Ctrl+CD (n = 7), Irr+CD (n = 7), Ctrl+HFD (n = 8), and Irr+HFD (n = 8).

Body weight and accumulated food intake were measured every week. In the 5th and 9th week after head-neck irradiation, we measured changes of blood glucose using the intraperitoneal glucose tolerance test (IPGTT). At the end of the ten-week study, a total of 25 mice (five mice from various groups died unexpectedly) were sacrificed under anaesthesia (33 mg/kg pentobarbital, intraperitoneal injection) for histological analysis. Hypothalamus, plasma, BAT, thyroid, liver and WAT tissues were collected and fixed in 4% paraformaldehyde or stored at -80°C for later use. The timeline of the study is found in Figure 7b.
4.2 Body Weights and Adipose Weights

Body weights were measured using an electric balance (Mettler Toledo, PL1501-S, Shanghai, China) every Monday at 8 am. Mice were sacrificed following the 10-week study. BAT from the interscapular region and WAT from inguinal and epididymal regions were weighed using an electronic precision scale (TE412-L; Sartorius, Göttingen, Germany).

4.3 Accumulated Food Intake

Every Tuesday during the study, we changed the bedding and withheld food at 8 am. Then, accumulated food intake after refeeding was measured using an electronic precision scale (TE412-L; Sartorius, Göttingen, Germany) from 8 pm to 8 am the next day. All initial and the remaining food was weighed every hour.

4.4 Intraperitoneal Glucose Tolerance Test (IPGTT)

The beddings were changed and feed were withheld at 8 pm. After 14 hours, all mice were treated with 20% glucose (2 g/kg body weight) by intraperitoneal injection. Blood glucose concentration was measured at 0, 15, 30, 60, 90, 120 minutes by tail vein blood using a tail vein prick and a glucometer (B, Braun, Meusungen AG, Germany).

4.5 Hematoxylin-Eosin (H&E) Staining

Thyroids, BAT, and WAT were fixed in 4% formaldehyde for 24 h and then in 70, 80, 85, 90, 95, 100, 100% ethanol for 30, 30, 30, 30, 20 and 20 minutes, respectively. Then tissues were transferred into dimethylbenzene for 5 minutes, following immersion in soft wax, hard wax, and mixed waxes for 90, 60, and 30 minutes, respectively. Tissues were embedded in mixed paraffin prior to sectioning. Thyroids, BAT, and WAT were cut into 6-μm sections using a paraffin slicing machine (RM2016; Leica, Wetzlar, Germany). Sections were baked for 4 hours in a 60 degree oven. After dewaxing with dimethylbenzene for 15 minutes and hydration with 100, 100, 90, 80 and 70% of ethanol for 5, 5, 4, 3 and 2 minutes, respectively. Sections were stained using a H&E Staining Kit (G1120; Solarbio, Beijing, China). Cell numbers were counted using Image J (version 1.8.0).

4.6 Western Blot Analysis

BAT and livers were homogenized in ice-cold standard RIPA buffer (Beyotime, P0013B, China), followed by centrifugation at 4°C for 15 minutes at 12,000 r/minutes. A BCA Protein Assay Kit (P0012; Beyotime, Shanghai, China) and a microplate reader (M5; MD-SpectraMax, Molecular Devices, San Jose, CA, United States) were used to determine protein concentrations. Equal amounts (35 μg/10 μl) of protein were subjected to SDS-PAGE, followed by transfer to polyvinylidene fluoride membranes (PVDF, IPVH00010: Millipore, Burlington, MA, US), that were activated with methanol. After blocking with 5% skimmed milk powder in TBST (pH = 7.6–7.9) for 2 h at room temperature, we used primary antibodies in 3% foetal bovine serum (9048-46-8, Solarbio) incubated at 4°C overnight (UCP1: rabbit IgG, 1:2,000, ab10983, Abcam, US; GLUT2: rabbit IgG, 1:1,000, Abcam, ab54460, US; β-actin: rabbit IgG, 1:2,000, Cell Signaling Technology, D6A8, #8457, USA; GAPDH: rabbit IgG, 1:1,000, Cell Signaling Technology, #5174, USA). The secondary antibody was goat anti-rabbit IgG H&L (HRP) (1:8,000, ZB-2306 ZSBG-BIO, China) incubated at room temperature for 1 h. The protein bands were developed using the Immobilon Western Chemiluminescent Substrate (Millipore, WBKLS0100) [39].

4.7 Plasma TSH and T3 Level Measurement
Plasma TSH and T3 levels were measured using a Mouse TSH ELISA Kit (F2564-A, FANKEL, CN) and a Mouse T3 ELISA Kit (F2574-A, FANKEL, CN) following manufacturer’s instructions. A standard curve was established along with actual samples. After data acquisition using a plate reader (M5, MD-SpectraMax, Molecular Devices, San Jose, CA, USA) at 450 nm absorption, the plasma concentrations of TSH and T3 were calculated according to the standard curve.

4.8 Statistical Analysis

All data were presented as means ± standard error of the means (SEMs). ANOVA followed by a post hoc least significance difference test was used for comparisons between Ctrl+HFD group and Ctrl+CD group, Irr+CD group and Ctrl+CD group, Irr+HFD group and Ctrl+HFD group, Irr+HFD group and Irr+CD group. The least significance difference test was used as the post hoc test. P < 0.05 denoted statistical significance. GraphPad Prism 22.0 and SPSS 7.0 (Statistical Product and Service Solutions) were used to create graphs and perform statistical analyses.

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Author Contributions
Conceptualization, Q.L. and J.D.; methodology, Q.L. and C.Z.; software, Q.L. and M.L.; validation, Q.L., J.D. and P.W.; formal analysis, H.W.; investigation, Q.L. and K.S.; resources, Q.L. and D.L.; data curation, Q.L., J.Z. and P.J.; writing—original draft preparation, Q.L.; writing—review and editing, J.D.; visualization, Q.L. and C.Z.; supervision, J.D. and P.W.; project administration, J.D.; funding acquisition, J.D.. All authors have read and agreed to the published version of the manuscript.

Competing interests
The authors declare no competing interests.

Figure legends
Figure 1. Single head-neck irradiation intervention decreases body weight and increases accumulated food intake in HFD mice. (a): Changes in body weight over 10 weeks. (b): Gross morphology. (c): Cumulative weights of food intake were counted 9 weeks post irradiation. (d): Cumulative calories of food intake were counted 9 weeks post irradiation. Data are presented as means ± SEM (Ctrl+CD: n = 4; Irr+CD: n = 6; Ctrl+HFD: n =7; Irr+HFD: n = 8). ∗P < 0.05 for the effect of irradiated mice fed an HFD vs. non-irradiated mice fed an HFD, †P < 0.05 for the effect of irradiated mice fed an HFD vs. irradiated mice fed a CD, ‡P < 0.01 for the effect of irradiated mice fed an HFD vs. irradiated mice fed a CD. Ctrl, non-irradiated; Irr, Irradiated; CD, chow diet; HFD, high-fat diet.

Figure 2. Effects of single head-neck irradiation intervention on the structure of thyroid follicles and TH levels. (a): Fresh thyroid samples. (b): H&E staining of thyroid follicles (400×). (c): Plasma T3 levels measured using a Mouse T3 ELISA Kit. (d): Plasma TSH
levels measured using a Mouse TSH ELISA Kit. Data are presented as means ± SEM (Ctrl+CD: n = 3; Irr+CD: n = 3; Ctrl+HFD: n = 3; Irr+HFD: n = 3). *P < 0.05 for the effect of irradiated mice fed an HFD vs. non-irradiated mice fed a HFD, †P < 0.05 for the effect of non-irradiated mice fed an HFD vs. non-irradiated mice fed a CD. Ctrl, non-irradiated; Irr, Irradiated; CD, chow diet; HFD, high-fat diet.

**Figure 3.** Single head-neck irradiation intervention changes the morphology of BAT cells and increases the expression of BAT Ucp-1 in HFD mice. (a): Fresh interscapular BAT gross samples. (b): The weight of BAT. (c): H&E staining of BAT cells (400×). (d): The number of BAT cells from the same unit areas were counted and recorded. (e): The level of BAT Ucp-1 was measured through the western blot, with β-actin as internal control. Data are presented as means ± SEM (Ctrl+CD: n = 3; Irr+CD: n = 3; Ctrl+HFD: n = 4; Irr+HFD: n = 4). *P < 0.05 for the effect of irradiated mice fed an HFD vs. non-irradiated mice fed an HFD, †P < 0.05 for the effect of irradiated mice fed an HFD vs. irradiated mice fed a CD, ‡P < 0.05 for the effect of non-irradiated mice fed an HFD vs. non-irradiated mice fed a CD. Ctrl, non-irradiated; Irr, Irradiated; CD, chow diet; HFD, high-fat diet.

**Figure 4.** Effects of single head-neck irradiation on weight and morphology of WAT. (a): The weight of inguinal fat. (b): The weight of epididymal fat. (c): H&E staining of WAT cells (400×). (d): The number of WAT cells from the same unit areas were counted and recorded. Data are presented as means ± SEM (a and b, Ctrl+CD: n = 4; Irr+CD: n = 6; Ctrl+HFD: n = 7; Irr+HFD: n = 8. c and d, Ctrl+CD: n = 3; Irr+CD: n = 3; Ctrl+HFD: n = 3; Irr+HFD: n = 3). *P < 0.05 for the effect of irradiated mice fed an HFD vs. non-irradiated mice fed an HFD, †P < 0.05 for the effect of irradiated mice fed an HFD vs. non-irradiated mice fed an HFD, ‡P < 0.05 for the effect of non-irradiated mice fed an HFD vs. non-irradiated mice fed a CD, ^P < 0.01 for the effect of non-irradiated mice fed an HFD vs. non-irradiated mice fed a CD. Ctrl, non-irradiated; Irr, Irradiated; CD, chow diet; HFD, high-fat diet.

**Figure 5.** Single head-neck irradiation intervention affects glucose tolerance and increases the expression of liver GLUT2 in HFD mice. (a): Fasting blood glucose was tested 9 weeks post irradiation. (b): IPGTT was tested 5 weeks post irradiation. (c): IPGTT was tested 9 weeks post irradiation. (d): The level of liver GLUT2 was measured through the western blot, with GAPDH as internal control. Data are presented as means ± SEM (a, b and c, Ctrl+CD: n = 4; Irr+CD: n = 6; Ctrl+HFD: n = 7; Irr+HFD: n = 8. d, Ctrl+CD: n = 3; Irr+CD: n = 4; Ctrl+HFD: n = 4; Irr+HFD: n = 4). *P < 0.05 for the effect of irradiated mice fed an HFD vs. non-irradiated mice fed an HFD, **P < 0.01 for the effect of irradiated mice fed an HFD vs. non-irradiated mice fed an HFD, †P < 0.05 for the effect of irradiated mice fed an HFD vs. non-irradiated mice fed a HFD, ‡P < 0.01 for the effect of irradiated mice fed an HFD vs. non-irradiated mice fed a HFD, ^P < 0.05 for the effect of non-irradiated mice fed an HFD vs. non-irradiated mice fed a CD, ^P < 0.01 for the effect of non-irradiated mice fed an HFD vs. non-irradiated mice fed a CD. Ctrl, non-irradiated; Irr, Irradiated; CD, chow diet; HFD, high-fat diet.

**Figure 6.** Mechanisms by which single head-neck irradiation intervention affects energy metabolism. CD, chow diet; HFD, high-fat diet; (-), have no influence; (+), improve; (↓), decrease; (↑), increase; TH, thyroid hormones; T3, 3,5,3′-triiodothyronine; TR, TH receptor; SNS, sympathetic nervous system; D2, type II deiodinase; Ucp-1, uncoupling protein 1; GLUT2, Glucose transporter 2.

**Figure 7.** Schedule diagram and irradiation intervention in this study. (a): Ten-week-old C57BL/6 mice received a single dose of 6 Gy X-ray head-neck irradiation. (b): The whole experimental design. CD, chow diet; HFD, high-fat diet.