Original Article

Different Effects of Obesity and Fasting on the Expression of Type 3 Deiodinase and Thyroid Hormone Receptors in the Liver and Visceral Adipose Tissue of C57BL/6 Male Mice

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Abstract

Introduction: Energy status can alter thyroid hormone signalling in different tissues. Little is known about the effect of fasting on the local thyroid hormone metabolism under high-fat diet (HFD)-induced obesity. We aimed to investigate the fasting effect on deiodinase type 3 (DIO3) and thyroid hormone receptors (TRs) expression in liver and visceral adipose tissue (VAT) of HFD-induced obese mice. **Methods:** The 30 male C57BL/6 mice were divided into three groups (n = 10/group): control (CON) group, obese (OB) group, and fasted obese (OBF) group. **Materials:** In a 14-week study, the expression levels of DIO3 and TRs in the liver and VAT of mice were measured by real-time polymerase chain reaction. Gene expression results were shown as fold changes defined by $2^{-\Delta\Delta ct.}$ Comparison between groups was performed by using one-way-ANOVA or Kruskal-Wallis ANOVA test. **Results:** In the liver, there was a significantly lower expression of DIO3 and higher expression of TRs in obese fasted mice compared to obese mice. Compared to the lean mice, OBF mice had significantly lower expression of DIO3 and higher expression of TRβ. In the VAT, mRNA expression of DIO3 was significantly increased in OBF and OB groups compared to the CON group. There were no significant differences in the mRNA expression of TRs between groups. **Conclusion:** Our findings suggest that fasting may be more effective in improving thyroid hormone metabolism in the liver rather than the VAT of obese mice.

Keywords: DIO3, fasting, male mice, obesity, thyroid hormone receptors

Introduction

Deiodinases are selenium-containing enzymes that regulate thyroid hormones signaling locally and independently of serum TH levels in tissue. Three types of deiodinase have been identified: deiodinase type 1 (D1) and type 2 (D2) convert T4 to the active form T3, and deiodinase type 3 enzyme (D3) converts T4 and T3 to the inactive forms rT3 and T2, respectively.[1] Most thyroid hormone actions are mediated by thyroid hormone receptors (TRs). The two major isoforms of TRs, TRα1 and TRβ1, are differentially distributed in tissues. TRβ1 is the main receptor in the liver, whereas TRα1 is prevalent in the adipose tissue.[2]

Obesity is a chronic, low-grade inflammatory disease associated with adipose tissue dysfunction, systemic inflammation, and insulin resistance.[3] It is well documented that unhealthy expansion of white adipose tissue (WAT) through hypertrophy leads to local adipose tissue hypoxia,[4] which increases adipose tissue inflammation in a hypoxia-inducible factor 1-alpha (HIF-1α)-dependent pathway.[3] Interestingly, induction of DIO3 expression through the HIF-1α-dependent pathway is sufficient to reduce tissue T3 content.[5]

Type 3 deiodinase is a physiological T3 inactivator, which is reactivated in several pathological conditions, such as cancer, chronic inflammation and critical illnesses, thus causing local

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reduction of T3 content in the target tissue.[1] The liver plays an essential role in the activation or inactivation of thyroid hormones. In a healthy liver under normal conditions, high expression of DIO1 and low expression of DIO3 are necessary to maintain normal thyroid hormone function.[6]

Visceral adipose tissue (VAT) is a metabolically active tissue that produces more inflammatory effects than subcutaneous adipose tissue.[3] It has been shown that VAT hypertrophy is associated with the onset and progression of non-alcoholic fatty liver disease (NAFLD) in moderate obesity.[7] Furthermore, Zhang *et al*.[8] reported that a high-fat diet (HFD) for 8 weeks caused fat accumulation and hypoxia in the liver.

Several studies have shown that weight loss can reverse obesity-induced changes in thyroid hormone levels in obese subjects.[9,10] However, changes in thyroid hormone levels alone may not reflect intracellular TH metabolism during fasting or obesity. In this context, the study by Pihlajamäki *et al*.[11] showed that liver fat accumulation is associated with decreased intrahepatic thyroid hormone levels in euthyroid obese patients. Thus, changes in the expression of deiodinases and receptors can provide an accurate assessment of thyroid hormone metabolism in the target tissue.[12] No studies have investigated fasting effects on local thyroid hormone metabolism in obesity induced by an HFD. We hypothesize that fasting can reverse the obesity-related changes in thyroid hormone metabolism. We, therefore, investigated obesity-induced alterations in the expression levels of DIO3 and TRs in the liver and VAT of C57BL/6 male mice, and the effect of fasting-induced weight loss on these changes.

Methods

Animal experiment

All experimental procedures were conducted according to the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. Thirty male C57BL/6 mice, 8 weeks old, weighing 26.6 ± 5.67g were purchased from the Pasteur Institute of Iran (Tehran). The C57BL/6 mice are a good model for mimicking human metabolic disorders because they show greater susceptibility to HFD-induced obesity. Due to the influence of physiological changes caused by the female cycle on the experimental results, we chose male mice for this study. All animals were housed in standard plastic cages (alone) at 23 ± 2°C with 50 ± 10% humidity and a 12-hour light/dark cycle at the laboratory animal-keeping centre in the institute. For acclimatisation, animals were fed *ad libitum* with a standard rodent diet and had free access to tap water for 1 week. After a week of adaptation, the mice were randomly divided into three groups: the control group (CON, n = 10) was fed a standard diet (SD), the obese group (OB, n = 10) was fed an HFD and the fasted obese group (OBF, n = 10) was fed an HFD and fasted for 36 h. The HFD contained 5.1 kcal/g (55% fat, 25% carbohydrate, and 20% protein), and the SD contained 3.7 kcal/g (10% fat, 70% carbohydrate, and 20% protein). The composition of HFD and SD is shown in Table 1. Both diets are prepared by the Royan Biotechnology Institute. The treatment lasted 14 weeks, and body weight and food intake were recorded weekly and daily, respectively. This study continued until the mice weight in the HFD groups was 20-25% higher than the control group, at the time the obesity model was induced.[13] At this time, all groups were weighed, and the OBF group was deprived of food for 36 hours (starting at 9:00 p.m.) with free access to water. After 36 hours, the three groups were weighed and sacrificed at 9:00 a.m. under chloroform anaesthesia. The liver and VAT were removed and immediately stored in liquid nitrogen and transferred to a −80°C freezer for further analysis [Figure 1].

RNA isolation, cDNA synthesis, and quantitative real-time polymerase chain reaction

Total RNA was isolated from liver and VAT using RNXplus solution according to the manufacturer's protocol (Cinaclone, Iran). The concentration of extracted RNA was estimated using spectrophotometric analysis using nano drops at 260 and 280 nm, with a ratio of OD260/OD280 > 1.7. Total RNA (1 µg) was used for cDNA synthesis, using First Strand cDNA Synthesis Kit (Cinaclone, Iran), according to the manufacturer's instructions. The real-time polymerase chain reaction (PCR) was performed in duplicate for each sample, including 10 µl BIOFACT™ 2X real-time PCR Mix (including SYBR Green I mixed with high Rox, BIOFACT, South Korea), 7 µl double distilled water, 0.5 µl forward primer, 0.5 µl reverse primer, and 2 µl cDNA (total volume 20 µl). To calculate relative gene expression, samples were normalised to TATA box binding protein (TBP) as an internal control. A real-time PCR system (Applied Biosciences, Paisley, UK) was used to perform PCR reactions of cDNA samples. PCR cycling was initiated at 95°C for 15 min followed by 50 cycles of 95°C for 20 s and 56°C for 30 s. At the end of the cycle, the melting curve was between 60°C and 95°C. Gene expression values were calculated as fold change defined by $2^{-\Delta\Delta ct}$. The sequence of primers used in this study is given in Table 2.

Statistical analyses

Statistical analyses were performed using SPSS software (version 21). All data were presented as means ± SD. The

Table 1: Diet Components

Diet nutrients	SD	HFD
Casein (g/kg)	220	310
Cornstarch (g/kg)	590	280
Sucrose (g/kg)	90	50
Soy oil (g/kg)	45	45
SFA (g/kg)	0	260
Fiber (g/kg)	55	50
Calcium	59	9.5
Phosphorus	4.1	6.5
As carbohydrate (%)	70	25
As fat (%)	10	55
As protein (%)	20	20
Energy (kcal/g)	3.74	5.02

SD; standard diet, HFD; high fat diet, SFA; saturated fatty acids

statistically significant differences were considered at $P < 0.05$. The Shapiro-Wilk test was used for the normality of the test, and Levene's test for the homogeneity of variances, followed by one-way analysis of variance (ANOVA) with a Tukey's *post hoc* test when the variances were equal, and the Brown-Forsythe with a Games-Howell *post hoc*, when the variances were not equal. If the data failed Shapiro's Wilk test, the Kruskal-Wallis one-way ANOVA on ranks test was used to compare all the groups. For normally distributed variables, the paired *t*-test was used for intra-group comparisons.

Ethical aspects

This study was approved by the Ethics Committee of the Shahid Beheshti University of Medical Science [IR.SBMU.NNFTRI.REC.1397.002].

Results

Body weight

In this study, all mice fully completed the study. At the beginning of the study, there was no significant difference in body weight between the groups [Table 3]. After 14 weeks, the mean body weight of the CON, OB, and OBF groups was 16.8% ($P = 0.001$), 42% ($P = 0.0001$), and 43.5% ($P = 0.0001$), respectively, above their start weight [Table 3]. The mean body weight of HFD-fed mice (OB and OBF) was significantly ($P = 0.001$ and 0.0001, respectively) higher than that of the SD-fed (CON) group. Moreover, the body weight gain in OB and OBF mice was considerably 19.48% ($P = 0.03$) and 22.3% ($P = 0.03$), respectively, higher than the control group [Table 3]. There was no significant difference in body weight gain and body weight between the OB and OBF groups ($P = 0.99$). To investigate the effect of fasting on local thyroid hormone metabolism, fasted obese mice (OBF) were starved for 36 hours with free access to water before euthanasia. The paired *t*-test showed that 36 hours of fasting led to a 12.6% reduction in body weight ($P = 0.0001$) in OBF mice. However, after 36 hours of fasting, there was no significant difference ($P > 0.05$) in the body weight between the OBF (33.63 ± 3.76 g) compared to the CON group (31.31 ± 4.4 g) [Table 3].

The expression of DIO3 and TRs in the liver

Here, we evaluated the alterations of DIO3 (by using one-way ANOVA, Brown Forsythe followed by Games-Howell

Table 2: Primer sequences used for PCR

Gene	Sequence (5'→ 3')	Product length (bp)
DIO3	F: CGC TGG TTC TAA AGT TCG	116
	R: GAT GTA GAT GAG GAA GTT G	
TRα	F: ATT CCT GCC CGA TTA CAT TG	96
	R: ATG AAC TTG GTA AAC TTG CTG	
TRβ	F: CAA AGT CAG GCG AAA TCA G	128
	R: CCC AGT TCT CCT CTA TCA G	
TBP	F: ACC GTG AAT CTT GGC TGT AAA C	86
	R: GCA GCA AAT CGC TTG GGA TTA	

Bp: base pair

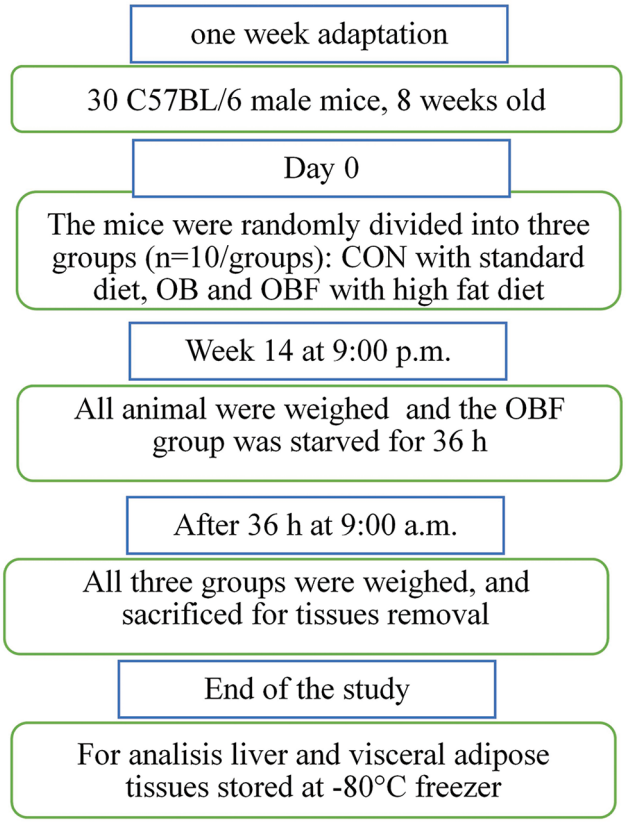

Figure 1: Schematic diagram of the experimental design

Table 3: Body weight change (g) in mice fed SD and HFD for 14 weeks, and mice with 36 hour-fasting

	SD	HFD		36-hour fasting
	CON	OB	OBF	OBF
Total (*n*)	10	10	10	10
Start weight	26.8±5.41	26.34±6.72	26.67±5.38	38.88±3.93
End weight	32.1±3.57*	38.31±2.59†‖	38.88±3.93‡¶	33.63±3.76**
Weight change	5.3±3.18	11.96±6.69§	12.21±7.02§	5.24±0.84

Values are represented as the mean±standard deviation. CON; control, OB; obese, OBF; obese fasted, HFD; high-fat diet, SD; standard diet. *P=0.001, †P=0.0001, ‡P=0.0001 vs start weight, the same column (Student's *t*-test). ‖P=0.001, ¶P=0.0001 vs. CON in the same rows (one-way ANOVA, Brown-Forsythe followed by Games-Howell *post hoc* test). §P=0.03 vs. CON in the same row (one-way ANOVA followed by Tukey's *post hoc* test). **P=0.0001 vs. start weight in the same column (36-hour fasting) (Student's *t*-test)

post hoc) and TRs (TRα and TRβ) (by using one-way ANOVA followed by Tukey's *post hoc*) expression in the liver of CON, OB, and OBF groups. Raw data real-time PCR were analysed, and gene expression results were shown as fold changes defined by $2^{-\Delta\Delta ct}$. The higher ΔCT values indicate lower gene expression. As shown in Figure 2a, the level of DIO3 gene expression decreased by 91% in the OBF group (3.52 ± 2.09) compared to the OB group (0.08 ± 2.74) (P = 0.015). Furthermore, there was a significant 4.8- and 3.8-fold increase in the TRα (3.54 ± 1.77, P = 0.032) and TRβ (3.32 ± 1.19, P = 0.005) expression, respectively, in the OBF group compared to the OB group (5.8 ± 1.99 and 5.25 ± 1.36, respectively) [Figure 2b and c]. The expression levels of DIO3 were elevated 1.9-fold in the OB group (0.08 ± 2.74) compared to the CON group (1.02 ± 0.92), but it is not significant (P = 0.5). Furthermore, there was a non-significant 56% and 22% reduction, respectively, in TRα (5.8 ± 1.99, P = 0.3) and TRβ (5.25 ± 1.36, P = 0.8) expression in the OB group compared to the CON group (4.62 ± 1.88 and 4.9 ± 1.21, respectively) [Figure 2a-c]. In the final analysis of real-time PCR results, we observed that liver DIO3 expression was significantly reduced by 82% (3.52 ± 2.09, P = 0.012) in the OBF group compared to the CON group (1.02 ± 0.92) [Figure 2a]. In addition, the expression of TRβ (3.32 ± 1.19, P = 0.02) but not TRα (P = 0.4) was significantly three-fold higher in the OBF group compared to the CON group (4.9 ± 1.21) [Figure 2b].

The expression of DIO3 and TRs in VAT

The one-way ANOVA followed by Tukey's *post hoc* test showed in the VAT, the level of DIO3 gene expression in the obese (OB) group was significantly 12-fold (−1.45 ± 2.61, P = 0.017) higher than the control (CON) group (2.197 ± 2.14) [Figure 3a]. In fasted obese (OBF) mice, mRNA expression of DIO3 was increased nine-fold (−1.12 ±3.39, P = 0.032) as compared to the CON group [Figure 3a]. In response to the question of whether fasting-induced weight loss reverses obesity-induced changes in DIO3 and TRs expression in VAT, as shown in Figure 3, 36-hour fasting led to a 21% decrease in DIO3 expression (−1.12 ±3.39) in VAT of the OBF group compared to the OB group (−1.45 ± 2.61), but it is not significant (P = 0.9). The Kruskal-Wallis nonparametric rank sum test was used to compare the expression level of TRα and TRβ genes in all groups. There was no significant difference in ΔCT mean values of TRα between the three groups, CON (1.61 ± 4.02), OB (4.59 ± 3.73), and OBF (2.48 ± 4.29). Furthermore, there was no significant difference in ΔCT mean values of TRβ between the three groups, CON (1.01 ± 5.07), OB (3.37 ± 3.69), and OBF (2.16 ± 2.62). Although not statistically significant, the mRNA expression of TRα and TRβ in OB mice was 87% and 80%, respectively, lower than the CON group [Figure 3b and c]. Furthermore, the mRNA expression of TRα and TRβ in OBF mice was 4.3- and 2.3-fold, respectively, higher than the OB group.

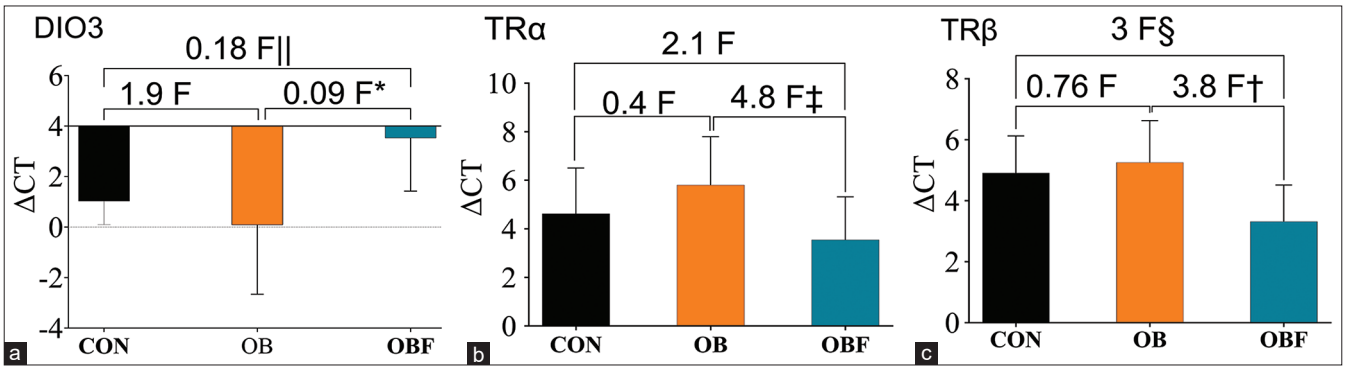

Figure 2: ΔCT mean ± SD values of DIO3 (a), TRα (b), and TRβ (c) gene expressions in the liver between control (CON), obese (OB), and obese fasted (OBF) groups. *P = 0.015; †P = 0.03; ‡P = 0.005; ||P = 0.012; §P = 0.02; N = 10/group

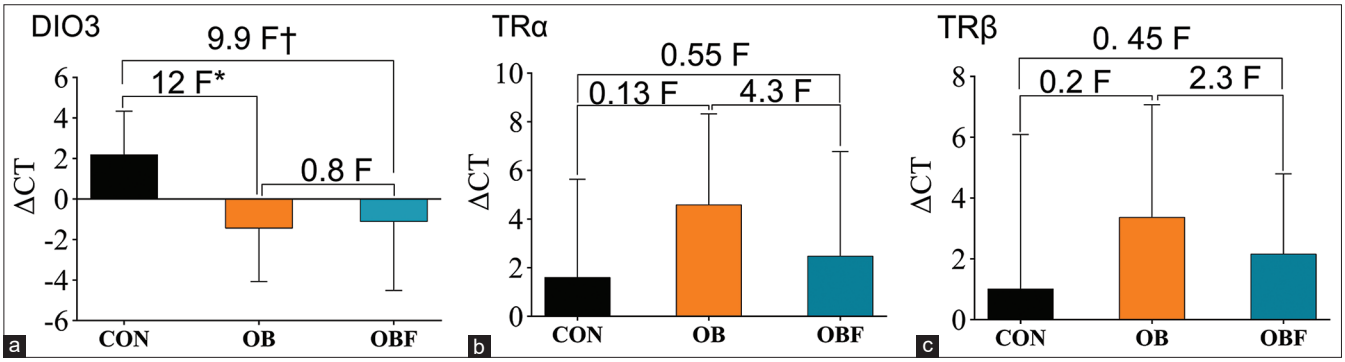

Figure 3: ΔCT mean ± SD values of DIO3 (a), TRα (b), and TRβ (c) gene expressions in the visceral adipose tissue between control (CON), obese (OB), and obese fasted (OBF) groups. *P = 0.017; †P = 0.032; N = 10/group

Discussion

The main finding of the present study is that fasting in HFD-induced obese mice improved thyroid hormone metabolism in the liver by decreasing the expression of DIO3 and increasing TRs expression. Our results also showed that in VAT, obesity was associated with increased DIO3 expression, while DIO3 expression showed a non-significant tendency to decrease during fasting.

Previous studies have shown increased hepatic DIO3 expression during fasting, but their results were inconsistent regarding hepatic TR expression.[14-16] A study by De Vries *et al*.[14] showed no change in TR expression during fasting. Another study conducted by Fontes *et al*.[15] indicated an increase in the expression of TRα but not TRβ, and Boelen *et al*.[16] found an increase in the TRβ expression but not TRα. Our results were in contrast to the findings of the above studies. These contradictions can be due to differences in study design and method. Under normal physiological conditions, D3 activity is low or undetectable after birth but can be reactivated under certain pathophysiological conditions, such as fasting, trauma, and tissue hypoxia.[1] Several studies have indicated that hepatic fat accumulation and liver hypoxia can occur in HFD-fed mice.[7,17,18] In addition, liver HIF-1α expression levels have been shown to increase in HFD-fed mice.[7,18] In our study, liver DIO3 expression in HFD-fed obese mice was 1.9-fold higher than the SD-fed mice (control), although this difference was not statistically significant. It seems that HFD for 14 weeks may cause fat accumulation and local hypoxia in the liver, as a previous study reported an HFD for 8 weeks induces hepatic steatosis and local hypoxia in mice.[8] In addition, Marschner *et al*.[19] demonstrated that fatty liver deposition caused by an HFD leads to liver inflammation and increased production of reactive oxygen species (ROS), which induces DIO3 expression and decreases hepatic T3 content. As seen in Table 3, before fasting, there was no significant difference in body weight between HFD-fed groups (obese vs. fasted obese). Therefore fasting-induced weight loss may affect HFD-induced alterations in thyroid hormone metabolism. Several studies have illustrated that fasting can reduce or reverse HFD-induced changes in the liver. For instance, Morgan *et al*.[20] showed that fasting decreased hepatic expression of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) in HFD-fed mice. Moreover, Nishikawa *et al*.[21] demonstrated that fasting reduced hepatic lipid accumulation in HFD-induced obese mice.

In this study, we observed a 13.6% weight loss in fasted obese mice, suggesting that by reducing hepatic TG (triglyceride) content and ROS levels, fasting may reverse obesity-related changes in hepatic TH metabolism. In addition, fasted obese mice showed a significant increase in TRα and TRβ mRNA levels compared to the obese group. One possible explanation is that reduced DIO3 expression increases hepatic T3 content to bind to TRs; therefore, the increased hepatic TRα and TRβ expression in our study may be associated with the increased hepatic T3 content.

In the present study, we also observed a significant increase in DIO3 expression in VAT of both obese and fasted obese groups compared with the control group. These findings agree with previous study that demonstrated DIO3 expression in WAT was higher in obese than non-obese subjects.[22] Furthermore, 36-hour fasting increased DIO3 expression in WAT of lean mice.[23] It is important to note that unlike the studies mentioned above, we induced fasting in HFD-fed obese mice, not lean mice. Fasting in lean mice probably imposes metabolic stress on the body and causes a rapid compensatory response. In contrast, obese mice have more fat storage than lean mice, which may show different responses to the intervention. One possible explanation for increased DIO3 expression in VAT of obese mice is hypoxia. Hypoxia is a consequence of adipose tissue expansion, especially the hypertrophy of VAT due to obesity.[3] It proposed that local hypoxia in adipose tissue is a potential trigger for obesity-related chronic inflammation, insulin resistance, and metabolic disorders.[24] A study by Hosogai *et al*.[4] indicated that an HFD (30% fat) for 8 weeks results in adipose tissue hypoxia. Previous studies also suggested that the VAT of morbidly obese patients is in a hypoxic condition[4,25] and that the increased adipose inflammation in obesity is a result of adipose tissue response to hypoxia.[26]

Here, we have shown that DIO3 expression decreased significantly in the liver and increased in VAT during fasting. A possible explanation is that the effects of fasting on improving obesity-related changes appear earlier in the liver than in the VAT. According to this explanation, in the study by Schmitz *et al*.,[27] weight loss was associated with improved insulin resistance and decreased pro-inflammatory cytokines only in the liver and did not affect VAT. Furthermore, they showed that rapid weight loss after bariatric surgery improved liver inflammation, but adipose tissue inflammation persisted for 12 months. Furthermore, one study by Zamarron *et al*.[28] showed that despite weight loss in HFD-fed mice, a proinflammatory CD11c + adipose tissue macrophage (ATM) content remained elevated. They suggested that weight loss does not effectively reverse obesity-induced ATM activation.

Finally, we observed, although not statistically significant, the expression of TRα and TRβ in VAT tended to be lower in the obese compared to the control group and to be higher in the fasted obese compared to the obese group. In this context, the study by Kurylowicz *et al*.[29] found that the expression of TRα and TRβ in the VAT of obese patients was significantly lower than that of non-obese patients. In contrast, in the study by Ortega *et al*.,[30] there was no significant difference in the expression of TRα between obese and non-obese women. More research is needed to clarify obesity-induced changes in thyroid hormone metabolism in VAT and the effects of fasting on these changes. Two of the limitations of our study that we suggest for future research are that we did not measure HIF-1α gene expression levels in the liver and VAT. Measurement of HIF-1α expression, which is upregulated by hypoxia, can be helpful to evaluate the relationship between tissue hypoxia and D3 activity during fasting or obesity.

Conclusion

Fasting decreased DIO3 expression and increased TRs expression in the liver of HFD-induced obesity. In VAT, fasting non-significantly decreased the expression of DIO3 and increased the expression of TRs of HFD-induced obese mice compared to non-fasted HFD-induced obese mice. We suggest that fasting may have positive effects on TH function in the liver and to some extent in VAT of HFD-induced obesity.

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Authors' contribution

Study concept and design: HZ, AG, GS, and AM; Definition of intellectual content: AM, FH, AG, and HZ;Literature search: AM, GS, and FH; Clinical and experimental studies: AM, GS, FH, AG, and HZ; acquisitionof data: AM, GS, and FH; analysis of data: AM, GS, FH, AG, and HZ; statistical analysis: AM, GS, FH, AG,and HZ; Manuscript preparation: AM; Manuscript editing and review: AM, GS, FH, AG, and HZ; Guarantor:AM, AG, and HZ.

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Conflicts of interest

There are no conflicts of interest.

Data Availability statement

The data pertaining to this manuscript is not publically available. The corresponding author may be contacted for specific queries in this regard.

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