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Evaluation of the Effect of Co-administration of IRAK Inhibitor and Pioglitazone on PPAR-γ, GLUT-4, TNF-α, and Leptin Genes Expression in Adipose Tissue of Insulin-resistant Mice

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Abstract

Background: The worldwide prevalence of diabetes is increasing. Diabetes is a complex disease that results from impaired secretion of insulin or insulin resistance. In adipose tissue, insulin increases glucose uptake by stimulating the transfer of glucose transporter type 4 (GLUT-4) to the plasma membrane. In this study, the effect of IRAK inhibitor (IRAKi) and pioglitazone on genes expression in adipose tissue of insulin resistant mice was evaluated.

Methods: Mice were randomly divided into 6 groups (n= 8 each), five groups of which were fed a high-fat diet and one group received a normal diet for 12 weeks. The treatment with pioglitazone and IRAKi was performed for 2 weeks. At the end of the study, the animals were sacrificed and the adipose tissue and blood samples were collected. The expression of GLUT4, TNF-α, peroxisome proliferator-activated receptor gamma (PPARγ), and Lepin were determined by real-time PCR in the adipose tissue. The malondialdehyde (MDA) level and total antioxidant capacity (TAC) in serum were measured. Results were analyzed by SPSS 22.

Results: The data showed that the combination of IRAKi and pioglitazone increased PPARγ expression, leptin and TAC levels in serum, and reduced TNF-α expression and MDA levels. The GLUT4 expression in adipose tissue was not significant between studied groups. Pioglitazone and IRAKi improved insulin function by inhibiting inflammation signaling.

Conclusion: According to the results of this study, IRAKi may be an appropriate target for inhibiting inflammation and related disorders, including insulin resistance.

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Introduction

Diabetes mellitus is a complex disease that results from impaired secretion of insulin or insulin resistance (1). The most common type of diabetes is type 2 diabetes (T2D), which is mainly caused by insulin resistance in the main target tissues, namely the liver, adipose tissue, and muscle (2). The prevalence of diabetes in the world is increasing sharply (3) and studies have shown that the prevalence of diabetes in Iran is 11.4% (4). In adipose tissue, insulin increases glucose uptake by stimulating the transfer of glucose transporter type 4 (GLUT-4) to the plasma membrane. In the liver, insulin also inhibits gluconeogenesis and glycogenolysis, and consequently, reduces the release of glucose into the blood. Insulin resistance in this tissue leads to increased hepatic glucose production and inhibition of glucose uptake by adipose tissue (5). Obesity is a known risk factor for developing insulin resistance and T2D (6).

On the other hand, free fatty acids (FFA) act as ligands of the Toll-like receptor (TLR) family. TLR2 and TLR4 play an important role in the immune system and the activation of inflammatory pathways and can bind to gram-negative bacterial lipopolysaccharides (LPS). Binding of ligands, such as FFAs, to the TLR4 complex, activates the pathway that leads to the NF-κB activation. By activating the NF-κB as a result of increasing the concentration of FFAs, transcription of inflammatory genes such as interleukin-6 (IL-6), cyclooxygenase-2 (COX-2), and tumor necrosis factor alpha (TNF-α) increases, that interfere with insulin signaling and develop insulin resistance (7).

Peroxisome proliferator-activated receptor gamma (PPARγ), which is mainly present in adipose tissue, intestines, and macrophages, is well known for its role in regulating the adipogenesis of insulin sensitivity and inflammation (8,9). Evidence suggest that PPARγ is involved in the etiology and pathogenesis of several diseases associated with metabolic syndrome, including obesity, diabetes, non-alcoholic fatty liver disease (NAFLD), and cardiovascular disease (10).

There are 4 Interleukin-1 receptor-associated kinase (IRAK) genes in the human genome, namely IRAK-1, IRAK-2, IRAK-3, and IRAK-4. IRAK1 acts as a key link in the inflammatory chain. The connection of ligand to TLR activates signaling pathways of inflammatory pathways. These receptors can be activated by a variety of stimuli, including microbial pathogens and the presence of reactive oxygen species. Abnormalities in the matrix caused by chronic inflammation, genetic factors, and other above-mentioned conditions can be stimulated and signaled by IRAK mediation (11). Synthetic IRAK inhibitors with the highest inhibitory effect on IRAK1 and IRAK-4 have antioxidant, anti-inflammatory, and anti-tumor effects and reduce the number of inflammatory cytokines as well as suppressing immunoglobulins and improving the inflammatory process in cancer. These inhibitors inhibit IRAK mulecules, directly by binding to it, thus, they inhibit the NF-κB activation. In the past, the therapeutic potential of IRAK inhibitors has been used to treat inflammatory autoimmune diseases such as asthma and rheumatoid arthritis, but today, its promising effects on melanoma have been identified. The above-mentioned inhibitor can also induce apoptosis in vivo and in vitro.

Due to the high prevalence of obesity, insulin resistance, and T2D in societies, that are the products of a sedentary lifestyle with high-calorie intake, the need to study in this field is inevitable. In this study, an inhibitor that has recently been

used in previous studies was used to inhibit both IRAK type 1 and 4. Therefore, the effect of co-administration of IRAK inhibitor and pioglitazone on genes expression in adipose tissue of mice with insulin resistance was evaluated.

Materials and Methods

Animals

In this experimental study, 48 male C57BL/6J mice with mean weight of 19 g and 6 weeks of age were used to induce obesity and insulin resistance. The animals were obtained from the animal house of Pasteur Institute of Iran. This study was approved by the Ethics Committee of Kerman University of Medical Sciences (Ethical code: IR.KMU.REC.1398.554). They were housed under standard conditions at controlled room temperature (23±2˚C) and a 12:12 light-dark cycle. Animals were randomly divided into 6 groups (n= 8 each), five groups of which were fed a high-fat diet and one group received a normal diet for 12 weeks. To make each kilogram of the high-fat diet, 365 g of powdered mouse food, 250 g of casein, 310 g of beef fat, 60 g of vitamin and mineral supplements, 10 g of cholesterol, 3 g of methionine garlic and colic acid were mixed with 2 g of each other, and then, the resulting dough was converted into standard pellets using a meat grinder, and after drying in air, it was kept in the refrigerator (12).

Treatment

After 12 weeks, the animals were treated for two additional weeks as follows:

The healthy control group was fed a standard diet without any drug. The control group received a high-fat diet without any drug. The sham group was fed a high-fat diet in the first 12 weeks and received DMSO (10 µl/kg) intraperitoneal in the last 2 weeks. Pioglitazone group who was fed a high-fat diet for 12 weeks, received pioglitazone (10 mg/kg) by gavage in the last 2 weeks. The IRAK inhibitor (IRAKi) group was fed a high-fat diet for 12 weeks and received IRAKi (2 mg/kg 3 times a week) intraperitoneal in the last 2 weeks. IRAKi was diluted in DMSO (5 mM) and further dissolved in sterile PBS (pH 7.2). The last group was fed a high-fat diet for 12 weeks, and received both pioglitazone (10 mg/kg by gavage) and RAKi (2 mg/kg intraperitoneal) in the last 2 weeks (13,14).

Blood and tissue collection

At the end of the treatment period, the mice were anesthetized with ether after 12 hours of fasting. After surgery, blood samples were collected from the hearts of the animals. All samples were flash-frozen in liquid nitrogen. Serum and tissue samples were stored in a freezer at -70˚C until the relevant tests were performed.

Measurement of total antioxidant status (TAS)

Randox Total Antioxidant Status kit was used to measure the antioxidant status. In this method, a combination of 2,2'-azino diethyl benzythiazoline sulfonate (ABTS) with H_2O_2 and peroxidase is incubated to produce the cationic radical ABTS, resulting in a color product with a maximum adsorption at 600 nm. Antioxidant capacity is equivalent to reducing the dye produced in the environment. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was also used as a standard.

Measurement of serum leptin

Serum samples were isolated from the blood using a centrifuge at 3000 rpm for 10 min at 4˚C. Leptin level was determined with a double-antibody radioimmunoassay (ZellBio GmbH kit, Germany) (15).

Measurement of malondialdehyde (MDA)

To measure MDA, a reaction mixture containing 150 µl of thiobarbituric acid, 20 µl of sodium dodecyl sulfate (SDS), 150 µl of 20% acetic acid (pH = 3.5), and 60 µl of distilled water was added to 20 µl of the serum sample. The resulting mixture was heated at 90˚C for 45 min, and after cooling at room temperature, was centrifuged at 10,000 rpm for 10 min to obtain a clear solution. Its absorption was then recorded at 532 nm. The standard diagram was drawn using 1,1,3,3-Tetramethoxypropane as the standard. So that solutions with a concentration of 2.5, 5, 10, 20, 30, 40, and 50 nanomoles/milliliter (nmol/mL) were prepared from this standard. After performing the above-mentioned steps, the standard curve was drawn and the amount of lipid peroxidation as MDA nmol/mL was measured using the standard curve.

Total RNA extraction

Total RNA was extracted from visceral adipose tissue samples using Bio Basic Kit (Bio basic, Canada). After extraction, the amount of RNA extracted (quantitative evaluation) and its purity (qualitative evaluation) was evaluated with a NanoDrop spectrophotometer (ND-1000 NanoDrop). RNA quality was assessed by ethidium bromide staining of 18S and 28S ribosomal RNA after electrophoresis on 1.5% agarose gel. Complementary DNA (cDNA) synthesis was performed using a cDNA synthesis kit (Pars Toos, Iran).

Real-time PCR

Real-time PCR amplification was performed with the Mic Biomolecular systems (BMS, Australia) using RealQ Plus Master Mix Green (Amplicon-Denmark), according to the protocol recommended by the manufacturer. PCR was run as follows: initial activation of Taq DNA polymerase at 95˚C for 5 min, denaturation at 95˚C for 20 s, annealing at 63˚C for 10 s, and elongation at 72˚C for 30 s. The number of cycles for all reactions was 40 cycles. At the end, the melting curve was plotted at a temperature of 65 to 95˚C. Primer sequences (Pishgam Biotech Co, Iran) are described in Table 1.

Table 1. Primer sequences used in this study

Gene	Primer		Size (bp)
	Forward	Reverse	
TNF-α	5′ATGAGCACAGAAAGCATGA3′	5′ AGTAGACAGAAGAGCGTGGT3′	152
PPAR-γ	5′ CATGCTTGTGAAGGATGCAAG3′	5′ TTCTGAAACCGACAGTACTGACAT3′	131
GLUT-4	5′ GATTCTGCTGCCCTTCTGTC3′	5′ ATTGGACGCTCTCTCTCCAA3′	106
Leptin	5′ AAGAAGATCCCAGGGAGGAA3′	5′ TGATGAGGGTTTTGGTGTCA3′	320
GAPDH	5′ TGGAGTCTACTGGCGTCTT3′	5′ TGTCATATTTCTCGTGGTTCA3′	138

Statistical analysis

All data were analyzed using SPSS version 22. After ensuring about the normal distribution of data using Kolmogorov-Simonov test, statistical analysis was performed by analysis of variance (ANOVA) and Tukey Post-Hoc test. All data are presented as Mean ± SE. $P<0.05$ was considered statistically significant.

Results

GLUT4 expression

The results of the present study showed that there was no significant difference between the control group and other groups in the expression of GLUT4. Also, the IRAKi alone and in combination with pioglitazone did not affect the transcription of the GLUT-4 expression in adipose tissue (Figure 1).

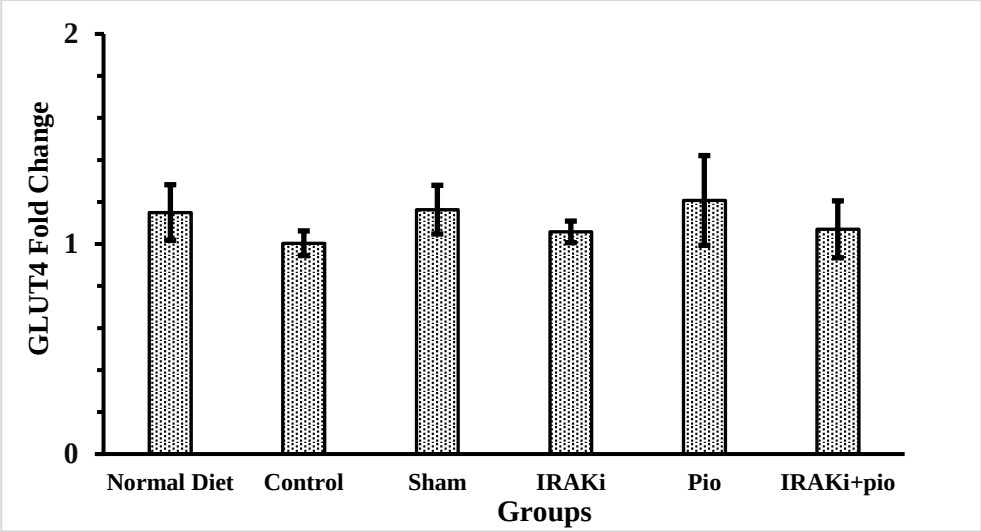

Figure 1. Transcription level of GLUT4. Insulin resistance was developed in mice by a high-fat diet, and then, the animals were treated with IRAK inhibitor (IRAKi), pioglitazone (Pio) or both of them (IRAKi+Pio) for 2 weeks. The level of GLUT4 gene expression was assayed by RT-qPCR.

The results showed that PPARγ expression significantly increased in the IRAKi- and pioglitazone-treated animals ($P<0.01$). Also, the combination of IRAKi and pioglitazone could up-regulate the expression of PPARγ obviously in comparison with the control group ($P<0.0001$) (Figure 2).

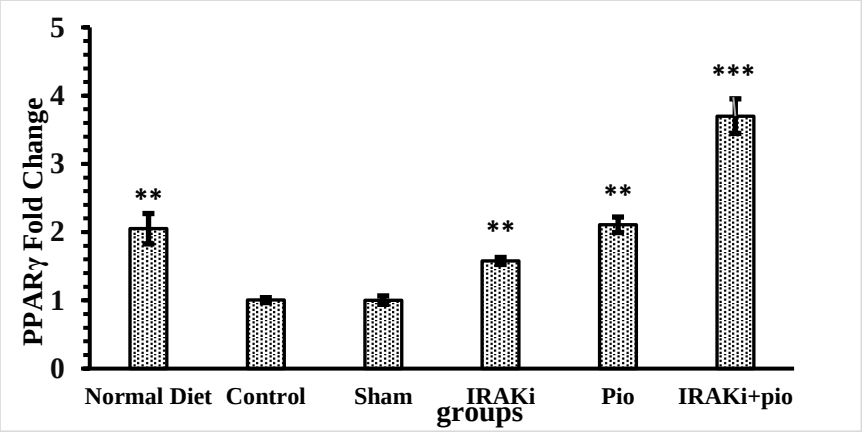

Figure 2. Transcription level of PPARγ. Insulin resistance was developed in mice by a high-fat diet, and then, the animals were treated with IRAK inhibitor (IRAKi), pioglitazone (Pio) or both of them (IRAKi+Pio) for 2 weeks. The level of PPAR.γ gene expression was assayed by RT-qPCR.

***P<0.0001, **P<0.01, in comparison with control group.

TNF-α gene transcription level was reduced in the animals treated with IRAKi and combination of IRAKi and pioglitazone compared to the control group (P<0.0001). Pioglitazone slightly decreased the TNF-α mRNA level expression compared to the control group (P<0.05) (Figure 3).

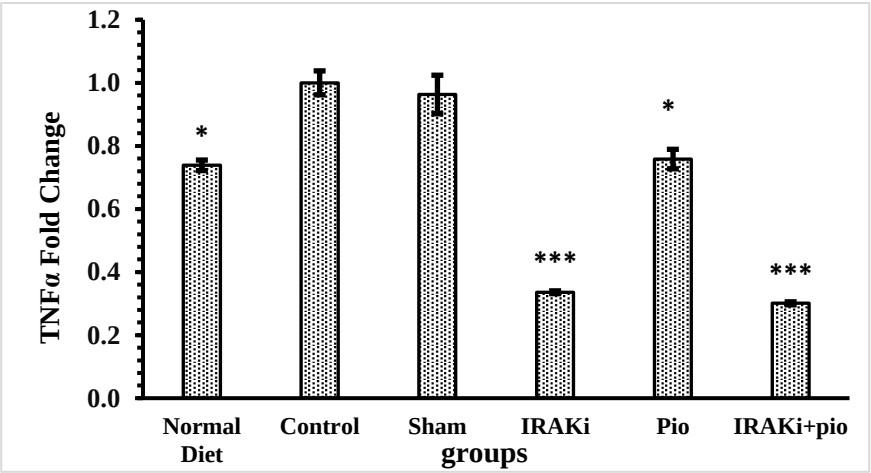

Figure 3. Transcription level of TNF-α. Insulin resistance was developed in mice by a high-fat diet, and then, the animals were treated with IRAK inhibitor (IRAKi), pioglitazone (Pio) or both of them (IRAKi+Pio) for 2 weeks. The level of TNF-α gene expression was assayed by RT-qPCR.

***P<0.0001, *P<0.05, in comparison with control group.

Leptin gene expression level was significantly increased in the groups treated with IRAKi (P<0.01) and IRAKi + pioglitazone (P<0.0001) compared to the control group. There was no significant difference in the pioglitazone group compared to the control group (Figure 4).

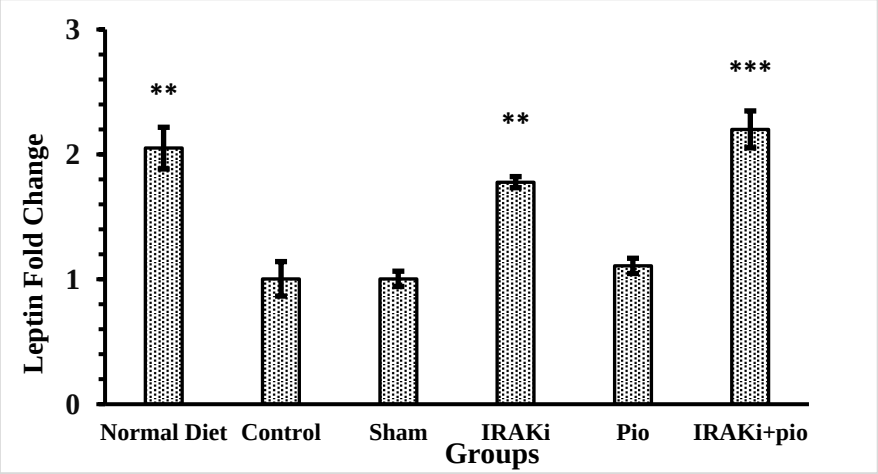

Figure 4. Transcription level of leptin. Insulin resistance was developed in mice by a high-fat diet, and then, the animals were treated with IRAK inhibitor (IRAKi), pioglitazone (Pio) or both of them (IRAKi+Pio) for 2 weeks. The level of leptin gene expression was assayed by RT-qPCR.

***P<0.0001, **P<0.001, in comparison with control group.

The total antioxidant capacity (TAC) was significantly decreased in the group fed a high-fat diet (HFD) compared to the normal diet (P<0.0001). But IRAKi treatment increased TAC compared to the control group (P<0.01). Also, in the group receiving pioglitazone and combination of pioglitazone and IRAKi, TAC increased in comparison with the control group (P<0.0001). The results are summarized in Figure 5.

Conversely, with increasing antioxidants, MDA levels were reduced in the group receiving IRAKi and pioglitazone compared to the control group (P<0.01). Also, in the group treated with IRAKi+Pioglitazone compared to the control group, MDA concentration significantly decreased (P<0.0001). The results are summarized in Figure 6.

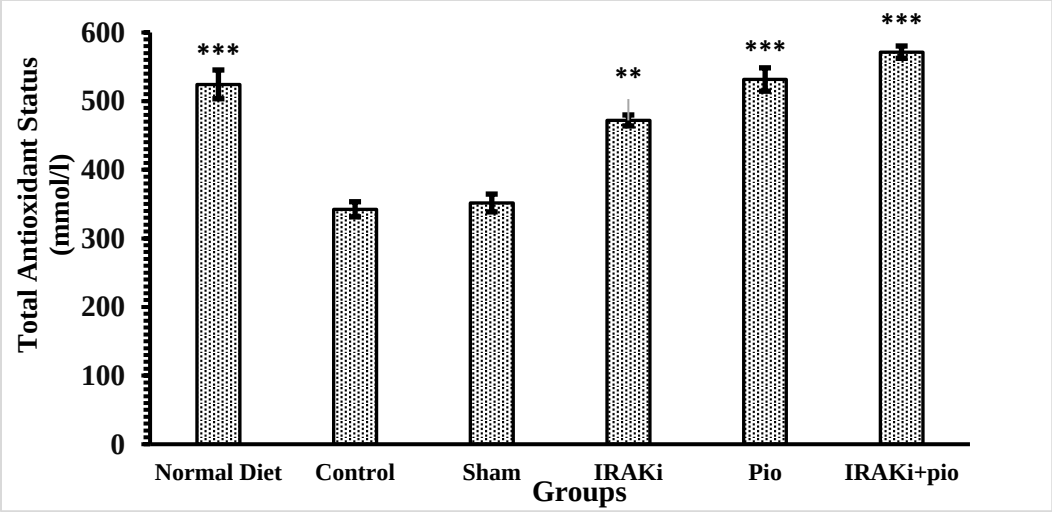

Figure 5. Total antioxidant status. Insulin resistance was developed in mice by a high-fat diet, and then, the animals were treated with IRAK inhibitor (IRAKi), pioglitazone (Pio) or both of them (IRAKi+Pio) for 2 weeks.

***P<0.0001, **P<0.001, in comparison with control group.

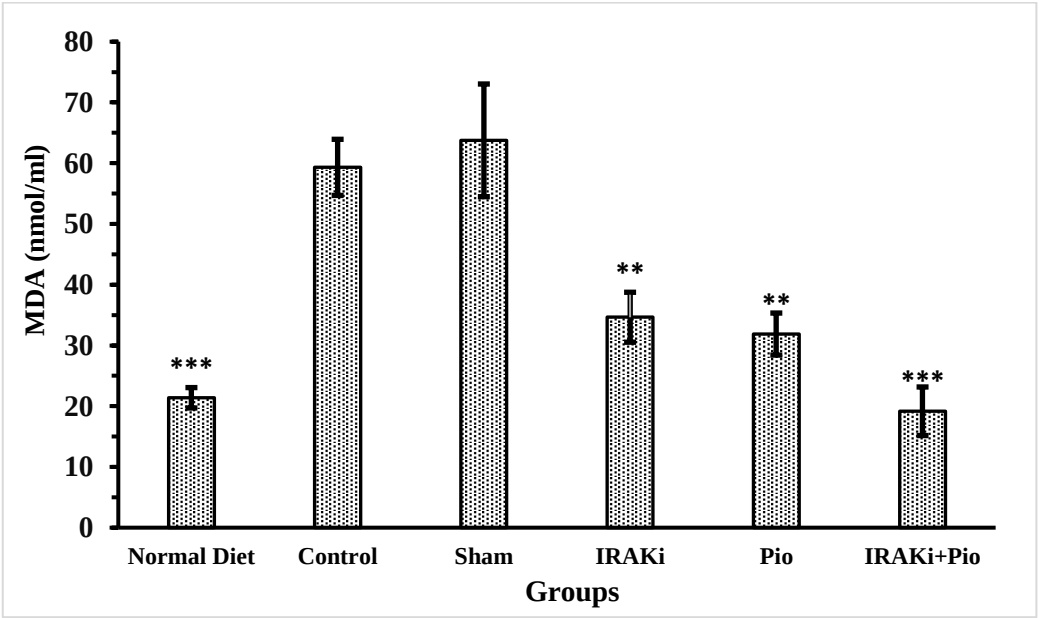

Figure 6. Concentration of MDA. Insulin resistance was developed in mice by a high-fat diet, and then, the animals were treated with IRAK inhibitor (IRAKi), pioglitazone (Pio) or both of them (IRAKi+Pio) for 2 weeks.

***P<0.0001, **P<0.01, in comparison with control group.

Discussion

Obesity induces insulin resistance via several mechanisms. Free fatty acids through Toll-like receptors (TLRs) and by mediatory effects of IRAKs, affect insulin function (16). Thiazolidinediones (pioglitazone) are ligands with a high affinity for PPAR-γ, which are currently used as insulin sensitizers for the treatment of T2D (17). Because of the key role of IRAKs in TLR

signaling, IRAK 1/4 inhibitor was used in this study to investigate its effects on insulin resistance.

GLUT4 expression showed no significant difference between groups in the present study. Kumari et al. (2016) found that IRF3 can induce inflammation and insulin resistance in mice adipose tissue, and inhibition of its expression increases insulin sensitivity and also GLUT4 expression (18). On the other hand, Kampman et al. (2011) showed that the expression of GLUT4 and UBC9 protein in skeletal muscle of patients with type 2 diabetes was significantly reduced compared to the control group (19). Discrepancies between the results of these studies, could be due to differences in the studied tissues or differences between protein and RNA expression.

As expected, in this study, decreased expression of TNF-α gene was observed in the IRAKi-treated groups compared to the control group. A study by Al-Hilali and Abduljaleel (2015) on the role of TNF-α and resistin in the development of insulin resistance in non-obese patients with type 2 diabetes, showed that TNF-α level was significantly higher in patients than the control group (20). The results of Xu et al. (2002) also showed that TNF-α levels increase in obese patients with type 2 diabetes (21). Also, in the study of Krogh-Madsen et al. (2004) found that TNF-α levels increase in insulin-resistant mice (22).

Ahmed et al. (2015) showed that increased expression of IRAK1 in adipose tissue was associated with increased inflammatory cytokines (23). Maitra et al. (2009) showed that IRAK-1 inhibits the nuclear factors PPARα and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (***PGC-1α***), that are essential for the expression of antioxidant enzymes such as catalase and glutathione peroxidase 3 (GPX3) (24). The results of a study by Rajaie et al. (2018) showed that IRAK inhibitors and pioglitazone increase adiponectin transcription levels (25).

The results of the present study showed that PPAR-γ expression was significantly increased in the group treated with IRAK inhibitor and pioglitazone. Increased expression of PPAR-α and PPAR-γ were inversely related to insulin resistance and increased insulin sensitivity, decreased hypertriglyceridemia, decreased blood pressure and heart rate. PPAR-α and PPAR-γ are inversely related to plaque volume and the rate of LDL oxidation in plaques (26). Also, increasing the expression of PPAR-α and PPAR-γ causes weight loss, reduces heart disease, and improves circulatory system function in obese insulin-resistant mice (26). So, the increased expression of PPAR-γ is a probable mechanism for increasing insulin sensitivity.

Transcription of leptin gene was increased in the animals that received IRAK inhibitor. Rajaie et al. (2018) reported that IRAK inhibitors and pioglitazone increase adiponectin transcription levels. Also, the concentration of adiponectin in the serum of insulin-resistant mice was increased by IRAK inhibitors and pioglitazone (25). Jialal et al. (2014) showed that leptin/adiponectin ratio was not associated with HOMA-IR. Also, in patients with metabolic syndrome, only the RBP4/adiponectin ratio increased significantly and leptin did not change (27). Decreased leptin production has been shown to be associated with increased susceptibility to infection. Conversely, immune disorders such as autoimmune

diseases are associated with increased leptin secretion and the production of proinflammatory cytokines. Thus, leptin may mediate the inflammatory response (28).

The results of the present study showed that IRAK inhibitor can increase the antioxidant capacity, and thereby, decrease MDA concentration. In metabolic diseases such as diabetes, nutritional factors such as sugar and free fatty acids can increase oxidative stress. Free radicals cause cell damage, induce apoptosis, and reduce insulin secretion (29). The antioxidant system in the body consists of several antioxidant enzymes and non-enzyme groups of antioxidant. Important enzymes in the antioxidant system include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GLPX) (30). Plasma total antioxidant capacity (TAC) indicates the body's potential for oxidants. In diabetes mellitus, the basal level of antioxidants decreases due to the production of oxidants (31). A study by Giacco et al. (2010) on diabetic mice with heart problems, showed that induction of antioxidant enzyme genes such as catalase and superoxide dismutase in these mice, can reduce diabetic complications (32). Athreya et al. (2017) shows that antioxidant therapy in patients with diabetes stops the production of ROS and eliminates oxidative stress in patients, resulting in ROS side effects and imbalance (33). Therefore, decrease of oxidative stress could be another mechanism for insulin sensitizing effect of IRAK inhibitor.

Conclusion

The therapeutic potential of IRAK inhibitors has been used to treat inflammatory diseases such as asthma and rheumatoid arthritis (23). However, the results of this study show that IRAK inhibitor increases the expression of PPAR-γ gene, which may increase the expression and secretion of adiponectin. This inhibitor also reduces the expression of TNF-α, which could be another possible mechanism of action of this compound in increasing insulin sensitivity. On the other hand, this compound increases the secretion of leptin, which has anti-inflammatory effects. The effect of this inhibitor in increasing antioxidant capacity and reducing lipid peroxidation, has also been shown to be another beneficial effect of this inhibitor. These results show that IRAK inhibitor have beneficial effects on insulin resistance, and it can also strengthen the pioglitazone effects.

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