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Antioxidant Effects of Pioglitazone and Interleukin-1 receptor-associated kinase inhibitor on Oxidative Stress Indices in obese C57BL/6J Mice

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Abstract

Background: Pioglitazone possesses antioxidant properties, whereas the antioxidant effects of IRAKi (Interleukin-1 receptor-associated kinase inhibitor; an anti-inflammatory agent) remain less established. Therefore, we investigated the antioxidant properties of IRAKi and pioglitazone in the liver of obese C57BL/6J mice.

Methods: Thirty-five mice were randomly assigned to five groups (n = 7); Control, high-fat diet (HFD), HFD-IRAKi, HFD-pioglitazone (PIO), and HFD-IRAKi-PIO. After 12 weeks on an HFD, IRAKi (2 mg/kg, intraperitoneally, three times a week) and pioglitazone (10 mg/kg, orally, daily) were administered for two weeks. The expression levels of Superoxide dismutase (SOD) and glutathione peroxidase (GPx) genes were evaluated through Real-time PCR analysis. Malondialdehyde (MDA), SOD, total antioxidant status (TAS), GPx, and total oxidant status (TOS) levels were quantified.

Results: HFD increased oxidative stress. IRAKi and pioglitazone, administered separately, increased SOD and TAS while reducing MDA and TOS compared to the HFD group. However, neither treatment significantly altered GPx activity. The combination of IRAKi and pioglitazone exhibited superior antioxidant effects compared to their separate administrations. Specifically, the combined treatment improved both GPx and SOD levels as well as their gene expression.

Conclusion: Our findings demonstrate the antioxidative effects of IRAKi and pioglitazone in C57BL/6J mice. Notably, their combination produced additive effects, enhancing the antioxidant defense system in the liver. These results suggest that IRAKi and pioglitazone, when used together, may serve as a promising therapeutic approach for managing oxidative stress in liver disease. By providing a dual mechanism of action, this combination highlights its potential for broader clinical applications in chronic disease management.

Keywords: High-fat diet, Superoxide dismutase, Glutathione peroxidase, Pioglitazone, Interleukin-1 receptor-associated kinase, Oxidative stress

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Introduction

Free radicals are highly reactive due to the presence of an unpaired electron. Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the biological systems' capacity to eliminate them, often accompanied by reduced antioxidant levels (1,2). Free radicals can induce lipid peroxidation and cause damage to DNA and biological membranes (3). Oxidative stress leads to cellular damage and cell death through necrosis or apoptosis (2,4). However, these compounds are not inherently harmful, as the body has mechanisms to neutralize free radicals. Oxidative stress contributes to liver damage not only by causing irreversible changes in lipids, proteins, and DNA but also by disrupting key biological pathways. These pathways regulate hepatic stellate cell activation, gene transcription, translation, and cell death. Consequently, oxidative stress plays a central role in the development of various liver diseases, including

steatohepatitis, fibrosis, and hepatocellular carcinoma (HCC) (2,5).

Non-alcoholic fatty liver disease (NAFLD) is a significant global health concern, affecting approximately 25% of the general population (6,7). Its prevalence increases dramatically among obese individuals and those with diabetes (8,9). Liver diseases range from simple steatosis to severe complications such as steatohepatitis, fibrosis, and cirrhosis, with further progression leading to HCC (10). The "two-hit hypothesis" has been proposed to explain the underlying mechanisms driving the onset and progression of NAFLD. The first hit involves insulin resistance, while oxidative stress, driven by ROS, constitutes the second insult triggering NAFLD development (9,11,12).

Research indicates that environmental, nutritional, genetic, and epigenetic factors contribute to NAFLD pathophysiology (8). Among these, oxidative stress plays a pivotal role as an early response to hepatic and extrahepatic injury (2,13,14). Oxidative stress is both a consequence and a contributor to fatty liver disease, mainly by inducing lipid peroxidation, which subsequently triggers inflammation. Additionally, elevated ROS levels lead to the retention of very-low-density lipoprotein in the liver, exacerbating fatty liver progression (2,12). Thus, reducing ROS-induced oxidative stress and maintaining redox homeostasis in the liver may be an effective strategy for NAFLD prevention and management (2,15). NAFLD is directly linked to obesity, which is closely associated with oxidative stress. Bełtowski et al demonstrated that a high-fat diet (HFD) reduced total antioxidant status (TAS), superoxide dismutase (SOD), and Glutathione peroxidase (GPx) levels (16). Comparisons between obese and normal-weight individuals revealed that malondialdehyde (MDA) levels increased while TAS decreased in obesity (17). Additionally, studies have reported that HFD significantly increased the risk of NAFLD development (18).

Interleukin-1 receptor-associated kinase (IRAK) inhibitors (IRAKi) target the inflammatory pathway. Toll-like receptors (TLRs) can be activated by various stimuli, including chronic inflammation, pathogens, and ROS. However, the antioxidant properties of IRAKi require further investigation, as they have not been extensively studied. IRAK proteins are key regulators of inflammatory pathways (19-21). In the liver, cellular damage typically leads to the release of proinflammatory mediators. Since IRAK is directly involved in inflammation, it may also affect the oxidative status of cells through inflammation-induced damage (19).

Thiazolidinediones (TZDs) are insulin sensitizers commonly used to treat type 2 diabetes mellitus (T2DM), with pioglitazone being a well-known example. Studies suggest that TZDs reduce hepatic steatosis by enhancing fatty acid oxidation while suppressing de novo lipogenesis (22,23). Pioglitazone has been shown to alleviate insulin resistance and reduce biochemical and histological damage in HFD-induced fatty liver models (22). Mice treated with pioglitazone displayed increased lipid accumulation alongside improved hepatic lipid profiles compared to controls. TZDs have demonstrated the ability to ameliorate steatosis and inflammation. Furthermore, they promote fat storage in adipose tissue, thereby enhancing insulin sensitivity in both the liver and peripheral tissues (23).

Given these studies, our study aimed to evaluate the effects of pioglitazone, IRAKi, and their combination on TAS, MDA, GPx, SOD, and total oxidant status (TOS) levels in the liver of C57BL/6J mice on an HFD.

Methods

Materials

Measurements of SOD, GPx, and TAS were conducted using specific kits provided by RANDOX Laboratories (TAS, NX2332; SOD, SD125; and GPx, RS 504). MDA levels were quantified using 2-thiobarbituric acid (CAS #: 504-17-6, Merck Chemicals GmbH). TOS was measured using the TOS Colorimetric Assay Kit from Elabscience (Cat. No. E-BC-K802-M). Additional materials included a total RNA extraction kit (Bio Basic, EZ-10 Spin Column Cat. No. BS82312), cDNA synthesis kit (TAKARA, Cat. No. RR037A), 2X SYBR Green (Ampliqon, Cat. No. A325402), and primers obtained from Metabion, Germany.

Animals

Thirty-five male C57BL/6J mice (Weight: 19 g; Age: 6 weeks) were used in this study. The animals were obtained from the animal house at the Pasteur Institute and maintained under standard conditions: a controlled room temperature (23 ± 2°C), a 12-hour light/dark cycle, and free access to food and water. Fatty liver was induced via a 12-week administration of HFD. The study protocol was approved by the Animal Care Committee under the Ethics Committee of Kerman University of Medical Sciences (Approval Code: IR.KMU.REC.1400.431). All experimental methods were conducted according to the guidelines established by this committee and the ARRIVE (Animal Research: Reporting of in Vivo Experiments) guidelines (24).

Study procedure

In this experimental investigation, 35 male C57BL/6J mice were randomly assigned to five groups (n = 7). The control group received a standard chow diet, while the other four groups were subjected to HFD for 12 weeks. The HFD was prepared by mixing 365 g of powdered diet, 310 g of beef fat, 10 g of cholesterol, 250 g of casein, 60 g of mineral and vitamin supplements, 3 g of methionine, and 3 g of colic acid. The mixture was processed using a meat grinder, air-dried, and stored in a refrigerator as standard pellets (15).

Treatment

Following the 12-week feeding period, the animals were

treated for 2 weeks as follows: The control group continued on a standard diet without intervention. The HFD group remained on the HFD without additional interventions. The pioglitazone (PIO) group received PIO (10 mg/kg daily) via gavage for two weeks following the 12-week HFD period. The IRAKi group continued the HFD for 12 weeks and was administered IRAKi (2 mg/kg intraperitoneally, three times a week) for the last two weeks. The IRAKi-PIO group received both PIO (10 mg/kg by gavage) and IRAKi (2 mg/kg intraperitoneally) during the final two weeks. The treatment duration was determined based on pilot studies, which indicated that two weeks were sufficient to observe significant changes in oxidative stress markers and liver function parameters. At the end of the experiment, the animals were fasted overnight, anesthetized with a ketamine/xylazine combination (80/5 mg/kg), and decapitated. The abdominal region was shaved and incised to facilitate liver dissection. The liver tissue was snap-frozen in liquid nitrogen and stored at -80°C for further analysis (25-27).

Measurement of liver triglyceride (TG) and cholesterol levels

We quantified TG and cholesterol levels in liver tissue using a lipid extraction method adapted from Haug and Høstmark. Briefly, 50 mg of liver tissue was homogenized in 1000 µL of isopropanol and incubated at 4°C for two days to facilitate lipid extraction. After the incubation, the samples were centrifuged (3000 rpm, 15 minutes) to separate soluble lipids from residual solid tissue. The resulting supernatant, containing TG and cholesterol, was collected for subsequent analysis. We quantified the TG and cholesterol levels using commercially available assay kits specifically designed for lipid measurement (28).

SOD and GPx quantification

GPx activity was assessed by monitoring the oxidation of glutathione, which is subsequently reduced to its active form by glutathione reductase. The decrease in absorbance at 340 nm, reflecting NADPH conversion to $NADP^+$, is directly proportional to GPx activity. SOD activity was determined based on its ability to convert superoxide radicals into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). In this assay, xanthine and xanthine oxidase generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to produce a red formazan dye. SOD activity is measured by evaluating the inhibition of this reaction (29,30).

Measurement of MDA

The MDA levels were quantified using the thiobarbituric acid-reactive substances (TBARS) method, measuring absorbance at a wavelength of 534 nm through a colorimetric approach. A calibration curve was established using 1,1,3,3-tetramethoxypropane to ensure accurate quantification. For the assay, a reaction mixture was prepared containing 150 µL of thiobarbituric acid, 20 µL of sodium dodecyl sulfate, 150 µL of 20% acetic acid (pH = 3.5), and 60 µL of distilled water, combined with 20 µL of the sample. This mixture was heated at 90 °C for 45 minutes, cooled to room temperature, and centrifuged at 10 000 rpm for 10 minutes to obtain a clear solution. Absorbance was recorded at 532 nm. The standard curve was generated using 1,1,3,3-tetramethoxypropane as the standard, with concentrations of 2.5, 5, 10, 20, 30, 40, and 50 nanomoles/milliliter (nmol/mL). The concentration of lipid peroxidation as MDA (nmol/mL) was calculated using the standard curve (29,30).

Measurement of TAS and TOS

Quantification of antioxidants was performed according to the protocol of the TAS RANDOX kits, with a concise overview provided for each parameter. TAS was measured by assessing the blue-green color of the radical cation ABTS (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) at 600 nm, with the degree of suppression proportional to antioxidant concentration (29,30).

In acidic environments, oxidizing agents present in the sample can convert Fe^{2+} ions into Fe^{3+} ions, leading to the formation of a stable blue-purple complex when combined with xylenol orange. The optimal colorimetric response was observed within a pH range of 2-3, with peak absorption observed at around 590 nm. The color intensity directly correlates with the concentration of oxidizing agents, enabling an indirect assessment of the sample's total oxidant capacity. The oxidative stress index (OSI) was calculated using the formula: $OSI = TOS/TAS$ (29,30).

Real-time PCR

We employed a comprehensive real-time PCR protocol to analyze gene expression in liver tissue samples. A total of 15 mg of liver tissue was utilized for RNA extraction. To ensure thorough disruption of the tissue, the samples were homogenized in a lysis buffer using a sonicator (Hielscher H200, Germany). Following homogenization, total RNA was extracted by adhering to the manufacturer's protocol of the total RNA extraction kit (Bio Basic, EZ-10 Spin Column, Cat. No. BS82312), ensuring the highest yield and purity of RNA. Once the RNA extraction was completed, cDNA was synthesized from about 500 ng of the extracted RNA using a cDNA synthesis kit (TAKARA, Cat. No. RR037A). The real-time PCR reactions were performed using specific primers designed for the target genes (Table 1). The reaction setup included 10 µL of 2X SYBR Green (Ampliqon Master Mix, Cat. No. A325402), 1 µL of each forward and reverse primer, and 100 ng of synthesized cDNA. The final volume of each reaction was brought to 20 µL with deionized water (dH_2O). The thermal cycling protocol was established as follows: an

initial denaturation step at 95 °C for 10 minutes, followed by 40 cycles comprising a 20-second denaturation at 95 °C, an annealing phase at the respective temperature for each primer lasting 45 seconds, and concluding with a melt curve analysis to confirm the specificity of the amplified products. For relative quantification, 18S rRNA was selected as the reference gene, and the relative expression levels of the target genes were determined using the $2^{-\Delta\Delta Ct}$ method, which facilitates normalization against the reference gene (31).

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). The normality of the data distribution was evaluated using the Shapiro-Wilk test. For group comparisons, a one-way ANOVA was conducted, followed by a post-hoc Tukey's test; if the data did not follow a normal distribution, the Kruskal-Wallis test was applied instead. A *P* value of less than 0.05 was regarded as statistically significant.

Results

HFD significantly increased liver TG levels compared to the control group receiving a standard diet ($P=0.044$). Pioglitazone administration significantly reduced TG content compared to the HFD group ($P=0.005$). The combination treatment of IRAKi and pioglitazone significantly reduced both TG and cholesterol levels compared to the HFD group ($P=0.032$ and $P=0.035$, respectively) (Figure 1).

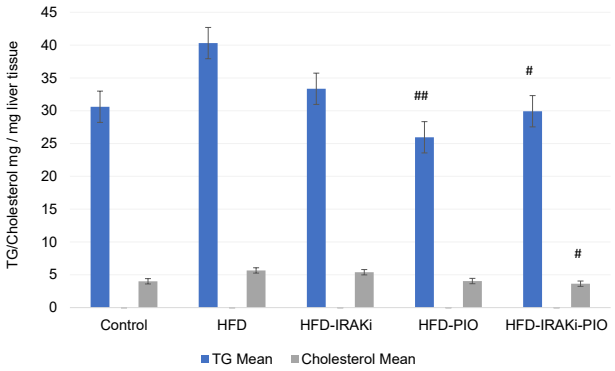

Figure 1. Quantification of TG and cholesterol levels in mice liver (mg/mg liver tissue). Study groups were defined as Control (Normal diet control), High-fat diet (HFD) received a 14-week HFD, HFD-PIO (received HFD for 12 weeks followed by 10 mg/kg pioglitazone for 2 weeks), HFD-IRAKi (received HFD for 12 weeks followed by 2 mg/kg IRAKi), HFD-IRAKi-PIO. Data are expressed as mean ± SEM, and $P<0.05$ was considered as significant. Statistically significant compared to the HFD group; # $P<0.05$, ## $P<0.01$

Our findings revealed that HFD induced oxidative stress by upregulation of TOS ($P<0.001$) (Table 2). IRAKi and pioglitazone, when administered separately, did not alter GPx activity compared to the HFD group (Figure 2). However, both treatments led to increased SOD ($P=0.001$, $P=0.011$) and TAS ($P=0.017$, $P=0.021$), while significantly reduced MDA ($P<0.001$) and TOS ($P=0.021$, $P=0.001$) levels compared to the HFD group (Figures 3 and 4, Table 2).

The combination of IRAKi and pioglitazone demonstrated superior antioxidant properties compared to individual administration. Specifically, this combination significantly increased GPx and SOD activity compared to the HFD group ($P<0.001$) (Figures 2 and 3), and led to a substantial reduction in TOS relative to HFD, HFD-IRAKi, and HFD-PIO groups ($P<0.001$, $P<=0.001$, and $P=0.001$, respectively) (Table 2). HFD significantly increased OSI levels compared to all other groups ($P<0.001$) (Table 2).

The analysis of *GPx* and *SOD* genes expression showed that the group received HFD reduced both genes expression compared to the control group ($P=0.011$ and $P<0.001$, respectively) (Figures 5 and 6). We found that the combination of IRAKi-PIO significantly reversed *GPx* and *SOD* genes expression compared to the HFD group ($P=0.001$ and $P<0.001$, respectively).

Discussion

We investigated the effects of pioglitazone, IRAKi, and their combination on various oxidative stress markers and antioxidant capacities in C57BL/6J mice subjected to an

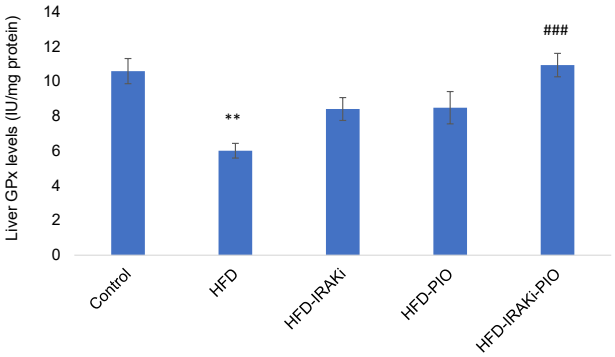

Figure 2. GPx activity in liver tissue of studied groups, normal diet control (Control), High-fat diet (HFD) received a 14-week HFD, HFD-IRAKi (received 2 mg/kg IRAKi for 2 weeks), HFD-PIO (received 10 mg/kg pioglitazone for 2 weeks), HFD-IRAKi-PIO, quantified by a specific kit. Data are expressed as Mean ± SD; $P<0.05$ was considered as significant. Statistically significant compared to the Control group; ** $P<0.01$. Statistically significant compared to the HFD group, ### $P<0.001$

Table 1. The primer sequences utilized for real-time PCR

Gene	Forward primer	Reverse primer
GPx	GGTGTTCCAGTGCGCAGAT	GGGCTTCTATATCGGGTTCGA
SOD	CCACTGCAGGACCTCATTTTAAT	TCTCCAACATGCCTCTCTTCATC
18S rRNA	CTTAGAGGGACAAGTGGCG	ACGCTGAGCCAGTCAGTGTA

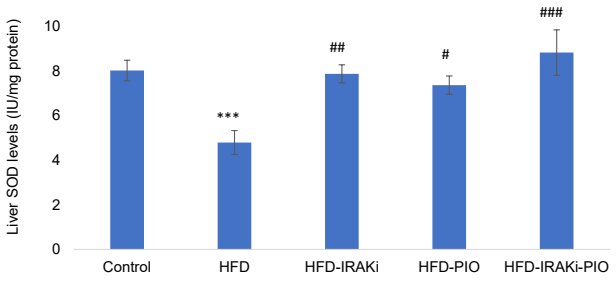

Figure 3. SOD activity in liver tissue of studied groups, normal diet control (Control), High-fat diet (HFD) received a 14-week HFD, HFD-IRAKi (received 2 mg/kg IRAKi for 2 weeks), HFD-PIO (received 10 mg/kg pioglitazone for 2 weeks), HFD-IRAKi-PIO, quantified by specific kit. Data are expressed as Mean ± SD; P < 0.05 was considered as significant. Statistically significant compared to the Control group; *** $P<0.001$. Statistically significant compared to the HFD group; # $P<0.05$, ## $P<0.01$, ### $P<0.001$.

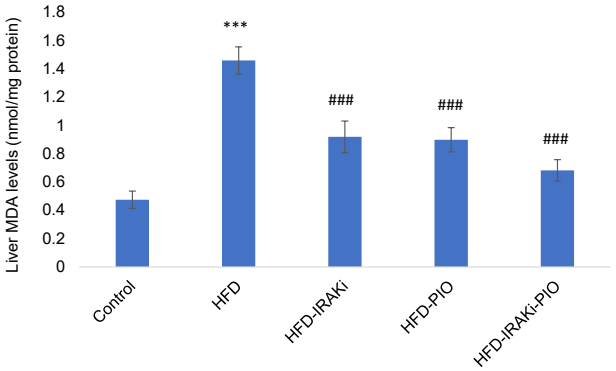

Figure 4. MDA levels in liver tissue of studied groups, normal diet control (Control), High-fat diet (HFD) received a 14-week HFD, HFD-IRAKi (received 2 mg/kg IRAKi for 2 weeks), HFD-PIO (received 10 mg/kg pioglitazone for 2 weeks), HFD-IRAKi-PIO, quantified by a specific kit. Data are expressed as Mean ± SD; $P<0.05$ was considered as significant. Statistically significant compared to the Control group; *** $P<0.001$. Statistically significant compared to the HFD group; ### $P<0.001$.

Table 2. TAS, TOS, and OSI levels in the liver of the studied groups

	TAS (uMol/mg protein)	TOS (uMol/mg protein)	OSI
Control	0.367 ± 0.034	0.034 ± .0039	0.091 ± .007
HFD	0.169 ± 0.019 **	0.081 ± .0066 ***	0.506 ± .069 ***
HFD-IRAKi	0.317 ± 0.033 #	0.06 ± .0037 **, #, $$$	0.195 ± .017 ###
HFD-PIO	0.313 ± 0.027 #	0.056 ± .0037*, ##, $$	0.187 ± .023 ###
HFD-IRAKi-PIO	0.398 ± 0.037 ###	0.028 ± .0032 ###	0.072 ± .009 ###

Groups defined as normal diet control (Control), High-fat diet (HFD) received a 14-week HFD, HFD-IRAKi (received 2 mg/kg IRAKi for 2 weeks), HFD-PIO (received 10 mg/kg pioglitazone for 2 weeks), HFD-IRAKi-PIO, quantified by a specific kit. Data are expressed as Mean ± SEM; $P<0.05$ was considered as significant. * Significant compared to the control group, # Significant compared to the HFD group, $ Significant compared to the HFD-IRAKi-PIO group. *#$ $P<0.05$, **##$$ $P<0.01$, ***###$$$ $P<0.001$. TAS, total antioxidant status; TOS, total oxidant status; OSI, oxidative stress index. Data were analyzed using one-way ANOVA (post-hoc, Tukey).

HFD. Our data indicate that HFD significantly increased oxidative stress levels, as evidenced by elevated MDA and TOS levels. Treatment with IRAKi, pioglitazone, and their combination exhibited significant protective effects against HFD-induced oxidative stress.

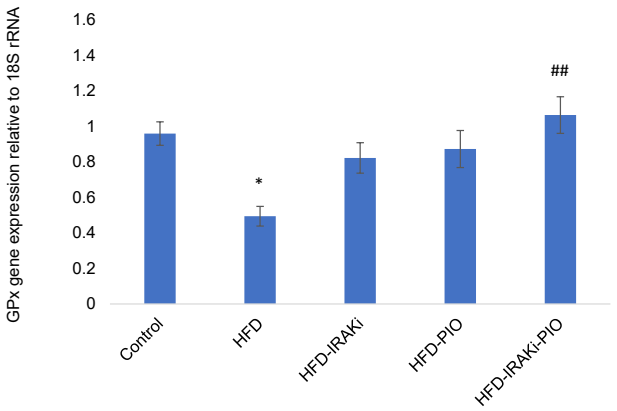

Figure 5. *GPx* gene expression in the liver tissue of the studied groups by real-time PCR. Groups: normal diet control (Control), High-fat diet (HFD) received a 14-week HFD, HFD-IRAKi (received 2 mg/kg IRAKi for 2 weeks), HFD-PIO (received 10 mg/kg pioglitazone for 2 weeks), HFD-IRAKi-PIO. Data are expressed as Mean ± SEM; $P<0.05$ was considered as significant. Statistically significant compared to the Control group; * $P<0.05$. Statistically significant compared to the HFD group; ## $P<0.01$.

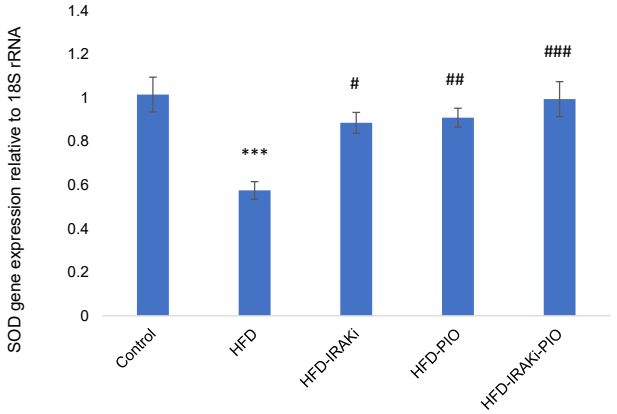

Figure 6. *SOD* gene expression in liver tissue of studied groups by real-time PCR. Groups: normal diet control (Control), High-fat diet (HFD) received a 14-week HFD, HFD-IRAKi (received 2 mg/kg IRAKi for 2 weeks), HFD-PIO (received 10 mg/kg pioglitazone for 2 weeks), HFD-IRAKi-PIO. Data are expressed as Mean ± SEM; $P<0.05$ was considered as significant. Statistically significant compared to the Control group; *** $P<0.001$. Statistically significant compared to the HFD group; # $P<0.05$, ## $P<0.01$, ### $P<0.001$.

Our findings indicate that HFD contributes to hepatic oxidative damage by increasing MDA levels and compromising the antioxidant defense system. These observations align with studies by Jarukamjorn et al (18), Noeman et al (32), and Mendes et al (33), collectively highlighting the crucial relationship between dietary composition and oxidative stress in the context of NAFLD.

Pioglitazone and IRAKi, when administered separately, demonstrated distinct effects on oxidative stress markers. Notably, pioglitazone has been shown to upregulate antioxidant defense mechanisms, as explained by Hsiao and colleagues, who reported that pioglitazone reduced oxidative DNA damage, likely through enhanced antioxidant activity (34). Another study indicated that pioglitazone lowered MDA while increasing TAS and TOS levels (35). Similarly, Al-Muzafar et al reported that a high-fat, high-carbohydrate diet elevated MDA and reduced GSH levels in rats, with pioglitazone reversing

these alterations (36). Our results confirm these findings, demonstrating that both treatments effectively reduced MDA and TOS levels compared to the HFD group. However, neither treatment significantly influenced GPx activity when administered separately, suggesting that despite exhibiting antioxidant effects, pioglitazone and IRAKi may operate through distinct mechanisms.

Interestingly, combination therapy with IRAKi and pioglitazone produced the most pronounced antioxidant effects, significantly enhancing GPx and SOD activity. This synergistic effect is important, as increasing GPx and SOD activity can more effectively attenuate HFD-induced oxidative stress than either treatment alone. The enhanced antioxidant capacity observed with combined therapy suggests potential mechanistic interactions between pioglitazone and IRAKi.

The current literature provides insight into the mechanisms underlying these effects. Maitra et al noted that IRAK signaling is associated with ROS generation, exacerbating liver inflammation and damage (37). The ability of IRAKi to attenuate both oxidative stress and associated inflammatory responses highlights its therapeutic potential in conditions such as NAFLD (21,37). Despite its promising antioxidant activity demonstrated in our study, the role of IRAKi in modulating oxidative disruptions related to inflammation warrants further investigation, as previous research established the intricate correlation between liver inflammation and oxidative stress (10).

Moreover, the observed reduction in oxidative stress indices, including OSI, following IRAKi and pioglitazone treatment highlights the importance of targeting both oxidative and inflammatory pathways to develop effective therapeutic strategies for metabolic liver diseases (1,3,10). Given that oxidative stress plays a crucial role in disease progression from simple steatosis to severe conditions such as steatohepatitis and HCC (2,6), the dual-action approach employed in this study may offer potential benefits in halting or reversing liver damage, as highlighted in previous studies.

As a limitation of this study, although histological parameters and ALT/AST levels were not quantified, the lipid measurements provide substantial evidence supporting fatty liver induction in our model, as depicted in Figure 1.

Conclusion

Our findings suggest that the combination of IRAK inhibitor and pioglitazone represents a promising therapeutic strategy for managing oxidative stress in fatty liver conditions. The observed increase in antioxidant enzyme activity and the reduction in oxidative markers indicate that combination therapy exerts a synergistic effect, improving oxidative balance more effectively than either treatment alone. Future studies should focus on dissecting the molecular pathways underlying these interactions, ultimately contributing to the development of targeted therapies for NAFLD and other related metabolic disorders.

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Authors' Contribution

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Methodology: Yaser Masoumi-Ardakani, Mahtab Pourkamalzadeh, Beydolah Shahouzehi, Hossein Fallah.
Project administration: Beydolah Shahouzehi.
Resources: Beydolah Shahouzehi.
Supervision: Beydolah Shahouzehi, Hossein Fallah.
Validation: Beydolah Shahouzehi, Yaser Masoumi-Ardakani.
Writing–original draft: Beydolah Shahouzehi, Hossein Fallah, Yaser Masoumi-Ardakani, Mahtab Pourkamalzadeh.

Competing Interest

All the authors declare that they have no conflict of interest.

Ethical Approval

The animal procedure was approved by the guidelines of the Animal Care Committee of the Ethics Committee of Kerman University of Medical Sciences (IR.KMU.REC.1400.431). All methods were performed according to the guidelines of the Animal Care Committee of the Ethics Committee of Kerman University of Medical Sciences and the ARRIVE guidelines 2.0.

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