

Leptin alleviated ovarian ischemia-reperfusion injury in rats via modulation of Sirt-1/Nrf2 and TLR4/NF-κB/caspase-3 signaling pathways

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Objective. Ovarian torsion is a gynecological emergency that occurs mostly during the female reproductive years due to ovarian masses or surgical manipulation. This work aims to explore the probable protective effect of leptin on rat ovaries due to ischemia-reperfusion (IR) injury.

Methods. Wistar albino rats were divided into four groups: 1) control group; 2) ovarian IR group (OVIR); 3) leptin group I [OVIR + leptin (10 µg/kg body weight, b.w.)]; and 4) leptin group II (OVIR + leptin (100 µg/kg b.w.)). Serum levels of estradiol and anti-Mullerian hormone (AMH) were measured. Levels of oxidative stress and inflammatory markers in ovarian tissue were determined along with the expression of sirtuin 1 (Sirt1), nuclear erythroid factor-2 (Nrf2), cyclooxygenase-2 (COX-2), nuclear factor kappa (NF-κB), toll like receptor-4 (TLR4), and caspase-3.

Results. Serum estradiol and AMH levels were decreased with increased expression of COX-2, TLR4, caspase-3, and NF-κB and decreased expression of Sirt1 and Nrf2 in ovary of the OVIR group, which were improved by exogenous administration of both leptin doses.

Conclusion. Leptin administration dose-dependently reduced the severity of OVIR injury via modulation of Sirt-1/Nrf2 and TLR4/NF-κB/caspase-3 signaling pathways. Thus, leptin may be used as an adjuvant measure to prevent ovarian damage and improve the outcomes. However, clinical studies are needed to evaluate these results in humans.

Key words: ischemia, reperfusion, ovary, leptin, sirtuin 1, toll like receptor-4

Ovarian torsion is an emergent surgical condition that is more common in women of childbearing age. Ovarian torsion is characterized by a twist of the ovary around its supporting ligaments so that it causes obstruction of the ovarian vessels and eventually ovarian ischemia, necrosis, and irreversible damage (Demir et al. 2021). Although detorsion of the ovary is the major line of treatment, it is associated with excess of reactive oxygen species (ROS) production,

which worsens the condition. Therefore, it is important to develop specific prophylactic strategies against ovarian ischemia-reperfusion (IR) injury to preserve the ovarian functions (Ali et al. 2021).

Leptin is a peptide hormone produced predominantly by the white adipose cells. It was the first adipokine that linked fat with reproductive physiology. Currently, leptin, in addition to its neuro-endocrine and immune functions, is considered to

be a multifunctional hormone because it does not regulate only body weight homeostasis, but also osteogenesis, chondrogenesis, and hematopoiesis (Perez-Perez et al. 2015). These effects of leptin are consistent with its production by different organs (Pico et al. 2021).

Emerging evidence has implicated leptin in reproductive physiology, such as the regulation of ovarian functions, oocyte ripening, embryo development, implantation, and placentation. This impact of leptin on reproductive functions has been indicated by the observed associations of leptin or leptin-receptor deficiency with compromised reproductive function (Perez-Perez et al. 2015; Malik et al. 2019; Salem 2021). Leptin has been suggested to regulate ovarian functions. However, most of these studies, either human or animal ones, are contradictory, since both stimulatory and inhibitory actions on the ovarian function have been described (Catteau et al. 2016).

In several animal models of IR injuries, leptin decreased the severity of these injuries via its antioxidant, anti-apoptotic, and anti-inflammatory activities (Sarsu et al. 2015; Zhang et al. 2019; Ragy and RaMzy 2021). There are many studies demonstrating that agents with antioxidant or anti-inflammatory activities may be beneficial in reducing ovarian IR injury (Colak et al. 2020; Ali et al. 2021; Demir et al. 2021). Thus, the current study was designed to investigate the probable protective effect of two doses of leptin (10 or 100 µg/kg body weight) in a rat model of ovarian IR injury and explore the underlying mechanisms that could be involved.

Materials and Methods

Animals and ethical issue. This experimental study was performed on 40 adults female Wistar albino rats (National Research Centre, Egypt) of age 6–8 weeks with body weight 200–250 g. The rats were housed in the laboratory environment in separate cages at standard temperature and light/dark cycle. Seven days before the experiment beginning, the animals received water and standard food *ad libitum*. The protocol was ethically approved by the Laboratory Animals Maintenance and Usage Committee of Faculty of Medicine, Minia University, Egypt (Approval No.147-2021), which adheres with the international guidelines (Council 2011).

Experimental induction of ovarian IR in rats. Rats were weighed and anesthetized with intraperitoneal injection (i.p.) of 25 mg/kg thiopental sodium (Refaie and El-Hussieny 2018). In sterile laboratory conditions, the skin of the lower abdominal wall

was cleaned with 2% iodine alcohol then a lower vertical abdominal incision (2.5 cm) was performed after shaving the abdominal area. A small peritoneal incision was made to locate the right and left adnexa. Bilateral ovarian ischemia was done for 3 h via a vascular clamp applied to the inferior part of the right and left ovary, where they join the uterus. Then the abdominal wall was closed with 3/0 silk suture. The abdomen wall was reopened and the clamp removed to achieve a regular reperfusion for another 3 h. Then the abdomen was closed again with 3/0 silk suture (Ekinci Akdemir et al. 2021). The rats were randomly divided into the following groups (n=10/each): 1) control group of rats underwent only to laparotomy and had no treatment; 2) ovarian ischemia reperfusion (OVAR) group of rats was exposed to ovarian IR and had also no treatment; 3) OVAR+leptin group I of rats received i.p. leptin of 10 µg/kg of body weight (b.w.) (Erkasap et al. 2004, 2017) for three days. In the first two days, the leptin was administrated before ischemia induction (Colak et al. 2020). The last leptin dose was administrated 30 min before reperfusion on the third day after ischemia induction (Yildirim et al. 2015). 4) OVAR+leptin group II of rats received i.p. leptin of 100 µg/kg b.w. (Ragy and RaMzy 2021) for three days. In the first two days, the doses were administrated before ischemia induction (Colak et al. 2020). The last leptin dose was administrated 30 min before reperfusion, on the third day after ischemia induction (Yildirim et al. 2015).

Treatment protocol. Leptin (Sigma-Aldrich, USA) was freshly prepared by dissolving it in sterile saline (Korinkova et al. 2020). A preliminary study was done to determine the effect of the vehicle, but there were insignificant differences between the non-treated OVAR group and the OVAR group treated with the vehicle. Both leptin doses (10 and 100 µg/kg) were selected based on the literature data (Erkasap et al. 2004, 2017; Sarsu et al. 2015; Ragy and RaMzy 2021) indicating that these doses could improve oxidative stress, apoptosis, and inflammation associated with IR injuries. In addition, these doses are within the physiological range (Feng et al. 2013; Pico et al. 2021) because high leptin dose may suppress the ovarian functions (Shaikat et al. 2021).

Collection of samples. At the end of the reperfusion period, all rats were euthanized and sacrificed under a light ether anesthesia. Blood samples were collected and left to clot. Then they were centrifuged for 10 min at 5,000 rpm. Sera were stored at –20 °C until the estradiol (Sigma Aldrich, USA) and anti-Mullerian hormone (AMH) (Sunlong Biotech Co., Ltd., China) were assayed.

Chemical analysis of ovarian tissues. The lower abdominal wall was opened, ovaries removed, and liberated from the surrounding soft tissues. Each ovary was divided into 2 portions. One portion was placed in a 10% formalin for histopathological evaluation, while the other one was preserved at -80°C for chemical analysis. Then, 0.1 g of the tissue was homogenized in 900 μL of ice-cold phosphate-buffered saline at pH 7.4 (10% w/v) and centrifuged at 4,000 rpm for 15 min at 4°C (Yilmaz et al. 2020). The supernatants were stored at -80°C for colorimetric assay of malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and nitric oxide (NO) levels (Bio-diagnostic, Egypt). Enzyme-linked immunosorbent assay (ELISA) kits were used for measurement of tumor necrosis factor-alpha (TNF- α) (Boster Biological Technology, USA), interleukin 10 (IL-10) (Sigma Aldrich, USA), B-cell lymphoma 2 (Bcl-2), and Bcl-2 associated $\times$ protein (Bax) (Cusabio Biotech Co, China).

Ovarian histopathology. Ovarian specimens were fixed in 10% formalin solution for 72 h. Paraffin-embedded samples (5 mm) were cut and stained with hematoxylin and eosin (H&E). All the sections (multiple sections/rat) were examined under an Olympus (Japan) light microscope (Yilmaz et al. 2020). According to the histopathological evaluation system, 5 microscopic fields were examined to assess the degree of congestion, hemorrhage, edema, inflammatory cells infiltration, and follicular degeneration. Each ovarian tissue was scored from 0 to 3 (0=no pathologic findings, 1=mild pathologic findings (less than 33%), 2= moderate pathologic findings (33–66%), and 3=severe pathologic findings (more than 66%). The pathologist examined all the ovarian sections in a blind fashion (Demir et al. 2021).

Immunohistochemistry for nuclear factor-kappa B (NF- κ B) and cyclooxygenase-2 (COX-2). Streptavidin-biotin peroxidase complex was used for staining. 5 μm -thick sections were cut from tissue blocks, de-paraffinized with xylene, and rehydrated through graded alcohol (100, 90, 70, and 50%). Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide/methanol buffer for 30 min. Immunohistochemistry was performed, using a polyclonal anti-COX-2 antibody obtained from Abcam (Toronto, ON, Canada) diluted 1:500 for 2 h and NF- κ B antibody ready-to-use (Santa Cruz Biotechnology, INC, Dallas, USA). Before incubation with specific antiserum, the background blocking was performed with normal goat serum (1:20; Dako, Glostrup, Denmark) in 0.05 M Tris-HCl

buffer, 0.5 M NaCl, pH 7.6 (TBS). Then the slides were incubated with a biotinylated secondary antibody for 30 min at room temperature followed by AB complex. The positive immunoreaction was detected by using a diaminobenzidine chromogen. Finally, the slides were washed, counterstained with Mayer's hematoxylin, dehydrated, and mounted. In each experiment, the negative controls were included with antibody dilution buffer instead of the primary antibody (Karimzadeh et al. 2013; Yilmaz et al. 2018).

For NF- κ B immunohistochemistry, the staining was classified by a semiquantitative method of Yilmaz et al. (2018) by multiplying the percentages of the stained cells (0–100%) by their score of staining intensity (weak=1, moderate=2 or strong=3 staining).

For COX-2 immunohistochemistry, semiquantitative assessment of the percentage of stained cells and staining intensity was carried out. Staining intensity was graded by using 4 stages grading scale: negative (–), weakly positive (1+), moderately positive (2+), and strongly positive (3+). The percentage of stained cells was defined in 4 categories: 0=no staining; 1=less than 25%; 2=26–50%; 3=51–75%; 4=more than 75%. For each case, the immunohistochemical score was produced by multiplying parameters for staining intensity by the percentage of stained cells. Immunohistochemical scores were categorized as low (1–3), moderate (4–6), and high (7–9) (Gipsyanti et al. 2021).

RT-PCR of SIRT-1, Nrf2, TLR-4, and caspase-3 in ovarian tissue. Total RNA was extracted from homogenized ovarian tissues using ribozol RNA extraction reagent (Amresco, Solon, USA) following the manufacturer's instructions. RT-PCR was performed with 50 ng RNA template per reaction using Thermo Scientific one-step kits in 25 μL reaction volume containing 70 nM of specific primers in the Real-Time PCR Detection System (Kappa Biosystems, USA). Gene expression was examined

Table 1
Primers for gene expression by RT-PCR.

Gene	Primer sequence
Sirt1	Sense: 5'-CAGCAACACCTCATGATTGG-3' Antisense: 3'-TCCACAGGAAACAGAAACC-5'
Nrf2	Sense: 5'-TGTCAGCTACTCCCAGGTTG-3' Antisense: 3'-ATCAGGGGTGGTGAAGACTG-5'
TLR-4	Sense: 5'-TGGCACTGTGACATCCTCAGA-3' Antisense: 3'-GCAACGGACATGCCAACATA-5'
Caspase 3	Sense: 5'-GGACAGCAGTTACAAAATGGATTA-3' Antisense: 3'-GGCAGGCCTGAATGATGAAG-5'
GAPDH	Sense: 5'-TCAAGAAGGTGGTGAAGCAG-3' Antisense 3'-AGGTGGAAGAATGGGAGTTG-5'

for Sirt1, Nrf2, TLR4, and caspase3. The SYBR green data were analyzed with a relative quantification to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a reference gene (Table 1). Then, the samples were placed in a thermal cycler (Applied Biosyst 7500 fast, Techne, Cambridge, LTD, UK). The relative expression level of gene was calculated using the formula $2^{-\Delta\Delta Ct}$ according to (VanGuilder et al. 2008). It was scaled relative to controls, whereas the control samples were set at a value of 1.

Statistic methods. The mean $\pm$ standard error of the mean (SEM) were determined for each group and the statistical differences among the different groups were analyzed by one-way ANOVA test followed by multiple comparison Tukey post hoc test to estimate the significant differences among the groups using GraphPad Prism software, version 6. The values were considered statistically significant if the probability $p < 0.05$.

Results

Changes in serum levels of estradiol and AMH in different groups. Serum levels of estradiol and AMH were significantly decreased in the OVIR group relative to the control group. Administration of low leptin dose to OVIR group significantly increased serum estradiol and AMH levels relative to the non-treated OVIR group, but their levels did not reach the control level. Administration of high leptin dose to OVIR group significantly elevated serum

estradiol and AMH levels as compared to OVIR group and OVIR group treated with low leptin dose and reached the control level (Figure 1).

Oxidant/antioxidant parameters in ovarian tissue. In OVIR group, ovarian tissue levels of MDA and NO were significantly increased and ovarian tissue level of antioxidants (GSH, SOD and CAT) was significantly decreased relative to the control group. Administration of low leptin dose to OVIR group significantly decreased the levels of MDA and NO and increased antioxidants levels relative to the non-treated OVIR group, but their levels did not reach the control level. Administration of high leptin dose to OVIR group significantly decreased MDA and NO levels and increased the antioxidant levels as compared to OVIR group and OVIR group treated with low leptin dose and reached the control level (Table 2).

Levels of inflammatory and apoptotic markers in ovarian tissue. The proinflammatory TNF- α and the pro-apoptotic Bax were significantly elevated, while the anti-inflammatory IL-10 and the anti-apoptotic Bcl2 were significantly decreased in the OVIR group when compared to the control group. Both doses of leptin reversed the measured parameters; however, it reached with the high dose the control level (Table 3).

Ovarian sections stained with H&E. In Figure 2, the histopathological examination of rat ovaries revealed that ovaries taken from rats of the control and OVIR groups treated with high leptin dose showed

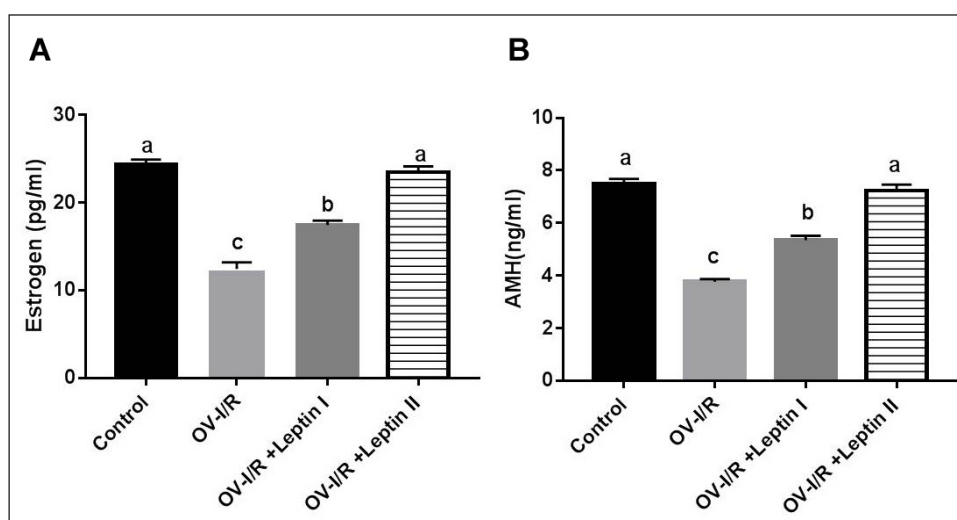


Figure 1. Serum levels of estradiol (A) and anti-Mullerian hormone (AMH) (B) in different groups. Data are presented as mean $\pm$ SEM (n=10). Different superscripts indicate significant differences. Abbreviations: OV-I/R – ovarian ischemia reperfusion group; Leptin I – low leptin dose; Leptin II – high leptin dose.

Table 2
Changes of oxidative parameters in the ovarian tissue in the different groups.

Parameter	Group			
	Control	OVIR	OVIR+Leptin I	OVIR+Leptin II
MDA (nmol/mg tissue)	4.30±0.13 ^c	16.30±0.13 ^a	9.50±0.18 ^b	4.50±0.15 ^c
NO (μmol/mg tissue)	5.10±0.0 ^c	26.40±0.09 ^a	12.30±0.15 ^b	5.10±0.08 ^c
GSH (mg/mg tissue)	100.80±1.00 ^a	78.06±0.75 ^c	91.83±0.67 ^b	99.13±1.20 ^a
CAT (U/mg tissue)	77.34±0.90 ^a	33.67±1.06 ^c	62.34±0.64 ^b	76.98±1.32 ^a
SOD (U/mg tissue)	13.29±0.18 ^a	6.32±0.23 ^c	8.86±0.16 ^b	12.75±0.33 ^a

Abbreviations: OVIR – ovarian ischemia-reperfusion; Leptin I – low leptin dose; Leptin II – high leptin dose; MDA – malondialdehyde; NO – nitric oxide; GSH – reduced glutathione; CAT – catalase; SOD – superoxide dismutase. Data are presented as mean±SEM (n=10). Different superscripts indicate significant differences.

Table 3
Changes of inflammatory and apoptotic markers in ovarian tissue in different groups.

Parameter	Group			
	Control	OVIR	OVIR+Leptin I	OVIR+Leptin II
TNF-α (pg/mg tissue)	67.71±0.69 ^c	126.50±0.97 ^a	98.86±0.83 ^b	70.69±0.94 ^c
IL-10 (pg/mg tissue)	102.90±1.00 ^a	40.10±0.90 ^c	56.25±1.48 ^b	101.40±0.59 ^a
Bcl2 (ng/mg tissue)	6.09±0.29 ^a	2.11±0.20 ^c	3.23±0.19 ^b	6.05±0.28 ^a
Bax (ng/mg tissue)	2.45±0.13 ^c	9.14±0.21 ^a	5.16±0.18 ^b	2.49±0.15 ^c

Abbreviations: OVIR – ovarian ischemia-reperfusion; Leptin I – low leptin dose; Leptin II – high leptin dose; TNF-α – tumor necrosis factor-alpha; IL-10 – interleukin 10; Bcl2 – B-cell lymphoma-2; Bax – Bcl2 associated x protein. Data are presented as mean±SEM (n=10). Different superscripts indicate significant differences.

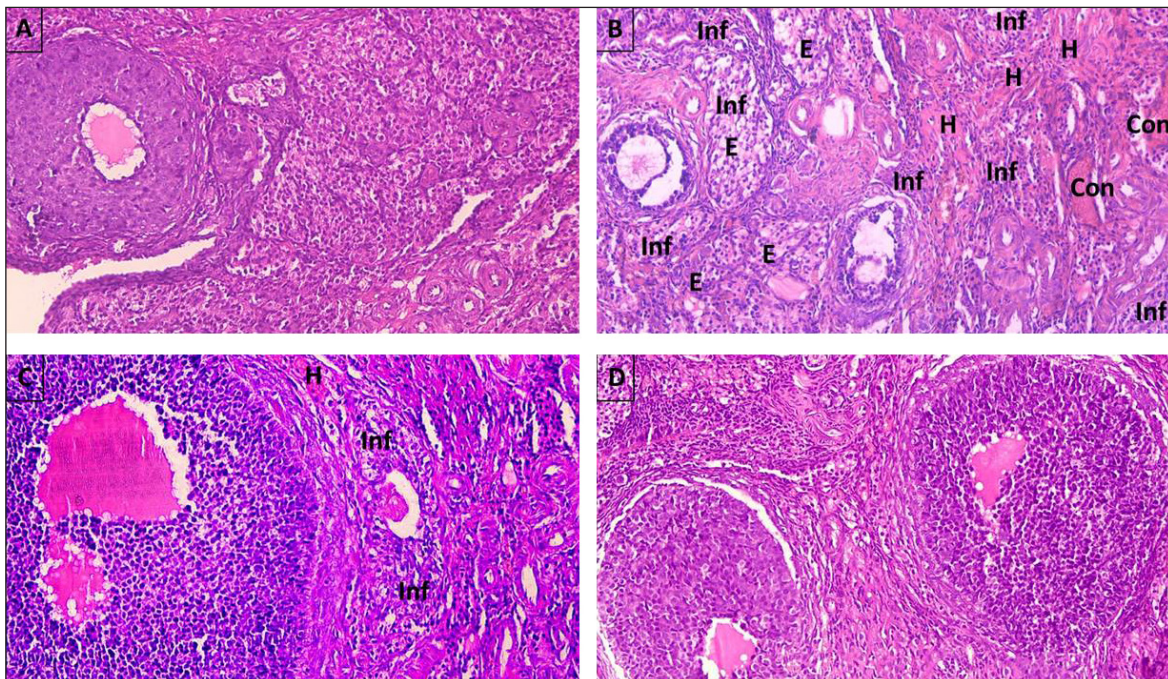


Figure 2. Histological evaluation of the rat ovary samples stained with hematoxylin and eosin (H&E) (magnification 200×). **A)** Section from the rat ovaries of control group shows normal ovarian tissue structure. **B)** Section from ovarian ischemia reperfusion (OV-I/R) group shows atrophy of ovarian follicles, severe edemas (E), severe vascular congestions (Con), numerous areas with hemorrhage (H), and marked neutrophils infiltration (Inf). **C)** Section from the rat ovaries of OV-I/R group treated with leptin I (low dose) shows normal ovarian follicles with mild inflammation (Inf) and hemorrhage (H). **D)** Section from rat ovaries of OV-I/R group treated with leptin II (high dose) shows preservation of normal ovarian tissue structure.

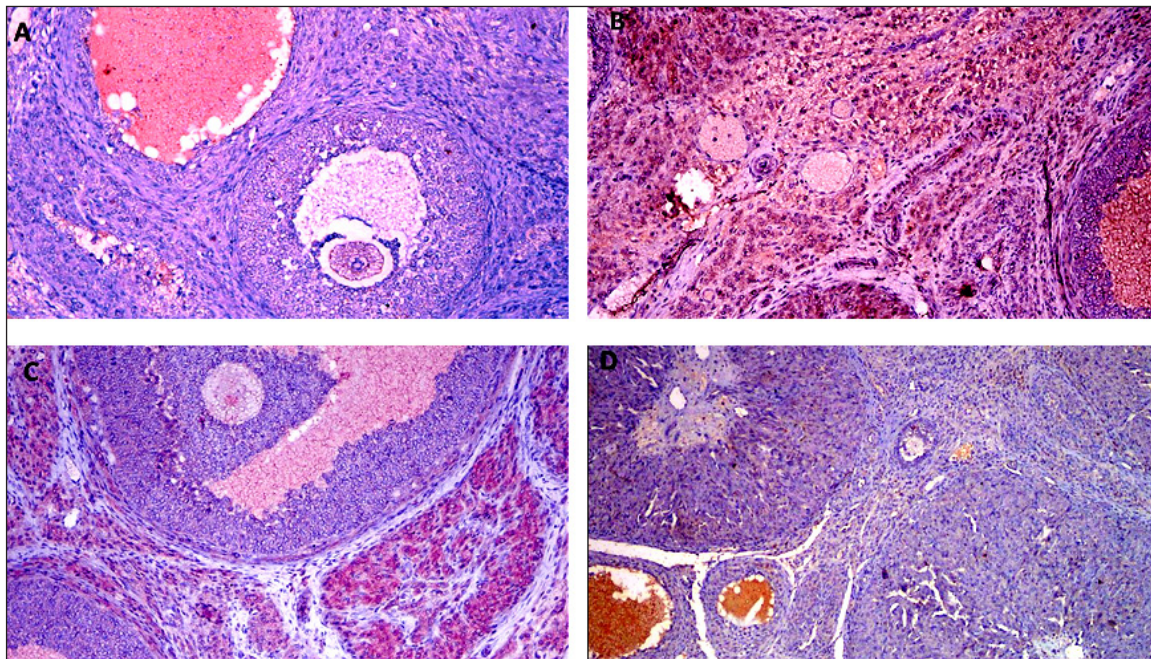


Figure 3. Immunohistochemically stained sections of rat ovaries for nuclear factor-kappa B (NF- κ B) (magnification 200 $\times$). **A)** Section of rat ovaries from control group shows negative NF- κ B expression. **B)** Section of rat ovaries from ovarian ischemia reperfusion (OV-I/R) group shows strong positive NF- κ B expression. **C)** Section of rat ovaries from OV-I/R group treated with leptin I (low dose) shows moderate positive NF- κ B expression. **D)** Section of rat ovaries from OV-I/R group treated with leptin II (high dose) shows negative NF- κ B expression.

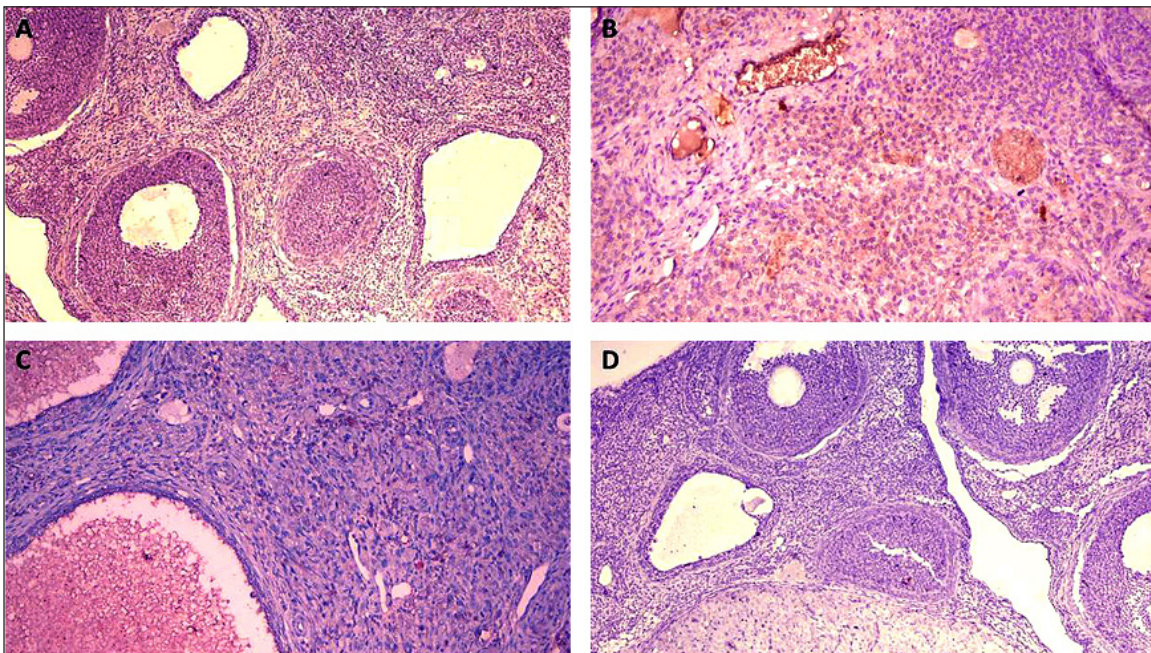


Figure 4. Immunohistochemically stained sections of rat ovaries for cyclooxygenase-2 (COX-2) (magnification 200 $\times$). **A)** Section from rat ovaries of the control group shows negative COX-2 expression. **B)** Section of rat ovaries from ovarian ischemia reperfusion (OV-I/R) group shows strong positive COX-2 expression. **C)** Section of rat ovaries from OV-I/R group treated with leptin I (low dose) shows low positive COX-2 expression. **D)** Section of rat ovaries from OV-I/R group treated with leptin II (high dose) shows negative COX-2 expression.

normal ovarian tissue structure. Ovaries examined from the OVIR group showed significant atrophy of ovarian follicles, severe edema, severe vascular congestions, numerous areas of hemorrhage, and marked neutrophil infiltration. Sections examined from rats of OVIR group treated with low leptin dose showed nearly normal ovarian tissue structure with mild inflammation and hemorrhage.

Immunohistochemistry assessment of NF- κ B and COX-2. Immunohistochemical staining of the studied rat ovaries using NF- κ B and COX-2 antibodies showed negative expression in ovaries that belong to both control and OVIR groups treated with high leptin dose. NF- κ B (Figure 3) and COX-2 (Figure 4) expressions were low positive in OVIR rats treated with low leptin dose and were strong positive in ovaries of rats that belong to OVIR group.

Morphometric results. The scoring of histopathology and immunohistochemistry of NF- κ B and COX-2 was significantly higher in the OVIR group as compared to the control rats. The scoring of

histopathology and immunohistochemistry significantly decreased in the OVIR treated with low leptin dose as compared to the non-treated OVIR group, but they did not reach the control level. In the OVIR group treated with high leptin dose, the scoring of histopathology and immunohistochemistry significantly decreased reaching the control level (Table 4).

Relative expression of Sirt1, Nrf2, TLR4, and caspase-3 by RT-PCR. Relative to the control rats, TLR4 and caspase-3 mRNA levels were significantly increased, while Sirt1 and Nrf2 mRNA levels significantly decreased in the OVIR group. In the OVIR group treated with low leptin dose, TLR4 and caspase-3 mRNA levels were significantly decreased and Sirt1 and Nrf2 mRNA levels significantly increased, but their levels did not reach the control level. However, in the OVIR group treated with high leptin dose, the mRNA levels of TLR4 and caspase-3 were significantly decreased and Sirt1 and Nrf2 mRNA levels significantly increased and reached the control level (Figures 5A–D).

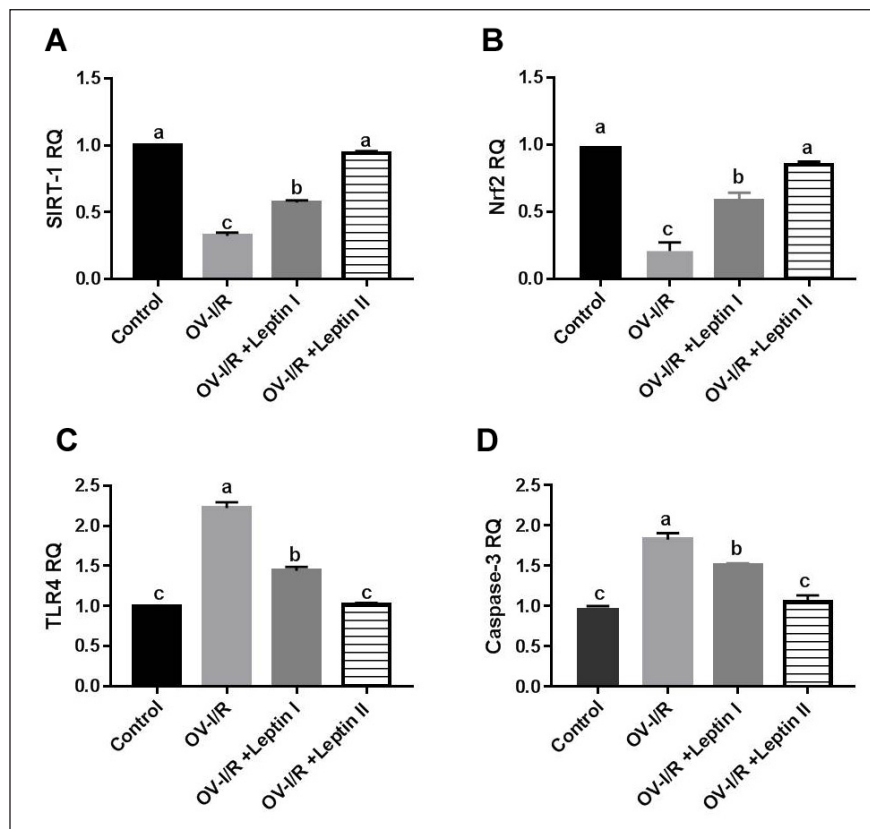


Figure 5. Relative expression (RQ) of sirtuin 1 (Sirt1) (A), nuclear erythroid factor-2 (Nrf2) (B), toll like receptor-4 (TLR4) (C), and caspase-3 (D) in ovarian tissues of different groups. Data are presented as mean $\pm$ SEM (n=10). Different superscripts indicate significant differences. Abbreviations: OV-I/R – ovarian ischemia reperfusion group, Leptin I – low leptin dose, Leptin II – high leptin dose.

Table 4
Scoring of histopathology and immunohistochemistry of NF- κ B and COX-2.

Parameter	Group			
	Control	OVIR	OVIR+Leptin I	OVIR+Leptin II
Histopathology	0 ^c	2.83 $\pm$ 0.16 ^a	1.66 $\pm$ 0.20 ^b	0.33 $\pm$ 0.20 ^c
NF- κ B IHC	0 ^c	205.0 $\pm$ 9.2 ^a	53.3 $\pm$ 3.3 ^b	3.0 $\pm$ 1.4 ^c
COX-2 IHC	0 ^c	8.16 $\pm$ 0.80 ^a	3.50 $\pm$ 0.50 ^b	0 ^c

Abbreviations: IHC – immunohistochemistry; OVIR – ovarian ischemia reperfusion; Leptin I – low leptin dose; Leptin II – high leptin dose; NF- κ B – nuclear factor kappa B; COX-2 – cyclooxygenase-2. Data are presented as mean $\pm$ SEM (n=10). Different superscripts indicate significant differences.

Discussion

The role of leptin in the era of reproductive physiology has become a new matter of concern. Therefore, the present study was designed to evaluate the possible protective effect of two different doses of leptin on experimentally induced ovarian ischemia-reperfusion injury. The involved underlying mechanisms of their actions were also explored. To the best of our knowledge, this is the first study that has been addressed on the effect of leptin on experimentally induced OVIR injury in rats. Based on the present data, we can say that leptin guards against this type of injury via upregulation of Sirt1/Nrf2 pathway and downregulation of TLR4/ NF- κ B /caspase-3 pathway, which was observed in the improvement in the histopathological picture of the ovaries and its reserve beside the improvement of the measured biochemical parameters.

Sirtuin 1(Sirt1) gene is expressed in granulosa cells, cumulus cells, oocytes, and theca cells of the ovary. It has been linked to follicular reserve preservation and ovarian lifespan extension, and is an essential factor in the activation of the steroidogenesis associated with luteinization and terminal differentiation (Nishigaki et al. 2022).

Sirt1 expression may be reduced due to oxidative stress conditions associated with ovarian I/R injury (Refaie et al. 2022) and upregulated with exogenous leptin administration in a dose-dependent manner as found in the present and other studies (Marwarha et al. 2014; Erkasap et al. 2017). Previous study has reported that the Sirt1 gene regulates proliferation and apoptosis of granulosa cells and its down-regulation is associated with a reduced ovarian reserve and lipid peroxidation as found in the OVIR group in our study (Alam et al. 2021). It also modulates the stress response to oxidative stress in granulosa cells by targeting transcription factors vital for ovarian functions and maintenance. Sirt1 gene

could stimulate expression of antioxidants, repairs cells damaged, and prevents the cell dysfunction by oxidative stress (Di Emidio et al. 2014; Alam et al. 2021; Nishigaki et al. 2022). This is compatible with the study, which has reported that the absence of oocyte-Sirt1gene resulted in a reduction of oocyte development or quality (Iljas et al. 2020). Thus, Sirt1 gene-mediated amelioration of oxidative stress and apoptosis plays important parts in the protective actions of leptin.

Previous study has confirmed that Sirt1 gene serves as the upstream regulator of Nrf2 signaling pathway, which mediates the antioxidant effect of leptin (Song et al. 2018; Zhang et al. 2018). Nrf2 plays a crucial role in cellular antioxidant defense mechanisms (Ren et al. 2011; Hepp et al. 2021; Ali et al. 2022). This is in agreement with a previous study, which has reported that leptin attenuated the development of oxidative stress (Bilbao et al. 2015) and enhanced the transcription of antioxidants, which neutralized free radicals (Fernandez-Martos et al. 2012; Buldak et al. 2015; Kornowski et al. 2021) as indicated by up-regulation of the gene expression of Nrf2 along with the elevation of level of antioxidants in ovarian tissue in the present study (Alshaheen et al. 2021).

Modulation of the pro-inflammatory TLR4/ NF- κ B signaling pathway to alleviate ovarian injury has become an area of clinical interest. The toll-like receptor 4 (TLR4) is strongly activated during reperfusion injury and participates in neutrophil activation, cytokine releasing, and ROS overproduction (Refaie et al. 2022). Its activation is functionally associated with the upregulation of the nuclear factor-kappa B (NF- κ B) signaling pathway causing the generation of pro-inflammatory cytokines and the expression of COX-2 and the inducible nitric oxide synthase (iNOS). Elevated levels of TNF- α and other interleukins also directly activate NF- κ B signaling and thus lead to the amplification of the initial inflammatory response (Sun et al. 2021).

The upregulation of Sirt1 gene negatively regulates the TLR4/NF- κ B pathway (Kauppinen et al. 2013; Iside et al. 2020). Sirt1 gene could protect ovaries against TNF- α -induced cytotoxicity not only by suppressing TLR4/NF- κ B gene expression (Refaie et al. 2022), but also by increasing the anti-inflammatory IL10 protein level (Zhu et al. 2021). Moreover, Sirt1 gene could block the TNF- α -induced expression of iNOS and COX-2, which subsequently leads to suppression of the apoptotic cascade and the formation of NO (Joudah et al. 2021). These findings agree with Erkasap et al. (2017) who have reported that leptin could decrease pro-inflammatory cytokines expression. Moreover, Faustmann et al. (2016) have reported the inhibitory effects of leptin on NF- κ B gene expression and the inverse relationship between NF- κ B activation and leptin concentrations. Our results suggest that the protective effects of leptin as an antioxidant and anti-inflammatory agent may involve the upregulation of the Sirt1 gene.

The antioxidant and the anti-inflammatory effects of leptin treatment may suppress apoptosis in ovarian tissue as evident by the decreased gene expression of caspase-3 and the increased Bcl2/BAX ratio in the present study. This is in the line with the data of Cavalcante et al. (2013), who have reported that leptin could accelerate follicular maturation and attenuate follicular atresia via apoptosis suppression. Moreover, it has been reported that leptin deficiency in mice was associated with an increase in ovarian granulosa cell apoptosis and suppression of folliculogenesis (Hamm et al. 2004). In addition, Menezes and colleagues (2019) have found that the leptin adding to a culture of isolated sheep follicles is capable of follicular endurance, oocyte growth, mitochondrial activity, and meiotic resumption improving due to its antiapoptotic effect. This means that leptin administration could elevate the serum marker of ovarian reserve, the AMH as detected in the present study and other investigations (Bilbao et al. 2015; Erkasap et al. 2017). Previous literature has reported that the reduction in AMH levels with ovarian injury may reflect the ovarian follicle atrophy, the increase in free oxygen radicals production, the accumulation of activated neutrophils, and the cellular membrane lipid peroxidation (Eken et al. 2019), which is consistent with our findings.

Herein, the significant decrease in estradiol level in the OVIR group was reversed with leptin treatment, with a significant increase in their level in a dose-dependent manner. The decreased serum estradiol seen in OVIR group could be explained by

the ROS-induced oxidative stress that may negatively affect granulosa cell and suppress steroidogenesis (Melekoglu et al. 2018). Hence, the antioxidant effect of leptin treatment may improve estradiol production by granulosa cells (Becerril et al. 2019; Kumar et al. 2019). This agrees with atrophic ovarian follicles appearance in the microscopic picture of the OVIR group, which was improved by both doses of leptin treatment. Certain study has reported that leptin acts directly on the ovaries and regulates follicular steroidogenesis (Hausman et al. 2012), while another one has reported that leptin increases estradiol production in a dose-dependent manner as it accelerates follicular maturation by attenuating follicular atresia and increases the ratio of BCL-2/Bax (Cavalcante et al. 2013).

It is worth to note that results of some previous either rat or human studies do not match with our findings. Certain studies have noticed that leptin suppressed estradiol production by granulosa cells in culture (Zachow et al. 1999; Caprio et al. 2001), while others have reported that in conditions with metabolic disturbances, such as obesity and polycystic ovarian syndrome, leptin has an inhibitory effect on the gonads (Naseri et al. 2021). So, these contradictory results regarding the effect of leptin may be due to the differences in the administrated doses of leptin, circulatory leptin levels, the duration of treatment, and the *in vivo* or *in vitro* study model used.

Conclusion

Leptin can protect both ovarian tissue and its reserve from the detrimental effects of OVIR injury. The underlying mechanisms are involved in the upregulation of Sirt-1/Nrf2 pathway, which suppress TLR4/NF- κ B/caspase-3 signaling pathway. Thus, leptin can be used as an adjuvant therapy to prevent ovarian damage and improve the outcomes. However, clinical studies are needed to ascertain these results in human. Further studies are also needed to explore the changes in leptin level in ovarian tissue in the model of OVIR.

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