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Underlying mechanisms behind the protective effect of angiotensin (1–7) in experimental rat model of ovarian ischemia reperfusion injury

Fatma F. Ali[a,*], Amira F. Ahmed[b], Doaa M. Elroby Ali[c]

[a] Medical Physiology Department, Faculty of Medicine, Minia University, Minia, Egypt
[b] Histology and Cell Biology Department, Faculty of Medicine, Minia University, Minia, Egypt
[c] Biochemistry Department, Faculty of Pharmacy, Deraya University, Minia, Egypt

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ABSTRACT

Aims: Ovarian ischemia as a consequence of torsion constitutes a gynecologic emergency affecting females during reproductive age. Its management by detorsion results in ovarian ischemia-reperfusion (IR) injury. Thus, a conservative treatment with detorsion is highly recommended. Therefore, we attempted to investigate the effect and underlying mechanisms of angiotensin 1–7 (Ang-(1–7)) treatment against ovarian IR injury.

Main methods: Female rats were included into: Sham group; Ang-(1–7) (300 μg/kg, i.p.) group; ovarian IR groups with and without Ang-(1–7) treatment. We determined ovarian Ang-(1–7), malondialdehyde (MDA) and nitric oxide (NO) in addition to serum total anti-oxidant capacity (TAC) levels. Ovarian gene expressions of angiotensin converting enzyme 2 (ACE2), Mas receptor, tumor necrosis factor alpha (TNF-α) and B-cell leukemia/lymphoma-2 (BCL-2) were estimated. Furthermore, histopathological changes and ovarian expressions of nuclear factor kappa B (NF-κB), inducible and endothelial nitric oxide synthases (iNOS and eNOS) were done.

Key findings: Treatment of ovarian IR rats with Ang-(1–7) led to marked improvement of ovarian damage through histological examination which was accompanied with marked increase in ovarian Ang-(1–7) level and expressions of ACE2 and Mas receptor, decrease in MDA and NO levels and expressions of NF–kB, iNOS and TNF-α with increase in serum TAC levels and ovarian expressions of eNOS and BCL-2.

Significance: Our results proved the protective effect of Ang-(1–7) against ovarian IR injury in rats and this may be attributed to ACE2/Ang (1–7)/Mas axis which showed anti-oxidant, anti-inflammatory and anti-apoptotic effects. Therefore, Ang-(1–7) can be used in the future for treatment of ovarian IR injury.

1. Introduction

Ovarian torsion is the total or partial rotation of the ovary, the fallopian tube, or both, around its vascular axis [1]. The majority of the cases are women of reproductive age. Therefore, early diagnosis and treatment is necessary for the preservation of the affected ovary, and hence that of fertility. So, management with detorsion of the pedicle is preferable than adnexectomy [2]. Unfortunately, detorsion surgery is often delayed and ischemia progresses because the signs of ovarian torsion are nonspecific. Developing novel agents to prevent IR injury in delayed ovarian torsion cases is of great concern [3].

The main pathological incident in ovarian torsion is ischemia followed by reperfusion. Thus, ovarian torsion–detorsion is an ischemia reperfusion (IR) injury to the ovaries [4].It leads to the occurrence of morphological, histological, and biochemical alterations within the ovarian tissue [5]. Ischemia is the death of cells which occurs by inadequate tissue perfusion, depletion of cellular energy storages, and cumulation of toxic metabolites. Ischemic tissues need to recover blood supply for cells renewal and elimination of toxic metabolites. But, reperfusion paradoxically leads to much more damage to the tissue than the ischemic damage. Reperfusion-related cell damage can occur by numerous factors, mostly including oxygen-derived free radicals, [3]. Reactive oxygen species (ROS) cause DNA damage, cell membrane, and mitochondrial damage *via* lipid peroxidation and cytokine production from activated neutrophils [6].

Nitric oxide (NO) is produced by nitric oxide synthase (NOS) enzymes; endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS). iNOS and eNOS are the predominant enzymes in rat ovaries. The excessive rise of the intracellular NO concentration initiates the toxic events that lead to cell death especially with ROS formation and generation of more harmful products such as peroxynitrite [7].

* Corresponding author.
E-mail address: fatmafarrag2008@gmail.com (F.F. Ali).

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Nuclear factor kappa B (NF-κB) comprises a family of pleiotropic transcription factors which play an essential role in regulating the expression of genes implicated in different cell processes, including inflammation [8]. Activation of NF-κB can be induced by different molecules such as cytokines and ROS [9]. Upon NF-κB activation, it triggers the transcription of proinflammatory mediators like interleukin-6, tumor necrosis factor alpha (TNF-α), and iNOS [10].

Angiotensin 1–7 (Ang-(1–7)) is an established bioactive peptide of the renin-angiotensin system (RAS). It has been known that Ang-(1–7) can oppose Ang II-exerted responses under different pathological conditions [11]. Ang-(1–7) can be formed both directly from Ang II and indirectly from Ang I *via* angiotensin converting enzyme 2 (ACE2) and its effects are believed to be mediated through a G protein-coupled receptor; Mas. The presence of ACE2/Ang-(1–7)/Mas axis in the ovaries of different species has been shown [12].Therefore, our study was a trial to estimate the effect and underlying mechanisms of exogenous administration of Ang-(1–7) against ovarian IR-induced damage. This was done through measuring the markers of oxidative stress; malondialdehyde (MDA), NO and total anti-oxidant capacity (TAC), ovarian gene expressions of ACE2/Ang-(1–7)/Mas axis, TNF-α and anti-apoptotic marker; B-cell leukemia/lymphoma-2 (BCL-2) in addition to evaluating the ovarian histopathological changes and expressions of NF-κB, iNOS and eNOS.

2. Materials and methods

2.1. Animals

32 female Wistar albino rats weighing 260–280 g were included in our study. Rats were allowed to acclimatize for one week before inclusion in the experiment. They were kept in suitable standard housing conditions. Rats were supplied with laboratory chow and tap water. Our experiment protocol was approved by Minia Faculty of Medicine Ethics Committee for animal research [protocol number 190:1/2019] which is compatible with the NIH Guide for Care and Use of Laboratory Animals [13,14]. Rats were divided into 4 groups (n = 8 each group):

Sham group: all operative procedures except vessels occlusion were performed in this group. Rats were i.p. injected with saline vehicle.

Angiotensin (1–7) group: sham operated rats were i.p. injected with Ang-(1–7) (300 μg/kg body weight).

Ovarian IR (OIR) group: rats were exposed to 3 h of ischemia followed by 3 h of reperfusion [5]. Rats were i.p. injected with saline vehicle 1 h before reperfusion.

OIR + Ang-(1–7) group: each rat was i.p. injected with Ang-(1–7) (300 μg/kg body weight) 1 h before reperfusion [15].

The dose of Ang-(1–7) was chosen according to previous studies [15,16] and our pilot experiment. Moreover, the effect of Ang-(1–7) has been evaluated in previous different acute and chronic studies with different doses which were similar to or even higher than the used dose in our study and proved to have protective effect with no documented side effects [17–20].

2.2. Drug protocol

Ang-(1–7) (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in physiological saline on the experiment day.

2.3. Surgical and experimental technique

The rats were weighed and anesthetized using ip injection of 20% urethane hydrochloride. After anesthesia, rats were kept in a supine position and 2% iodine alcohol was used for antisepsis of the lower abdominal region. Then, 2.5 cm longitudinal incision was made in the lower abdominal region and the right ovary was visualized. For induction of ischemia, vascular clamps were applied on the right ovarian vessels. Thereafter, the anterior abdominal wall was sutured in two layers using 3/0 silk. At the end of 3-h ischemia period, the silk sutures were subsequently opened and the vascular clamps were removed and a 3-h reperfusion was continued [5,7,21].

2.4. Biochemical studies

At the end of our experiment and reperfusion period, rats were killed by decapitation. Blood samples were gathered in non-heparinized tubes. The clotted blood samples were centrifuged at 4,000 rpm for 15 min at 4 °C for sera separation. Sera were stored at −20 °C for analysis of TAC levels. Then, the right ovaries were dissected out and obtained.

2.4.1. Determination of serum TAC

Estimation of serum TAC was done according to manufacturer instructions using commercially available kit (Biogiagnostic, Egypt).

2.4.2. Estimation of ovarian MDA, NO and Ang (1–7)

Specimens of ovarian tissues were weighed and homogenized in ice cold phosphate buffered saline (PBS) solution. The homogenates were centrifuged and the supernatants were obtained for estimation of MDA and NO and Ang (1–7). Estimation of ovarian MDA levels was done according to manufacturer instructions (Biogiagnostic, Egypt). Determination of NO was performed using a colorimetric assay kit (Promega, USA). Ovarian levels of Ang (1–7) were measured using a commercial ELISA Kit (Wuhan Fine Biological Technology Co., china) following the instructions of the manufacturer.

2.5. Sample preparation and RNA isolation

Parts of rats' ovarian tissues were rinsed in liquid nitrogen, immersed and stored in −80 °C refrigerator for RNA extraction. Total RNA was isolated from frozen ovarian tissue according to RNA isolation kit (RiboZol™ RNA Extraction Reagents) AMRESCO, LLC Solon, Ohio USA. The concentration and purity of the purified RNA were determined by spectrophotometer Nanodrop1000, Thermo Scientific apparatus. High quality RNAs (A260/280 ≥ 1.6) were selected and were used for Quantitative real-time PCR synthesis.

2.6. Quantitative real-time PCR

ACE2, Mas receptor, TNF-α, BCL-2 and β-actin genes were selected as targets and internal reference gene. The sequences of interest genes were obtained from NCBI database and primer sets were designed *via* Thermo Fisher Scientific. Quantitative real-time PCR was performed using the One-Step TaqProbe qRT-PCR Kit abm. The sequences of the primers used in this study were as follows:

B-actin primers:
Forward: 5′-CCC GCG AGT ACA ACC TTC T-3′
Reverse: 5′-CGT CAT CCA TGG CGA ACT -3′
ACE2 primers:
Forward: 5′-GTG GAG CAC TGA CTG GAG C-3′
Reverse: 5′-GAC AGG AGG CTC GTA AGG TG-3′
Mas receptor primers:
Forward: 5′-TGA CAG CCA TCA GTG TGG AGA-3′
Reverse: 5′-GCA TGA AAG TGC CCA CAG GA-3′
TNF-α primers:
Forward: 5′-ACC ACG CTC TTC TGT CTA CTG-3′
Reverse: 5′-CTT GGT GGT TTG CTA CGA C-3′
BCL-2 primers:
Forward: 5′-GCG AAG TGC TAT TGG TAC CTG-3′
Reverse: 5′-ATA TTT GTT TGG GGC AGG TCT-3′.

TaqMan qRT-PCR gene expression assays were conducted in 0.1 ml fast tubes according to the manufacturer instructions, in a final volume of 50 μl. Thermal cycling was performed on the Bioer (Fully automated Real Time PCR) by using the following cycling conditions: 50 °C for

Table 1
Changes in oxidative stress parameters in different group.

Parameters	Groups			
	Sham	Ang-(1–7)	OIR	OIR + Ang-(1–7)
- Ovarian MDA (nmol/g)	78.57 ± 1.96	82.97 ± 2.11	120.8 ± 2.51[a,b]	98.46 ± 1.75[a,b,c]
- Ovarian NO (nmol/g)	60.62 ± 2.04	55.32 ± 1.89	89.52 ± 1.80[a,b]	71.57 ± 3.33[a,b,c]
- Serum TAC (mmol/g)	1.95 ± 0.12	2.06 ± 0.14	0.66 ± 0.08[a,b]	1.43 ± 0.09[a,b,c]

Ang-(1–7): Angiotensin 1–7; OIR: Ovarian ischemia reperfusion; MDA: Malondialdehyde; NO: Nitric oxide, TAC: Total antioxidant capacity.
[a] Significant difference from sham group.
[b] Significant difference from Ang-(1–7) group.
[c] Significant difference from OIR group, $p \leq 0.05$.Values are expressed as mean ± SEM of 8 rats in each group.

1 min, 95 °C for 10 min, and 40 cycles at 95 °C for 15 s, and 60 °C for 1 min.

2.7. Histological study

Ovarian tissue specimens from all groups were rapidly taken and fixed in 10% neutral-buffered formaldehyde for 24 h. Five-micrometer tissue sections were cut and stained with hematoxylin and eosin [22] and examined by U.TV0.5XC-3 light microscopy (Olympus, Japan). The histologist was blinded to the different groups.

2.8. Immunohistochemical evaluation

Immunohistochemical staining was done using polyclonal rabbit antibodies for NF-κB, iNOS and eNOS. Paraffin blocks were cut into 4 μm thick. Xylene was used to deparaffinize the paraffin embedded tissue sections, tissues gradually rehydrated through descending grades of ethyl alcohol. Tissues were treated with endogenous peroxidase in 0.3% H_2O_2 for 30 min to block the endogenous peroxidase activity. For antigen retrieval, the slides were boiled in 10 mM citrate buffer solution (pH 6.0) for 10–20 min followed by cooling at room temperature for 20 min. Then, the sections were incubated with the primary antibody rabbit polyclonal anti-NF-κB antibody (Cat #RB-9034-R7), anti-eNOS antibody (Cat #RB-9072-R7) and anti-iNOS antibody (Cat#RB-9242-P) (Thermo Scientific, Lab vision, Kalamazoo, MI, USA) for 30 min at room temperature in a humified chamber. After washing with phosphate buffer solution (PBS), the slides were incubated for 10 min with biotinylated secondary antibody. Incubation of the slides with streptavidin peroxidase complex was done for 5 min followed by incubation with 3,3-diaminobenzidine tetrahydrochloride (DAB) for 3 min. All the slides were counterstained by Mayer's hematoxylin for 1 min then washing with water. Dehydration with ascending grades of ethyl alcohol was done, followed by clearance, mounting and finally covered with cover slips. With each run, negative controls were done, the sections were stained in the same technique but without the primary antibody and treating them with PBS instead. The light microscope was used to detect and localize the immunostaining of the three antibodies. For NF-κB, the positive cells were nuclear stained. While the cytoplasmic stained cells were considered positive in case of iNOS and eNOS.

2.9. Measuring area fraction of NF-κB, iNOS and eNOS immune-positivity

Image J 22 software (open source Java image processing program) used for area fraction measurement of NF-κB, iNOS and eNOS immune-positivity [23]. Area fraction was measured in a standard measuring frame per 5 random fields in each group by using magnification ×400 by light microscope. Areas with positive immunostained tissues were used for evaluation regardless the intensity of staining. A red binary color was used to mask these areas so it could be measured by the computer system as follows:

1- Software converted the image type to 8-bit grey scale.
2- The image was then color threshold to choose only the color of interest which is the brown color of the immune-positivity.
3- Then the color was masked by a red binary color to measure area fraction which is the percentage of the pixels in the brown color that had been highlighted in red.

2.10. Statistical analysis

Expression of data was in the form of mean ± standard error of the mean (mean ± SEM). One-way analysis of variance was used for statistical analysis followed by Tukey's multiple comparisons test to identify significant differences between groups. Data were considered statistically significant if $p \leq 0.05$. We used Graph Pad Prism Software Version 6 for all statistical analyses.

3. Results

3.1. Oxidative stress parameters

According to study results, there was significant increase in ovarian MDA and NO levels with significant decrease in serum TAC level in OIR rats compared to sham and Ang-(1–7) groups. Meanwhile, OIR group treated with Ang-(1–7) prior to reperfusion improved significantly oxidative stress parameters in comparison to OIR group (Table 1).

3.2. Ovarian Ang-(1–7) levels

As regards Ang-(1–7), there was significant decrease in ovarian Ang-(1–7) level in OIR rats compared to sham and Ang-(1–7) groups. Meanwhile, OIR group treated with Ang-(1–7) prior to reperfusion improved significantly Ang-(1–7) level as compared to OIR group (Fig. 1).

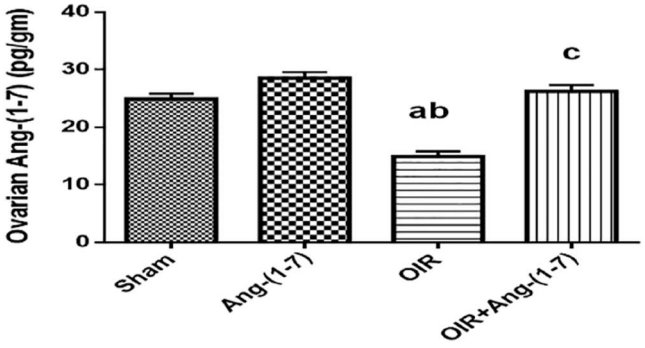

Fig. 1. Ovarian Ang-(1–7) levels of different groups.
Ang-(1–7): Angiotensin 1–7; OIR: Ovarian ischemia reperfusion; ACE2: Angiotensin converting enzyme 2. [a] Significant difference from sham group and [b] significant difference from Ang-(1–7) group and [c] significant difference from OIR group, $p \leq 0.05$.Values are expressed as mean ± SEM of 8 rats in each group.

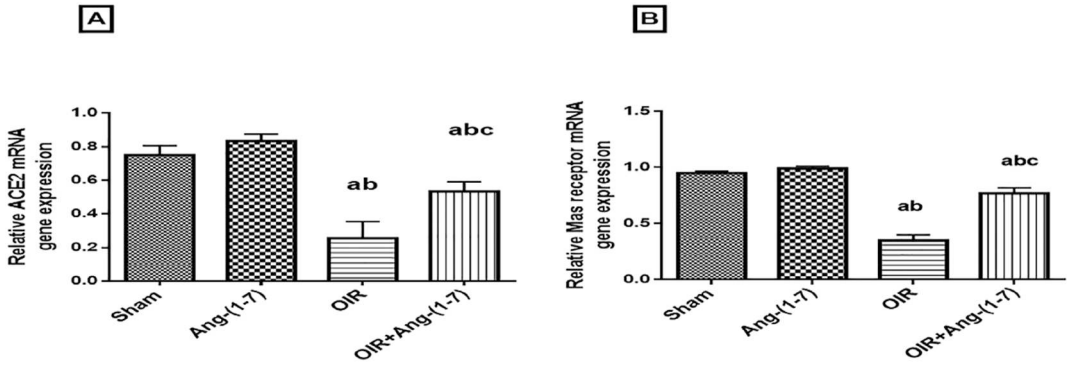

Fig. 2. Ovarian ACE2 and Mas receptor mRNA gene expressions of different groups.
Ang-(1–7): Angiotensin 1–7; OIR: Ovarian ischemia reperfusion; ACE2: Angiotensin converting enzyme 2. [a] Significant difference from sham group and [b] significant difference from Ang-(1–7) group and [c] significant difference from OIR group, $p \leq 0.05$.Values are expressed as mean ± SEM of 8 rats in each group.

3.3. Ovarian ACE2 and Mas receptor mRNA gene expressions

Our results showed that the ovarian gene expressions of both ACE2 and Mas receptor were significantly decreased in OIR group when compared to sham and Ang-(1–7) groups. Meanwhile, group received Ang-(1–7) before reperfusion showed a significant increase in their expressions in comparison to OIR group (Fig. 2A and B).

3.4. Ovarian TNF-α mRNA gene expression

The obtained results showed that ovarian expression of TNF-α was significantly increased in OIR rats compared to sham and Ang-(1–7) groups. On the other contrary, treatment of OIR rats with Ang-(1–7) prior to perfusion led to significant decrease in TNF-α expression as compared to OIR group (Fig. 3A).

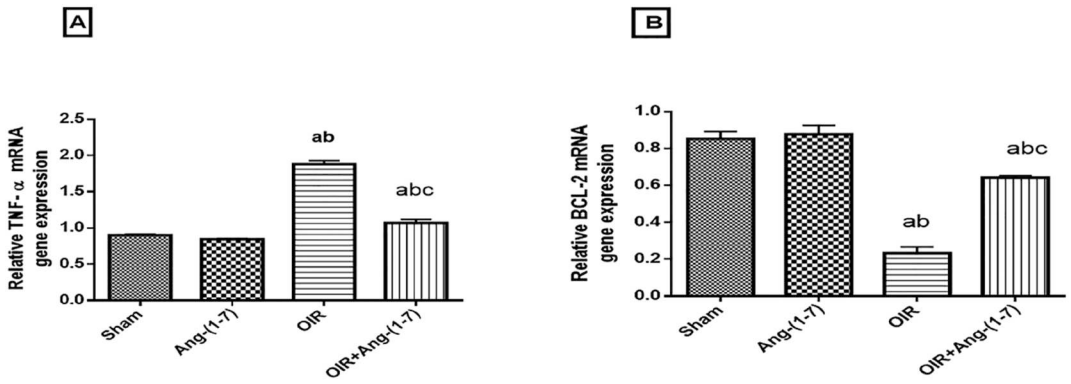

Fig. 3. Ovarian of TNF-α and BCL-2 mRNA gene expressions of different groups.
Ang-(1–7): Angiotensin 1–7; OIR: Ovarian ischemia reperfusion; TNF-α: Tumor necrosis factor alpha and BCL-2: B-cell leukemia/lymphoma-2. [a] Significant difference from sham group and [b] significant difference from Ang-(1–7) group and [c] significant difference from OIR group, $p \leq 0.05$.Values are expressed as mean ± SEM of 8 rats in each group.

3.5. Ovarian BCL-2 mRNA gene expression

Exposure of rats to OIR led to a significant decrease in BCL-2 gene expression in comparison with sham and Ang-(1–7) groups. Meanwhile, its expression was significantly improved when OIR rats treated with Ang-(1–7) before reperfusion (Fig. 3B).

3.6. Histological changes

Microscopic examination of the rat ovarian tissue of sham operated (A) and Ang-(1–7) (B) groups showed normal ovarian histological architecture with the presence of ovarian follicles in different stages in addition to normal luminal and mural granulosa cells, theca interna and theca externa. On the other contrary, degeneration of the ovarian follicles, vascular hemorrhage and congestion, inflammatory cell infiltrations, pyknotic nuclei and vacuolated cytoplasm of some ovarian cells with capillary dilatation and congestion were detected in ovarian IR (C) group. Meanwhile, administration of Ang-(1–7) to rats prior to reperfusion in ovarian IR (D) group led to marked improvement in the histological structure of the ovary which was evidenced by the presence of well-formed ovarian follicles, mild hemorrhage and congestion, mild inflammatory cell infiltrations and scattered cells with pyknotic nuclei and vacuolated cytoplasm (Fig. 4A–D).

3.7. Immunohistochemical results

3.7.1. NF-κB expression

Ovarian tissue of both sham operated (A) and Ang-(1–7) (B) groups showed negative NF-κB immune-reactivity of both the nucleus and cytoplasm of ovarian cells. While, OIR (C) group showed strong positive expression in both the nucleus and cytoplasm of ovarian cells. However, the expression was negative in the nucleus of many ovarian cells and faint expression in the cytoplasm of the ovarian cells in OIR + Ang-(1–7) (D) group (Fig. 5A–D).

3.7.2. iNOS expression

Rat ovarian tissues immunolabeled for iNOS showed negative immune-reactivity in either sham operated (A) or Ang-(1–7) (B) groups. In OIR (C) group, there was strong positive expression. Meanwhile, OIR + Ang-(1–7) (D) showed very faint iNOS expression in the cytoplasm of few ovarian cells (Fig. 6A–D).

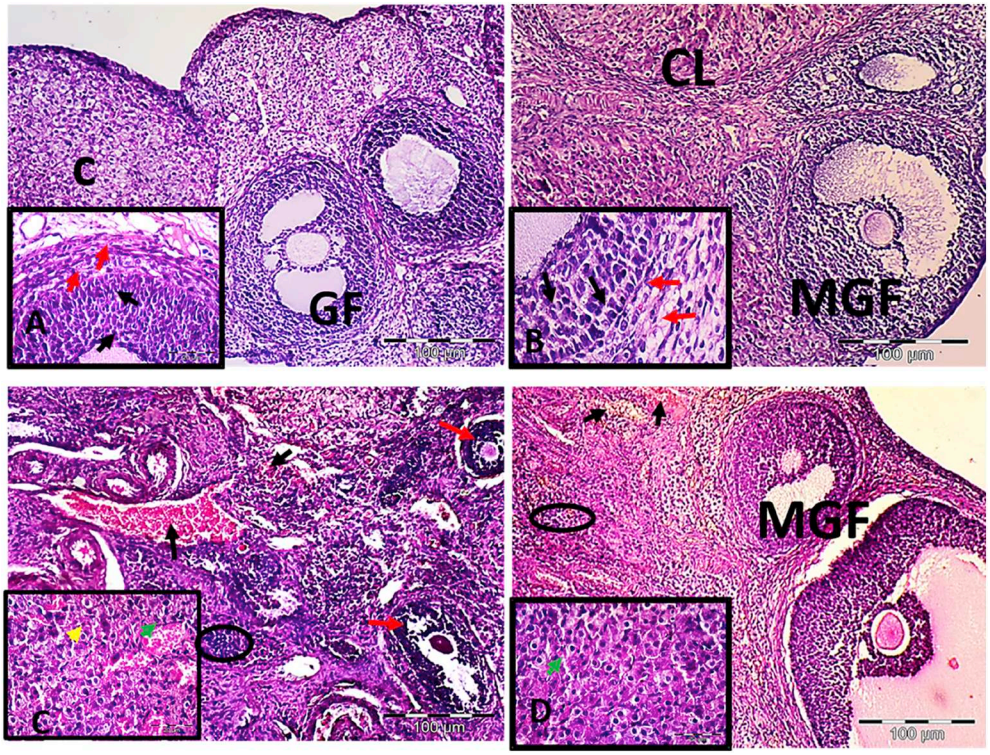

Fig. 4. Photomicrographs of the rat ovarian tissue showing: sham operated (A) and Ang-(1–7) (B) groups with normal histological architecture of the ovary with presence of the ovarian follicles in different stages, corpus luteum (CL), growing follicle (GF) and mature growing follicle (MGF) with normal luminal and mural granulosa cells (black arrows) and theca interna and theca externa (red arrows). Ovarian IR (C) group showing degeneration of the ovarian follicles (red arrows), vascular hemorrhage and congestion (black arrows) and inflammatory cell infiltrations (black circle). Notice pyknotic nuclei (green arrow) and vacuolated cytoplasm (yellow arrow) of some ovarian cells with capillary dilatation and congestion (inset). Ovarian IR + Ang-(1–7) (D) group showing marked improvement in the histological structure of the ovary with well-formed ovarian follicles (MGF), mild hemorrhage and congestion (black arrows). Mild inflammatory cell infiltrations (black circle). Notice scattered cells with pyknotic nuclei and vacuolated cytoplasm (green arrow) (inset). (H and E magnification was ×100 and inset magnification was ×400). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.7.3. eNOS expression

Ovarian tissue of both sham (A) and Ang-(1–7) (B) groups showed positive eNOS immune-reactivity of the cytoplasm of endothelial cells of ovarian vessels. Meanwhile, its immunereactivity in OIR (C) group was negative in both the cytoplasm of endothelial cells of many ovarian vessels and cytoplasm of most ovarian cells. Positive expression of the cytoplasm of the endothelium of few ovarian vessels was noticed. On the other hand, OIR + Ang-(1–7) (D) group showed positive eNOS expression of the cytoplasm endothelial cells of ovarian vessels and weak reaction in cytoplasm of ovarian cells (Fig. 7A–D).

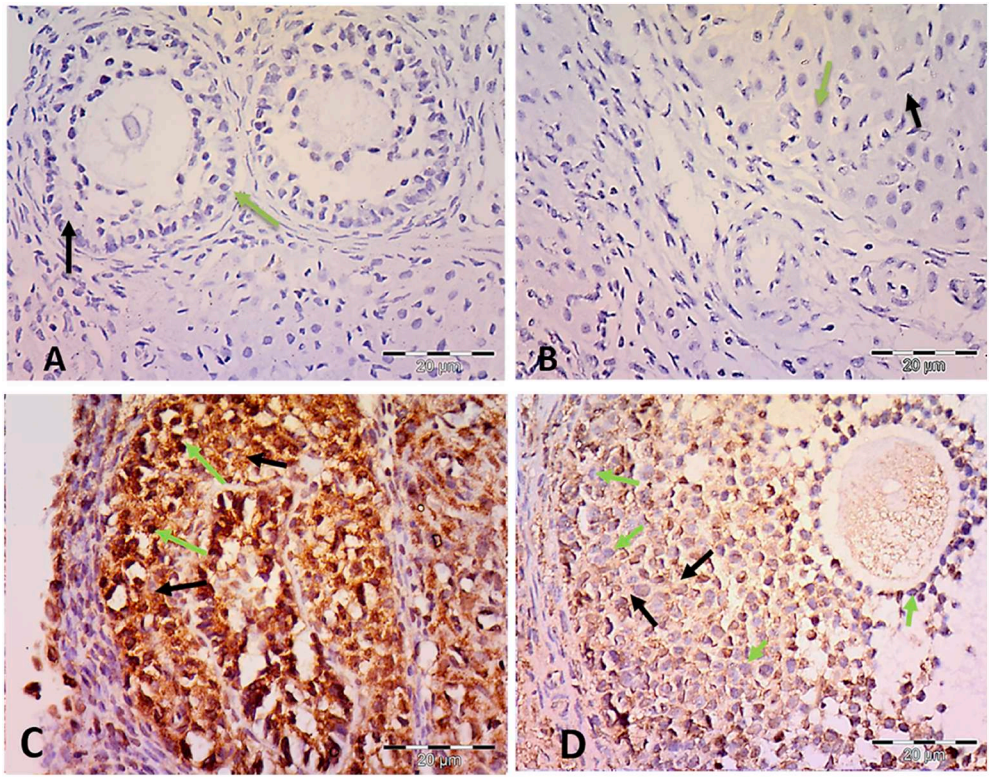

Fig. 5. Photomicrographs of the rat ovarian tissue showing: sham operated (A) group with negative NF-κB immune-reactivity of the nucleus (green arrow) and cytoplasm (black arrow) of the ovarian cells. Ang-(1–7) (B) group with negative NF-κB immune-reactivity of the nucleus (green arrow) and cytoplasm (black arrow) of the ovarian cells. Ovarian IR (C) group with positive NF-κB expression of the nucleus (green arrows) and cytoplasm (black arrows) of the ovarian cells. Ovarian IR + Ang-(1–7) (D) group with negative NF-κB reaction of the nucleus (green arrows) of many ovarian cells and faint expression of the cytoplasm (black arrows) of the ovarian cells. (NF-κB magnification was ×400). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

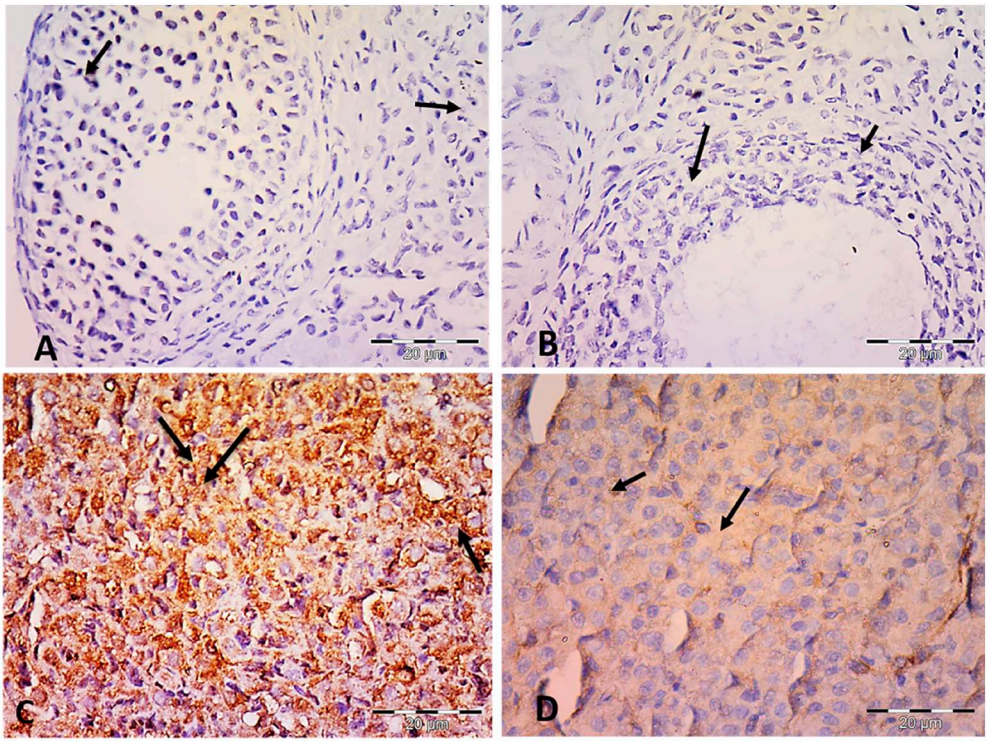

Fig. 6. Photomicrographs of the rat ovarian tissue showing: sham operated (A) and Ang-(1–7) (B) groups with negative iNOS immune-reactivity of the cytoplasm of the ovarian cells (black arrows). Ovarian IR (C) group with positive iNOS expression of the cytoplasm of the ovarian cells (black arrows). Ovarian IR + Ang-(1–7) (D) group with very faint iNOS reaction of the cytoplasm of few ovarian cells (black arrows). (iNOS magnification was ×400).

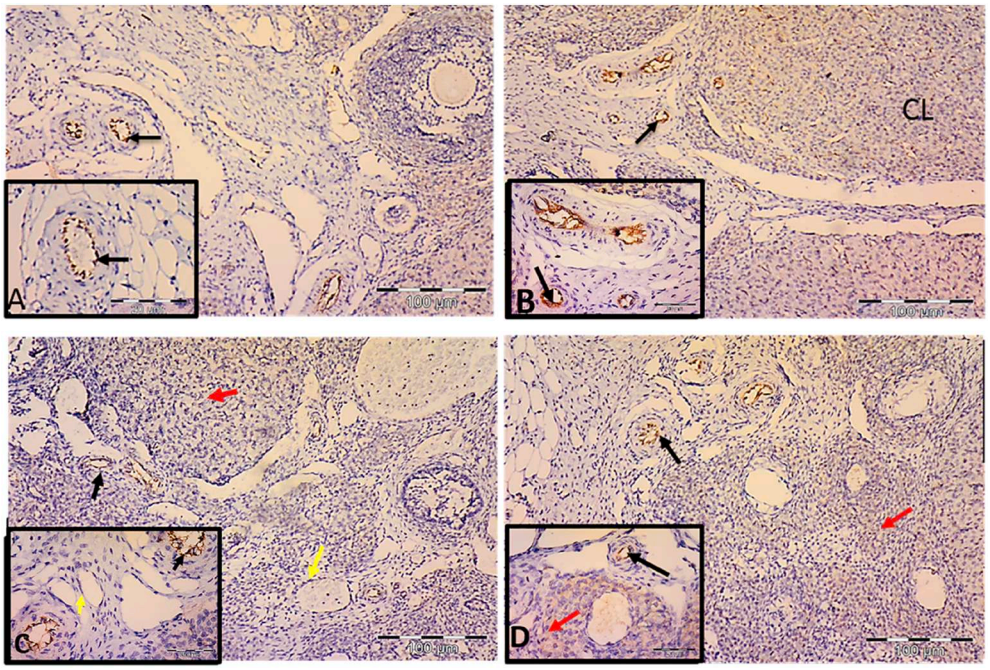

Fig. 7. photomicrographs of the rat ovarian tissue showing: sham operated (A) and Ang-(1–7) (B) groups with positive eNOS immune-reactivity of the cytoplasm of the endothelial cells of the ovarian vessels (black arrows).Ovarian IR (C) group with negative eNOS expression of the cytoplasm of the endothelial cells of many ovarian vessels (yellow arrows) and cytoplasm of most ovarian cells (red arrow). Notice positive expression of the cytoplasm of the endothelium of few ovarian vessels (black arrows). Ovarian IR + Ang-(1–7) (D) group with positive eNOS expression of the cytoplasm of endothelial cells of ovarian vessels (black arrows) and weak reaction in cytoplasm of ovarian cells (red arrows). (eNOS magnification was ×100 and inset magnification was ×400). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.8. Morphometric results of area fraction of NF-κB, iNOS and eNOS immune-positivity

As regards ovarian NF-κB and iNOS, there was significant increase of their expressions in OIR group in comparison with sham and Ang-(1–7) groups. Meanwhile, administration of Ang-(1–7) prior to reperfusion resulted in significant decrease in their expressions compared to OIR group. Concerning ovarian eNOS, our results showed that there was a significant decrease in eNOS expression in OIR group as compared to sham and Ang-(1–7) groups. However, its expression was significantly increased in OIR group treated with Ang-(1–7) in comparison with OIR group (Table 2).

4. Discussion

Ovarian reserve is more important these days as maternal age for pregnancy is older than previous [3]. Ovarian ischemia is a serious gynecological case which occurs due to ovarian torsion for various reasons like surgical manipulation and ovarian masses. Management with detorsion reperfuses the ischemic ovaries but leads to reperfusion injury. Therefore, a conservative treatment option is increasingly recommended with detorsion [21]. Oxidative stress, free radicals formation and inflammation are considered the main pathways in the occurrence of ovarian IR injury [7].

The present study provided an evidence for a pathophysiological

Table 2
Mean area fraction of ovarian tissues immunostained for NF-κB, iNOS and eNOS expressions.

Parameters	Groups			
	Sham	Ang-(1–7)	OIR	OIR + Ang-(1–7)
- NF-κB	3.87 ± 0.29	4.63 ± 0.38	18.50 ± 0.80[a,b]	10.50 ± 0.42[a,b,c]
- iNOS	4.00 ± 0.33	4.25 ± 0.31	20.38 ± 0.98[a,b]	10.63 ± 0.60[a,b,c]
- eNOS	21.50 ± 0.87	23.38 ± 0.60	12.63 ± 0.89[a,b]	24.25 ± 0.59[c]

Ang-(1–7): Angiotensin 1–7; OIR: Ovarian ischemia reperfusion; NF-κB: Nuclear factor kappa B; iNOS: inducible nitric oxide synthase; eNOS: endothelial nitric oxide synthase.

[a] Significant difference from sham group.

[b] Significant difference from Ang-(1–7) group.

[c] Significant difference from OIR group, $p \leq 0.05$.Values are expressed as mean ± SEM of 8 rats in each group.

role for the counter-regulatory RAS axis; ACE2/Ang-(1–7)/Mas in an experimental rat model of ovarian IR injury. Our results showed the association of ovarian damage which was confirmed with histological examination with the significant lowered ovarian expression of the ACE2/Ang-(1–7)/Mas axis in ovarian IR group as compared to sham rats. This is compatible with previous studies that showed decreased expression of ACE2/Ang-(1–7)/Mas axis during other animal models of IR in the kidney and testes [15,24].

According to our study results, marked oxidative stress damage was detected in the ovarian IR group compared to sham operated rats. This was manifested by the significant increase in the ovarian MDA and NO levels and decrease in serum TAC levels which is in line with other studies [6,21,25,26].

Ischemia is a decrease in blood supply of an organ. It results in the breakdown of ATP and lipid peroxides so that the generation of lactic acid and hypoxanthine is enhanced. During reperfusion, xanthine oxidase converts hypoxanthine to uric and superoxide radicals. These radicals consist of hydrogen peroxide, hydroxyl radicals, and superoxide anions which cause lipid peroxidation [1]. Lipid peroxidation is a free radical process that leads to the degradation of polyunsaturated fatty acids in cell membranes. Interaction with membrane lipids leads to an increase in membrane permeability and severe cell damage. MDA is the end product of lipid peroxidation and the elevation in tissue MDA concentration in case of IR shows that oxidative stress has developed [3].

Protection against oxidative stress involves a complex system of enzymatic antioxidants (SOD, GPX, glutathione reductase, catalase) and non-enzymatic antioxidants (glutathione (GSH), vitamins C and D) [27]. The oxidant/antioxidant balance altering in favor of oxidants leads to oxidative tissue damage [21].

The obtained results showed that there was significant increase in ovarian iNOS expression with significant decrease in eNOS expression in ovarian IR group as compared to sham rats which is as detected by [1,5,28,29]. Thus, the obtained increase in ovarian NO level in ovarian IR group in this study is attributed to the increased iNOS expression which is in agreement with [30] who found that NO could be protective or toxic during ischemia, relying on the NOS isoform involved. NO produced by eNOS reduced apoptosis and conferred protection. Whereas, pathological concentrations of NO from iNOS induced apoptosis and were toxic. In addition, [7] reported that NO is a source of free radicals formation as the high production of NO results in peroxynitrite formation which is an aggressive and powerful cellular oxidant system.

[29] indicated that phosphoinositide-3-kinase (PI3K) and Akt activation played a vital role in the process of IR injury salvage and as is reported eNOS is one of the major targets of Akt. They considered that the increased protein expressions of PI3K/Akt/eNOS signal pathway were a reason for the marked anti-IR injury effect. Meanwhile, both iNOS and oxidative stress resulted in NO uncoupling and inflammation which exacerbated IR injury. Furthermore, [31] found that eNOS overexpression significantly ameliorated IR injury in mouse livers which was evidenced by the correction of increased transaminase levels and improvement in histological signs of liver damage.

[33] reported that ROS can act through activation of NF-κB family of transcription factors which controls the production of proinflammatory cytokines such as TNF-α. TNF-α could in turn activate NF-κB and further aggravate proinflammation. This is in accordance with our study results as there was associated increase in oxidative stress parameters with the significant increase in ovarian expression of NF-κB and TNF-α in ovarian I/R group as compared to sham which is also as detected by [8–10]. In addition, [33] demonstrated that NF-κB activation was consistent with the expression of cytokines related with this transcription factor such as TNFα and iNOS that is in line with our study results. Moreover, [34] reported that activated NF-κB induced the synthesis of iNOS with subsequent production of NO in addition to cytokines.

Apoptosis is a form of cell death and provides tissue development and homeostasis [35]. [4] mentioned that when a tissue suffers from I/R, inflammatory cells cause generation of ROS, which increases leukocyte activation (particularly neutrophils) and leads to tissue damage and apoptosis that is as found in our study as there was significant decrease in ovarian expression of the anti-apoptotic marker; BCL-2 in ovarian IR group as compared to sham rats.

Anti-oxidant effects, anti-inflammatory and anti-apoptotic effects of Ang (1–7) in IR injury were demonstrated in experimental settings in previous studies [15,24,30].

Our study showed that acute treatment with Ang (1–7) prior to reperfusion in ovarian IR rats led to protective effects as demonstrated by marked attenuation of ovarian damage and inflammation as evident by histological examination. This was associated with significant increase in ovarian expression of ACE2/Ang (1–7)/Mas axis and decrease in oxidative stress parameters which was confirmed by the significant decrease in the ovarian levels of MDA and NO and increase in serum TAC levels. This indicates that one of the protective mechanisms of ACE2/Ang (1–7)/Mas axis was associated with its antioxidant effects which is in accordance with previous studies [36–39].

[38] reported that ACE2/Ang-(1–7)/Mas pathway is involved in the control of NADPH oxidase (Nox) expression which was well correlated with ROS production. They provided the evidence that activation of the ACE2/Ang-(1–7)/Mas axis produced a direct protective effect during ischemic injury *via* decreased Nox expression and resulting lower ROS production.

Our results demonstrated that treatment of ovarian IR rats with Ang (1–7) before reperfusion resulted in significant decrease in iNOS and increase in eNOS expression in ovarian tissue. This could be attributed to the increased ovarian expression of ACE2/Ang (1–7)/Mas axis and proves the involvement of eNOS in its protective effect in case of IR injury which is in agreement with other studies [37,39,40]. [43] found that ang-(1–7), through binding to the Mas receptor, activated Akt-dependent pathways including the stimulation of Akt phosphorylation *via* Akt kinase. These pathways ultimately led to activation of eNOS. Furthermore, [40] reported that eNOS activity was increased by either ACE2 overexpression or Ang-(1–7) infusion indicating that Ang-(1–7)

through Mas receptors could directly activate eNOS.

According to study results, there was significant decrease in ovarian expression of NF-kB and TNF-α in association with the significant increased expression of ACE2/Ang (1–7)/Mas axis in ovarian IR rats treated with Ang-(1–7) as compared to IR group which is in line with previous studies [42–44]. This confirms the anti-inflammatory actions of Ang-(1–7) and its axis which resulted in suppression of expression of NF–kB which facilitates the synthesis of several different pro-inflammatory cytokines such as TNF-α and this is compatible with other studies [44–46].

[47] found that Ang-(1–7) through promoting the inhibition of Nox and diminishing ROS generation in turn led to marked attenuation of the NF-κB/iNOS axis. [48] also reported that Ang-(1–7) could mediate its anti-inflammatory/antioxidative effect *via* activating Mas receptor which was evidenced by inhibition of NF-κB, Nox enzyme and consequently lipid peroxidation. All of these findings are in line with our study results.

The activation of the PI3K/Akt pathway is one of most relevant Mas-related signaling events [49] and might be involved in the protective effect of Ang-(1–7) against Nox-derived ROS formation and apoptosis [47,50]. Furthermore, [15] found that acute treatment with Ang-(1–7) reduced the DNA damage and consequently promoted the gene expression of BCL-2. This is as detected in our study as there was association between the increased ovarian expression of both ACE2/Ang (1–7)/Mas axis and BCL-2 in ovarian IR rats treated with Ang (1–7) as compared to IR group which confirms the anti-apoptotic effect of ACE2/Ang (1–7)/Mas axis in our experimental rat model of ovarian IR.

In conclusion, the study results showed the protective effect of up-regulation of ACE2/Ang (1–7)/Mas axis *via* Ang-(1–7) administration against a rat model of ovarian IR injury that was attributed to its anti-oxidant, anti-inflammatory and anti-apoptotic effects. This was confirmed by marked improvement of ovarian damage through histological examination which was associated with marked decrease in ovarian oxidative stress parameters (MDA and NO) and expressions of NF–kB, iNOS and TNF-α with increase in serum TAC levels and ovarian expressions of eNOS and anti-apoptotic marker; BCL-2.

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Authors' contribution

Dr. Fatma performed the study design, measured the biochemical studies, performed the statistical analysis and wrote the article. Dr. Doaa performed and wrote PCR part. Dr. Amira performed and wrote the histopathology and immunohistochemical parts.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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