

Ischemia–reperfusion induced injury of rat ovary and the possible protective effect of amlodipine: Histological, immunohistochemical and biochemical study

Original
Article

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ABSTRACT

Introduction: Ovarian torsion constitutes a gynecologic surgical emergency. However, early diagnosis of torsion may help to detorsion of the affected ovary and to preserve the fertility. Conservative therapy may lead to local and systemic deteriorating effects on the viability of the detorsioned ovary.

Aim: The present study was done to evaluate the possible protective effect of amlodipine on ischemia and ischemia reperfusion (I/R) induced ovarian injury in a rat model using histological, immuno-histolochemical, biochemical and morphometric studies.

Material and Methods: Forty adult female albino rats were used. They were equally divided into five groups. Group 1: Sham operation (control). Group 2: (ischemia) 3 hours duration of ischemia. Group 3: (ischemia/reperfusion) same as group 2 followed by 3 hours of reperfusion. Group 4: (ischemia and amlodipine) 5 mg amlodipine given orally one hour before ischemia then 3 hours of ischemia. Group 5: (ischemia, re-perfusion and amlodipine) 2 hours of ischemia then 5 mg amlodipine given orally then 3 hours of reperfusion. Then ovarian removal was performed and subjected to histological, immuno-histolochemical , biochemical and morphometric studies.

Results: In ischemia and (I/R) groups, the ovaries revealed exfoliation and desquamation of granulosa cells into the follicular cavity of degenerated follicles, with karyolysis and karyorrhexis of the oocyte nucleus, Congested dilated vessels, hyaline degeneration and vacuolation in the interstitial region, Massive cellular infiltrate, hemosiderin deposits and massive hemorrhage of the ovarian stroma. There was positive immunoreactivity for PECAM-I and CD68 and negative for estrogen receptor immunostain. Both superoxide dismutase (SOD) and glutathione (GSH) concentrations were significantly decreased while malondialdehyde MDA (LPO) concentration was significantly increased in ischemia and I/R groups as compared with the other groups. Amlodipine addition improves both histological findings and biochemical values

Conclusion: Amlodipine attenuates ischemia and (I/R) induced ovarian tissue injury

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Key Words: Amlodipine, exfoliation, hemorrhage, ischemia, reperfusion.

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INTRODUCTION:

Ovarian torsion constitutes a gynecologic surgical emergency. It results from twisting of the adnexa (ovary and / or fallopian tube) on its supporting ligaments^[1]. Childbearing age women and pre-menarche girls are the most common affected groups^[2] in whom, ovarian torsion often is misdiagnosed^[3]. In neglected or prolonged cases, necrosis develops in the ovarian tissue to which blood flow has been cut for a long time. In these cases, surgical removal of the ovaries is required^[4]. Before surgery, painkillers usually are used for pain control^[5]. Laparoscopic ovarian-torsion surgery generally is used for ovarian detorsion and to restore blood flow to the ovary. On the other hand, early diagnosis of torsion may help to preserve the fertility^[6]. Surgical management which include de-torsion of the affected segment is the treatment of choice in case of early

diagnosis^[1]. Meanwhile, this type of therapy may lead to local and systemic deteriorating effects on the functional capacity and viability of the de-torsioned ovary^[7].

Ovarian torsion leads to ischemia which results in cell death due to insufficient blood supply to ovarian tissue. Ischemic ovarian tissue needs to restore its blood supply which is essential for cell regeneration. De-torsion of the ovary helps to recover its perfusion leading to more worsen of the tissue injury than caused by ischemia^[8]. This patho-physiologic process called ischemia – reperfusion (I \R)^[9]. It leads to adnexal congestion, edema, ischemia with discoloration and finally necrosis^[10].

One of the main features of ischemia-reperfusion is oxidative stress which results in reactive oxygen species (ROS) production with subsequent apoptosis, antioxidant

system depression and lipid peroxidation affection (LPO)^[11, 12].

As a consecutive result of cell injury, catalase and glutathione (GSH) levels are reduced causing inactivation of various enzymes^[13]. Malondialdehyde (MDA) is the basic product of polyunsaturated fatty acid peroxidation and is quite a toxic molecule. Increased MDA level is a marker for LPO. Therefore, it is used to determine in vivo and in vitro oxidative stress levels^[14].

Ischemia and hypoxia induced caspase activation leading to apoptotic guided cell death^[15]. CD68 is a type of glycoprotein which has the ability to bind with LDL (low density lipoprotein). It is usually expressed in the cytoplasmic granules of various blood cells and monocytes^[16]. It is a useful marker of macrophage cell lineage as monocytes, giant cells, histiocytes, osteoclasts and Kupffer cells^[17]. It can be expressed in human ovaries, positive cells are expressed in the ovarian stroma and in the theca lutein cells of the corpus luteum and its vascular connective tissue^[18].

CD31 antigen (PECAM-1) is a single chain trans-membrane glycoprotein with a molecular weight of 130 kD. It is expressed on the surface of granulocytes, platelets and monocytes. It is suggested to be involved in some active processes during wound healing, thrombosis and angiogenesis^[9].

Amlodipine is a calcium channel blocker. It has also angio-selectivity and anti-oxidant property^[19]. In previous studies, It was reported that, amlodipine inhibits lipid peroxidation, enhances production of nitric oxide, maintain the activity of superoxide dismutase (SOD) and may cause decrease in oxidation of (LDL)^[20]. However, another study investigated the effect of amlodipine on ischemic heart model in dogs^[21]. Their results showed that, amlodipine can cause relaxation and decrease oxygen consumption in ischemic myocardial muscle associated with increase nitric oxide production. Various studies reported that, amlodipine had protective effect on ischemia-reperfusion on some organs as liver^[22] heart^[23] and brain^[24]. These successful results for amlodipine in some organs in different systems guide us to the use of this medication in the model of ischemia and ischemia reperfusion (I/R) injury of rat ovaries. Hence, this study was done to evaluate the possible protective effect of amlodipine on ischemia and ischemia reperfusion induced ovarian injury in a rat model using histological, immune-histochemical, biochemical and morphometric studies.

MATERIAL AND METHODS:

Animal :

Animals were bred in accordance with international guidelines. The studies were approved by the institutional animal care and use committee of Menoufyia University. In this study, Forty adult female albino rats were used. Their weight ranged between 200 to 240 gm. The animals were obtained from Menoufyia University experimental

animal house. The rats were housed in metal cages at suitable constant temperature and were exposed to day light from 10-12 hours.

Drugs and Chemicals:

Amlodipine:

It was used in tablet form. Each tablet contains 5 mg of besylate salt of amlodipine. It was purchased from Amirya Pharmaceutical Industries (Alexandria - Egypt). The used dose was dissolved in 10 ml of 0.9% normal saline for oral administration.

Thiopental sodium:

Each vial contains 500 mg of Thiopental sodium in 20 ml. It was obtained from Sigma – Tec. Pharmaceutical Industries – Egypt – S.A.E. Each 1 ml of vial was diluted in 4 ml of distilled water for intra-peritoneal injection of the calculated dose.

Surgical Technique:

After the acclimatization period, thiopental sodium was used for anesthesia of the experimental animals. It was injected intra-peritoneal in a dose of 25 mg/kg and repeated as needed. A midline longitudinal incision in the lower abdomen (2.5 cm) was made. A small peritoneal incision was performed, and the adnexa and uterine horns were identified. A sham operation was performed in eight of the rats as a control group. Bilateral adnexal (Ovarian) ischemia was performed by applying vascular clips below the ovaries of female rats. The histo-pathological and biochemical changes of ovarian torsion and vascular clamp usage were shown to be very similar^[7]. The incision was closed with 4/0 nylon sutures, and after an ischemic period of three hours, the bilateral ovaries were surgically removed for histological and biochemical examination. For I/R group the three-hour period of ischemia was followed by 3 hours of reperfusion, that was done by removal of the clips. After which bilateral ovaries were removed for histological and biochemical studies^[25].

The experimental animals were randomly divided into equal five groups as follows:

Group 1: Control (sham) group : female rats were subjected only for laparotomy.

Group 2: Pure ischemia group: Surgical removal of bilateral ovaries were performed after three hours of ischemia.

Group 3: Ischemia - reperfusion (I/R) group: female rats underwent the same three hours of ischemia followed by three hours of reperfusion then surgical removal of both ovaries was done.

Group 4: Ischemia– amlodipine group: amlodipine (5 mg/kg) was administered orally one hour before starting ischemia then 3 hours of ischemia was performed then surgical removal of both ovaries was done^[25].

Group 5: Ischemia - reperfusion (I/R) and amlodipine group: ischemia was performed in this group for 3 hours then amlodipine (5 mg/kg) was administered orally one hour before reperfusion then reperfusion was done for 3 hours followed by surgical removal of both ovaries.

From each animal, both right and left ovaries were dissected and removed. The ovarian tissues were divided and subjected to the following studies:

I- Histological evaluation:

The right ovary of each female rat was dissected then fixed in 10% formal saline. The specimens were processed to obtain paraffin blocks. Each block was cut to 5 μ m thick sections and subjected to haematoxylin and eosin stains to examine the general architecture of the ovary^[26].

II- Immuno-histochemical study:

Formalin-fixed ovarian tissue was embedded in paraffin blocks for immuno-histochemical examination. Sections were de-paraffinized in absolute alcohol. Using absolute methanol 0.5% containing hydrogen peroxide (100 volumes) and 0.4% hydrochloric acid (1M) at room temperature for 40 minutes to block endogenous peroxidase activity. The sections were washed in water followed by 0.05 M Tris-buffered saline, then incubated in 1% trypsin. Finally, the sections were washed in cold water, stained using CD68 as primary antibodies at a dilution of 1:200^[9] while the primary antibodies for estrogen receptor beta (PC168, Oncogene Research Products or PA1 310, Affinity Bio-Reagents, Inc.) were used at 1:2000 or 5 μ g/ml dilution^[27] and for PECAM-1 (CD31) at a dilution of 1:100 was used as primary antibodies^[9] and were incubated with ultraviolet block. For Caspase-3 immunostaining, activated Caspase-3 expression was evaluated using a peroxidase-conjugated rabbit monoclonal antibody IgG (Cell signaling Technology, Ipswich, MA) at dilution 1:200. Secondary antibodies (biotinylated) was applied for one hour^[28]. Slides were exposed to streptavidin peroxidase and chromogen then washed with PBS. The slides were counter stained with Mayer's haematoxylin.

III-Morphometric study:

Image analysis system (Leica Qwin 500 C Image analyzer computer system (Leica Imaging system LTD., Cambridge, UK) at Histology Department, Faculty of Medicine, Al-Azhar University, Cairo was used for assessment of the mean area percentage (%) of Caspase-3, quantification of the mean number of estrogen receptor beta immune-positive cells (granulosa cells) and mean number of CD68 immuno-reactive cells (activated macrophages) in the ovarian sections. Assessment of the mean area% of Caspase-3 immunopositive cells was carried out at a magnification of \times 400. Five serial sections per rat were examined; in each section, 10 non overlapping high-power fields were measured. Using estrogen receptor beta immunostained sections and CD68 immunostained sections respectively, the number of estrogen receptor beta positive

cells and CD68 positive cells within the ovarian sections, were counted in four high (X400) non overlapping fields. For each group, the results were expressed as mean number of estrogen receptor beta and CD68 positive cells.

IV- Biochemical study:

In the present study, specimens of the left ovary were stored and preserved frozen at -80° C until the day of chemical assay. To obtain tissue homogenate, ovarian tissue was homogenized using glass homogenizer in cold 0.9% NaCl. For determination of enzymatic activity of superoxide dismutase (SOD), glutathione (GSH) and Malondialdehyde (MDA), tissue homogenates were centrifuged then the supernatant was used. All enzymatic assays were estimated at room temperature^[29]. For estimation of SOD activity, Kinetic colorimetric method was used by measuring it at 460 nm, at temperature 25C. Its absorbance values were read at 0 and 8 minutes of illumination^[30]. For measuring of tissue glutathione (GSH), Sedlak and Lindsay's method was used, using spectrophotometer, absorbance was determined at 412 nm^[31]. Colorimetric estimation of MDA in the ovary homogenate was performed by boiling homogenate with thiobarbituric acid. The resulting colored material was collected and measured at 532 nm^[32].

V-Statistical analysis:

With respect to Caspase-3 immune-staining, estrogen receptor beta and CD68 immune-staining, quantitative data were summarized as mean and SD and compared using one-way analysis of variance (ANOVA) test. In addition, all the biochemical data were expressed as mean \pm SD. Differences between the means were tested using analysis of variance. Statistical analysis was performed using SPSS (version 9; IBM, Armonk; New York, USA). The difference was insignificant at P- more than 0.05, P-value less than 0.05 was considered significant and highly significant at p- less than 0.001

RESULTS:

Histological results:

The morphologic characteristics of the ovarian tissues were normal in the control group. The general architecture of the ovary was structurally normal. It consisted of outer cortex and inner medulla. The cortex showed ovarian follicles in various stages of development. Under the surface, there were primordial follicles and primary follicles. Secondary follicles with multiple layers of granulosa cells containing several antral cavities. The ovarian stroma between the follicles appeared normal (Fig. 1). Mature graffian follicle containing large single antrum, oocyte, zona pellucida, cumulus oophorous, granulosa cells and thecal cells (Fig. 2). In addition, the outer cortex contained normally appearing corpus luteum, having foamy acidophilic cytoplasm and pale-stained nuclei (Fig. 3). Histological examination of ovaries of ischemia group revealed loss of normal ovarian histoarchitecture with multiple distorted and degenerated follicles, having

dark pyknotic nuclei. Congested dilated blood vessels, acidophilic hyaline degeneration and a huge accumulation of vacuoles were also observed in the interstitial region (Fig. 4). Abnormal secondary follicle showed exfoliation and desquamation of granulosa cells into the follicular cavity with karyolysis and karyorrhexis of the oocyte nucleus. Massive cellular infiltrate of the ovarian stroma was seen (Fig. 5). Hemosiderin deposits and massive hemorrhage in the ovarian stroma was detected (Fig. 6). Moreover, vacuolations were obvious in the granulosa and theca lutein cells of the corpus luteum (Fig. 7).

The general architecture of the ovary in ischemia reperfusion group nearly had similar histological findings such as degenerated follicles, vascular dilatation, vacuolations and edema of the ovarian stroma were observed (Fig. 8). Congested blood vessels with extravasation of red blood cells were observed in corpus luteum of the ovary of this group (Fig. 9). Histopathological findings of ischemia amlodipine group was partially improved than ischemia and I/R (ischemia reperfusion) groups findings (Fig. 10). In ischemia reperfusion amlodipine group, the structural ovarian integrity was achieved significantly near normal in follicles and the stroma, although some degenerated follicles were still present. In interstitial region, some vacuolations, hyaline degeneration and minimal cellular infiltrate were observed while hemorrhage could not be detected (Fig. 11 and 12).

Immunohistochemical results:

CD 31 (PECAM-I) immunostaining

Mild positive reaction of CD31 expression in the small blood vessels within theca layer of the ovarian follicle and in the stromal region were observed in the control group. Endothelial cells in these vessels were stained with CD31 (PECAM-I). Ischemia and ischemia reperfusion groups, showed strong positive expression of PECAM-I mainly in the endothelial cells lining the blood vessels wall of the stroma. While, ischemia-amlodipine group showed moderate positive expression of PECAM-I in small vessels of stromal area and within corpus luteum. Ovarian tissues of ischemia- reperfusion amlodipine group showed mild positive expression of PECAM-I in small vessels of the stroma (Fig. 13 A-E).

Estrogen receptor immunostaining

Control ovary denoted strong positive immune-reactivity for beta estrogen receptors within the cytoplasm of zona granulosa cells of the ovarian follicle. Ischemia group showed negative immune-reactivity for beta estrogen receptors within granulosa cells. Ischemia reperfusion ovary demonstrated weak positive expression for beta estrogen receptors within granulosa cells. Ischemia amlodipine ovary denoted mild positive immune reactivity for beta estrogen receptors within granulosa cells. Ischemia reperfusion amlodipine ovary showed strong positive immunoreactivity for beta estrogen receptors within granulosa cells (Fig. 14 A-E).

CD68 immunostaining

Immunohistochemical labeling of anti-CD68 antibody, a specific marker for monocytes and macrophages revealed the presence of macrophages and weak positive expression of CD68 in the ovarian stroma of control group. While, ischemia group ovary showed presence of macrophages infiltrating the stromal area and wall of a degenerated follicle which showed strong positive expression of CD68. Ischemia reperfusion group ovary showed strong positive CD 68 expression in the ovarian stroma. Moderate positive expression of CD68 in ovarian stroma was observed in ischemia amlodipine group. Decreased activity of macrophages and weak CD68 expression in the ovarian stroma were demonstrated in the ischemia reperfusion-amlodipine group (Fig. 15 A-E).

Caspase-3 immunostaining

In the control group, a negative expression of caspase-3 immunoreaction was noticed in nuclei of granulosa cells of ovarian follicle. In ischemia group, most of cells lining the ovarian follicles showed intense positive immune expression of caspase-3. Ischemia reperfusion group revealed strong positive expression of caspase-3 in multiple granulosa cells of the follicle. Ischemia amlodipine group revealed moderate positive expression of caspase-3 immunoreaction in some granulosa cells and few theca cells. Ischemia reperfusion amlodipine group showed mild positive expression of caspase-3 immunoreaction in few granulosa cells while most of follicular cells are negative (Fig. 16 A-E).

Morphometric and statistical results

The mean area% of Caspase-3 immune-reaction was significantly increased ($p < 0.05$) in ischemia and ischemia reperfusion (I/R) groups in comparison with the other experimental groups (Table 1A and Graph 1A).

The mean number of estrogen receptor beta immune-positive cells in ischemia and I/R groups showed a highly significant decrease ($p < 0.001$) compared to the control group. Ischemia amlodipine group showed a significant decrease ($p < 0.05$), while ischemia reperfusion amlodipine group showed a non-significant increase compared to the control (Table 1B and Graph 1B).

Regarding the mean number of CD68 immune-reactive cells in the ovarian tissue, ischemia and I/R groups showed a significant increase ($p < 0.05$) compared to the control group, while ischemia amlodipine and ischemia reperfusion amlodipine groups showed a non-significant increase compared to the control group (Table 1C and Graph 1C).

Biochemical Investigations:

Levels of SOD were decreased in the ischemia and ischemia reperfusion (I/R) groups. However, administration of 5 mg/kg b.w. of amlodipine before ischemia and I/R treatment reversed the trend in the rat's ovary. Thus, ischemia and I/R treatments significantly decreased ($P < 0.05$) the levels of SOD activity in the

rat's ovary (Table 2, Graph 2). The tissue homogenates of Ovarian sections showed a highly significant decrease ($P < 0.001$) in the reduced GSH concentrations in ischemia and I/R groups in comparison to the other groups (Table 2, Graph 3). Also the homogenate contents showed a highly significant increase ($P < 0.001$) in MDA (LPO) concentrations in ischemia and I/R group when compared to the other groups (Table 2, Graph 4).

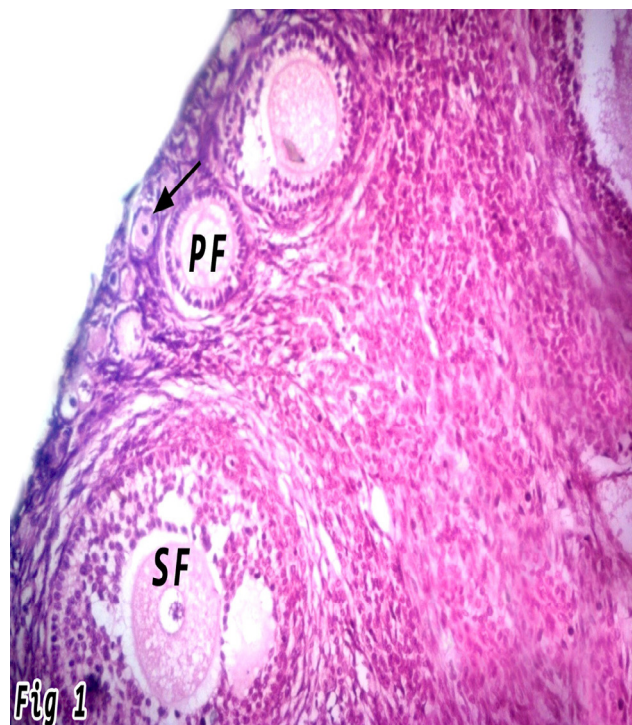


Fig. 1: photomicroGraph of control adult rat ovary showing primordial follicles under the capsule (arrow), primary follicles (PF) and secondary follicle (SF). Note: the normal appearance of the ovarian stroma. (H andE, x200)

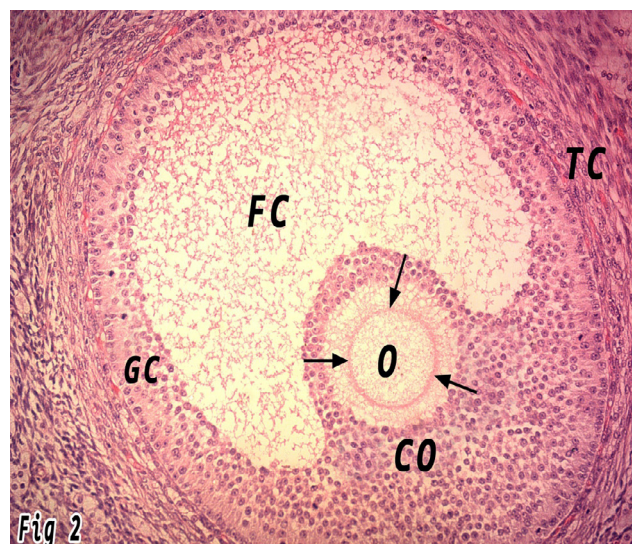


Fig. 2: photomicroGraph of control adult rat ovary showing mature graffian follicle containing follicular cavity (FC), oocyte (O), zona pellucida (arrow), cumulus oophorus (CO), granulose cells (GC) and thecal cells (TC). (H andE, x400)

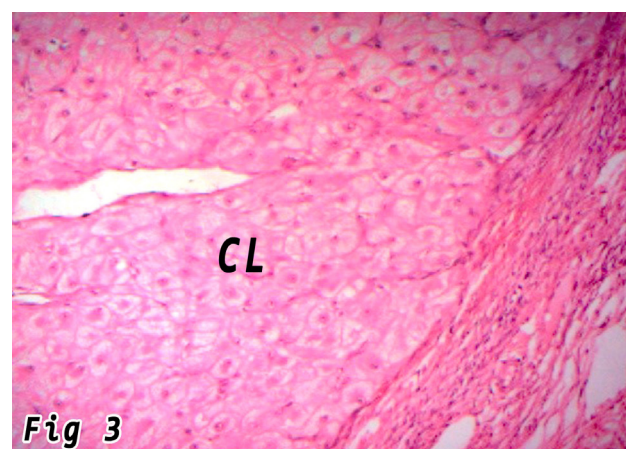


Fig. 3: photomicroGraph of control rat ovary showing normally appearing corpus luteum (CL). Cells having acidophilic foamy cytoplasm with pale nuclei. (HandE, x 400)

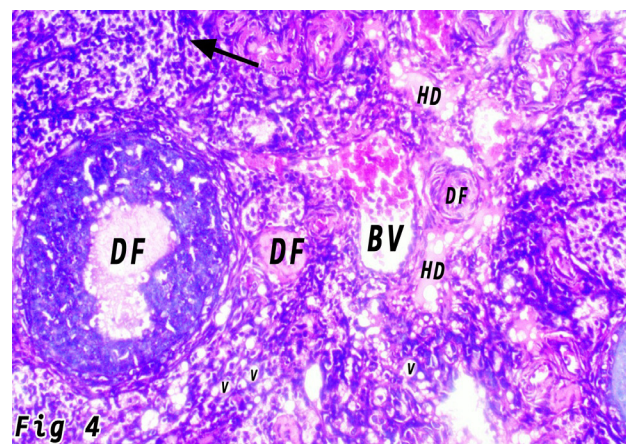


Fig. 4: photomicroGraph of ischemia group adult rat ovary showing loss of normal ovarian histoarchitecture with multiple distorted and degenerated follicles (DF), having dark pyknotic nuclei (arrows). Congested dilated blood vessels (BV), acidophilic hyaline degeneration (HD) and vacuolation of the stroma were also present (V). (H andE, x200)



Fig.(5): photomicroGraph of ischemia group adult rat ovary showing abnormal secondary follicle with exfoliation and desquamation of granulose cells (GC) into the follicular cavity with karyolysis and karyorrhexis of the oocyte nucleus (arrow). Note: the massive cellular infiltrate of ovarian stroma (arrow head). (HandE, x 400)

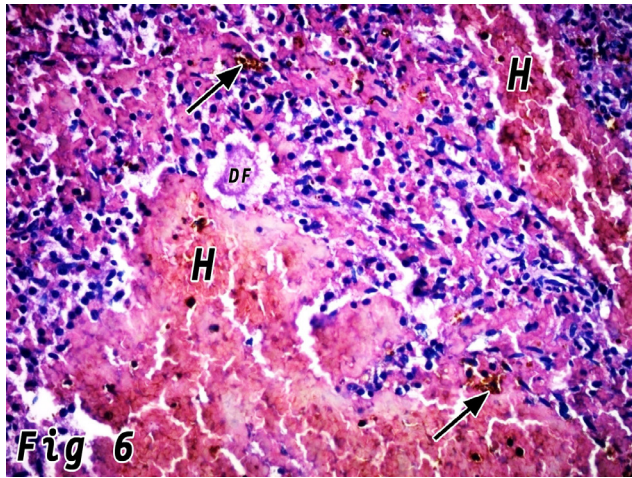


Fig. 6: photomicroGraph of a section in ovary of ischemia group showing hemosiderin deposits (arrow) and massive hemorrhage(H) in the ovarian stroma. Note presence of degenerated follicle (DF). (HandE, x 400)

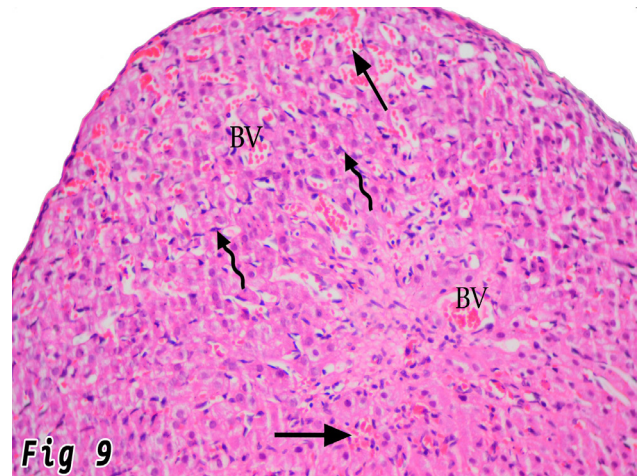


Fig.9: photomicroGraph of ovarian section of ischemia reperfusion (I/R) group with noted red blood cells extravasation (arrow), congested blood vessels (BV) and multiple dark nuclei (corrugated arrow) in corpus luteum. (HandE, x200)

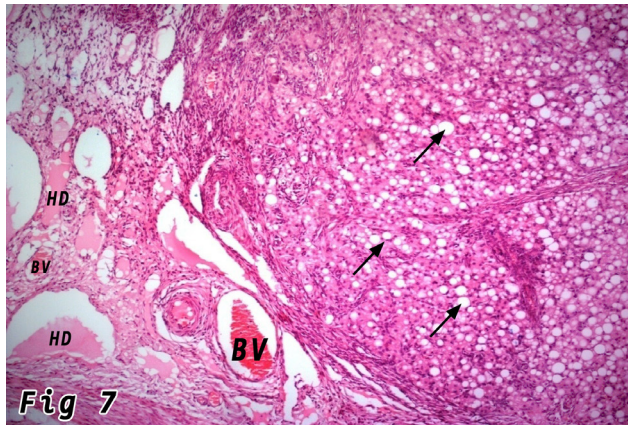


Fig. 7: photomicroGraph of a section in ovary of ischemia group showing vacuolation of granulosa and theca lutein cells of the corpus luteum (arrow). Congested blood vessels (BV) and hyaline degeneration (HD) of ovarian stroma were Observed. (HandE, x 200)

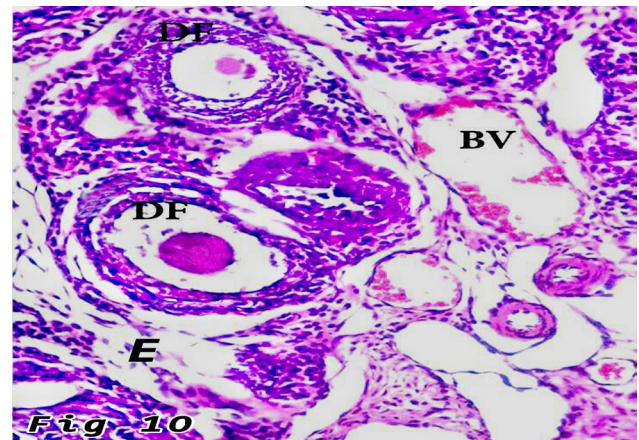


Fig.10: photomicroGraph of ovarian section of ischemia amlodipine group revealing degenerated follicle (DF), congested blood vessels (BV) and edema in ovarian stroma (E). (HandE, x200)

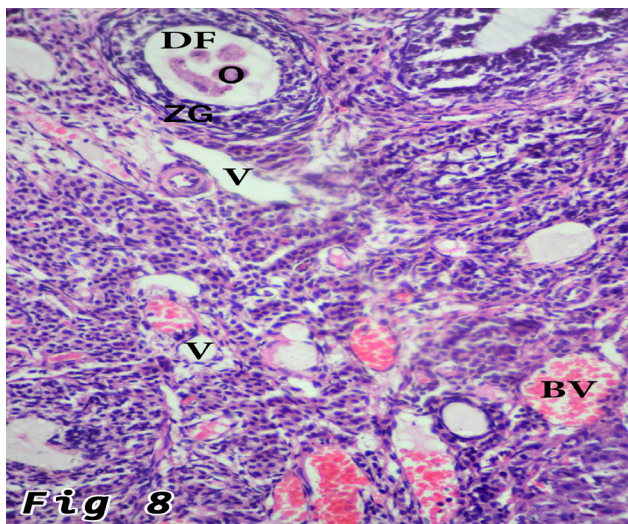


Fig. 8: photomicroGraph of ovarian section of ischemia reperfusion group revealing degenerated follicle (DF) and oocyte (O) surrounded by degenerated zona granulosa cells (ZG). Many vacuoles (V) and congested blood vessels (BV) can be seen in ovarian stroma. (HandE, x 400)

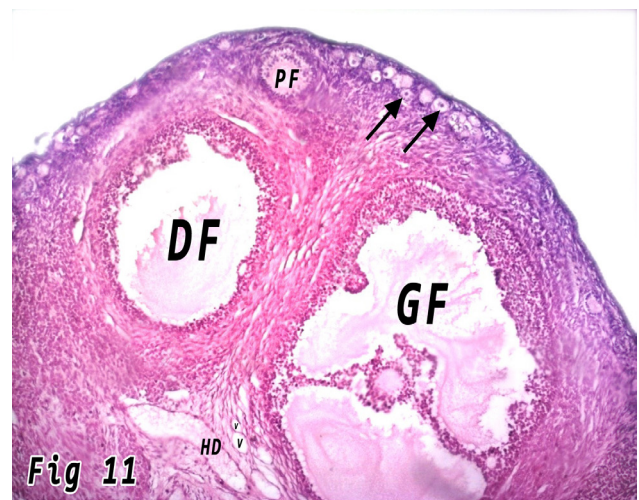


Fig. 11: PhotomicroGraph of section in rat ovary of ischemia reperfusion amlodipine group showing near normal ovarian stroma , primordial follicles (arrow) under the capsule, primary follicle (PF) , mature graffian follicle (GF) and degenerated follicle (DF). Note: vacuolation (V) and hyaline degeneration (H) in ovarian stroma. (HandE, x 100)

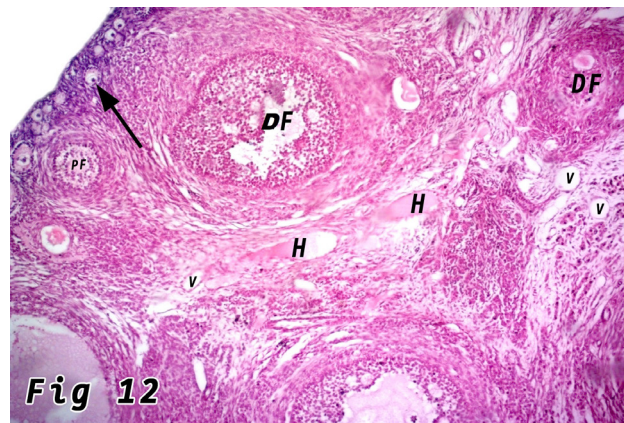


Fig. 12: PhotomicroGraph of section in rat ovary of ischemia reperfusion amlodipine group showing near normal ovarian stroma , primordial follicles (arrow) under the capsule, primary follicle (PF) and degenerated follicle (DF). Note: vacuolation (V) and hyaline degeneration (H) in ovarian stroma. (HandE, x100)

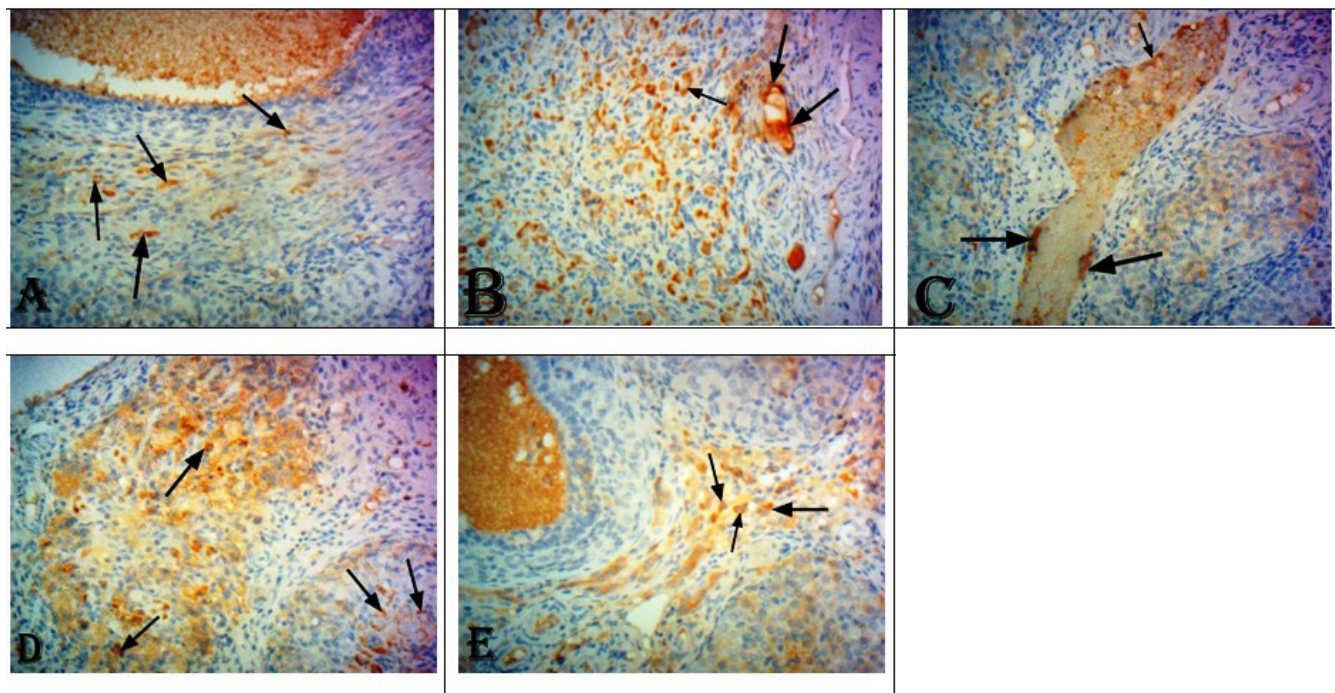


Fig. 13: A- control adult rat ovary showing part of ovarian follicle surrounded by theca layer and stromal area containing network of blood vessels giving mild positive PECAM-1 expression (arrow) B- ischemia group rat ovary, showing strong positive expression of PECAM-1 mainly in the endothelial cells lining the blood vessel of the stroma (arrow) C- ischemia reperfusion group rat ovary, showing strong positive PECAM-1 expression in endothelial cells in the expanded blood vessel wall in stromal area D-ischemia-amlodipine group rat ovary showing moderate positive expression of PECAM-1 in small vessels of stromal area and within corpus luteum E-ischemia- reperfusion amlodipine group rat ovary showing mild positive expression of PECAM-1 in small vessels of the stroma.

(PECAM-1 immunostaining, x 400)

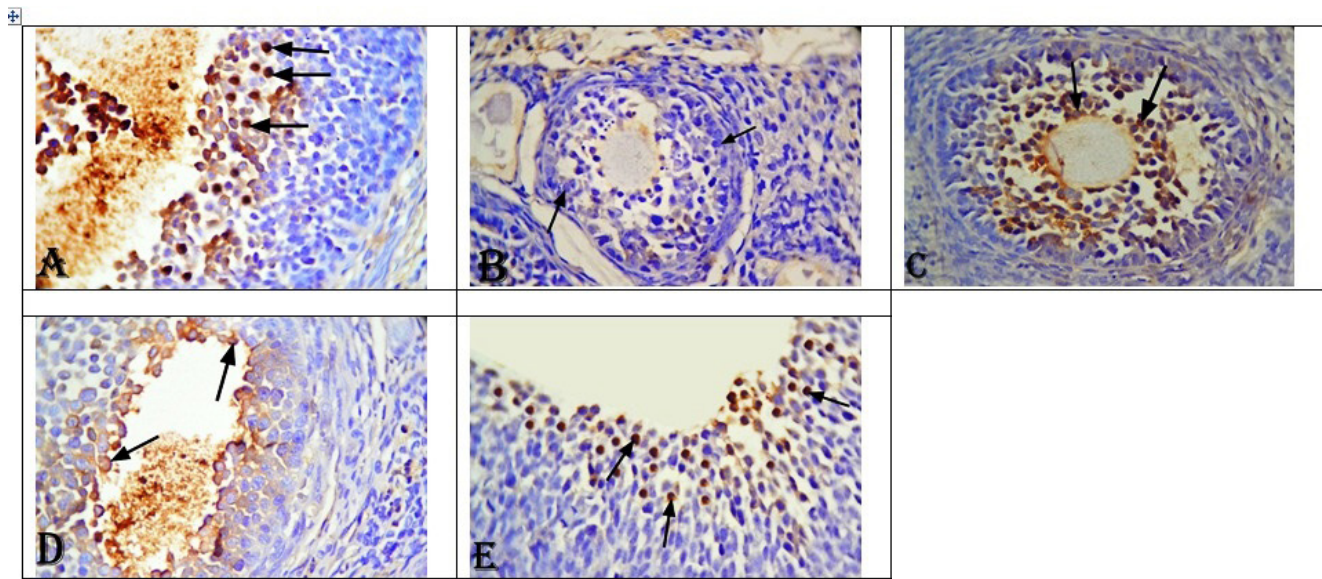


Fig. 14: A-control adult rat ovary denoting strong positive immune-reactivity for beta estrogen receptors within zona granulosa cells (arrow) (x 1000). B- ischemia group rat ovary showing negative immune-reactivity for beta estrogen receptors within granulosa cells (arrow) (x 400). C-ischemia reperfusion rat ovary showing weak positive immunoreactivity for beta estrogen receptors within granulosa cells (x 1000) D-ischemia amlodipine rat ovary denoting mild positive immunoreactivity for beta estrogen receptors within granulosa cells (x 400). E-ischemia reperfusion amlodipine adult rat ovary showing strong positive immunoreactivity for beta estrogen receptors within granulosa cells (x 1000). (Estrogen immunostaining, x 1000)

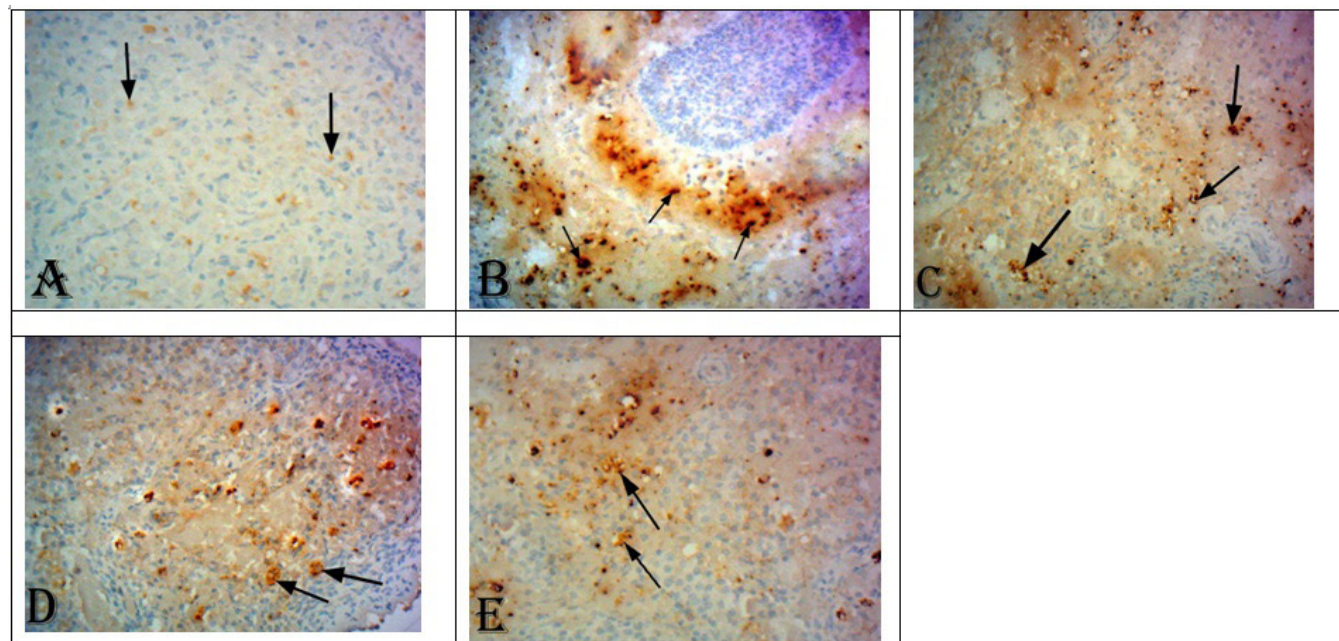


Fig. 15: A-control rat ovary revealing macrophage cells in ovarian stroma and weak expression of CD68 (arrow). B-Degenerated follicle and stromal area in ischemia group with infiltrated macrophages and strong positive CD68 expression (arrow). C-ischemia reperfusion group ovary showing strong positive Cd68 expression in ovarian stroma (arrow). D- ischemia amlodipine group ovary denoting moderate CD68 expression in ovarian stroma (arrow). E- ischemia reperfusion amlodipine ovary with decreased macrophage activity in stromal area and weak CD68 expression (arrow). (CD68 immunostaining, x 400)

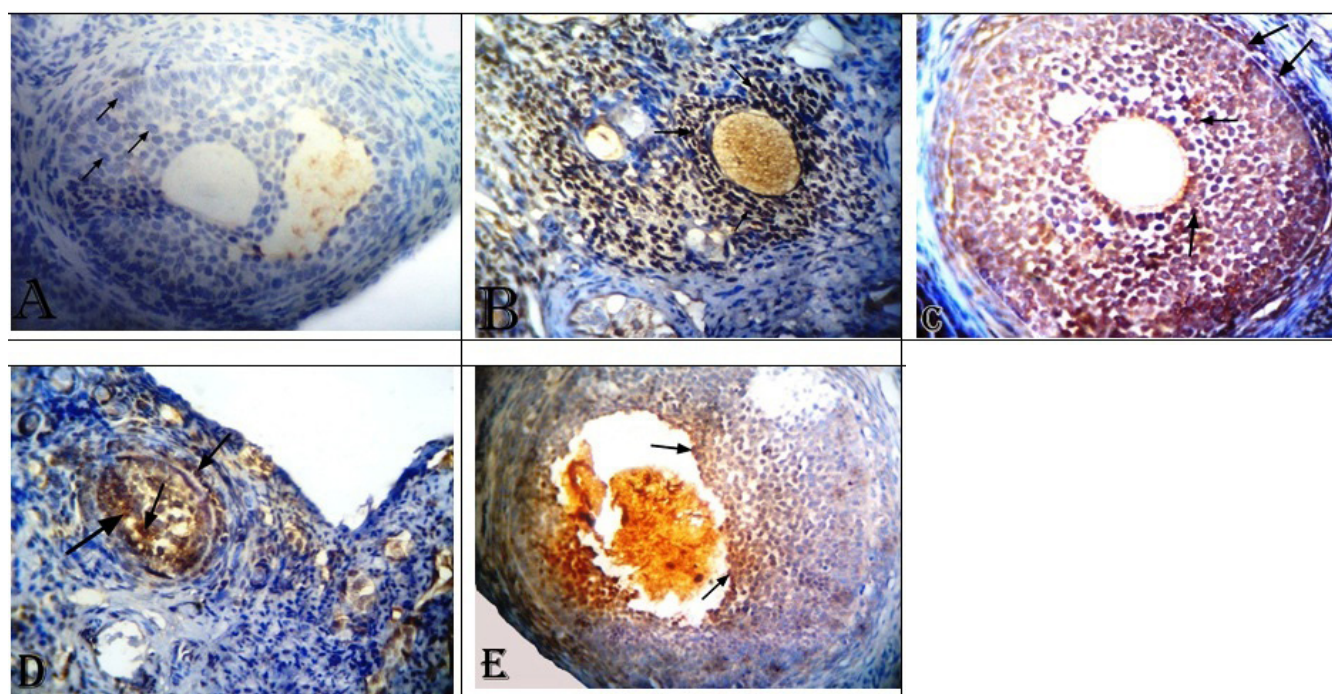


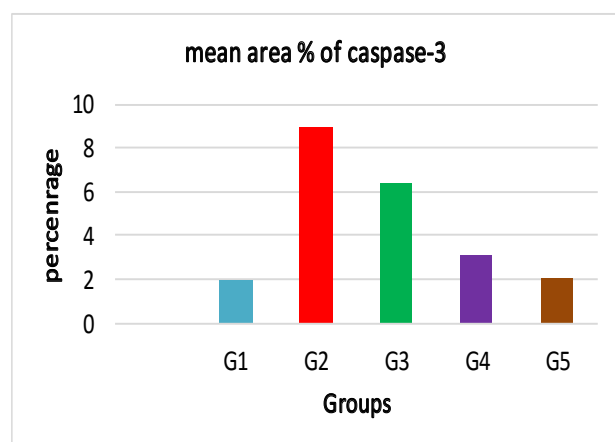
Fig. 16: A- control group ovarian section showing negative expression of caspase-3 immunoreaction in granulosa cells of ovarian follicle (arrow) B- ovarian follicle in ischemia group with intense positive caspase-3 immunoreaction in most of cells lining the follicle (arrow) C- ischemia reperfusion group revealing strong positive expression of caspase-3 immunoreaction within multiple granulosa cells of the follicle (arrow) D- ischemia amlodipine group revealed moderate positive expression of caspase-3 immunoreaction in some granulosa cells (arrow) and few theca cells (arrow head) E- ischemia reperfusion amlodipine group revealed mild positive expression of caspase-3 immunoreaction in few granulosa cells (arrow) while most of follicular cells are negative

(Caspase-3 immunostaining, x 400)

Table 1a: Mean area% of Caspase-3 immunopositive cells (±SD) in the different rat groups.

Group	Mean area % of caspase-3
Control group (G1)	1.95 ± 0.47
Ischemia group (G2)	8.96 ± 0.17*
Ischemia / reperfusion (G3)	6.35 ± 0.12*
Ischemia + amlodipine (G4)	3.12 ± 0.35
Ischemia / reperfusion amlodipine (G5)	2.09 ± 0.35

* $P < 0.05$ was considered significant



Graph 1a : Effect of ischemia , ischemia and reperfusion , ischemia amlodipine and ischemia reperfusion amlodipine on mean area percentage of Caspase-3 immunostain expression in the rat ovary.

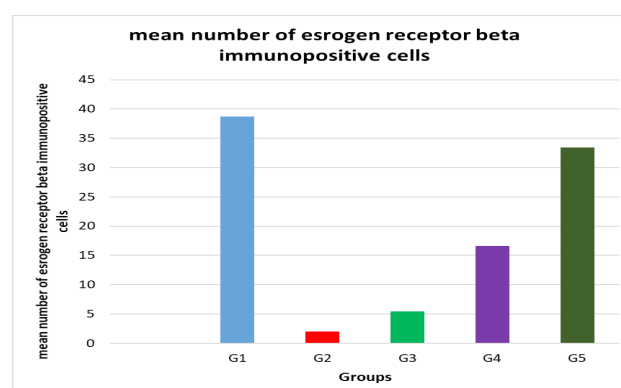
Table 1b: Mean number of estrogen receptor beta immunopositive cells (±SD) in granulosa cells in all studied groups.

Group	Mean number of estrogen receptor beta
Control group (G1)	38.74 ± 12.07
Ischemia group (G2)	1.97 ± 1.85**
Ischemia / reperfusion (G3)	5.48 ± 5.41**
Ischemia + amlodipine (G4)	16.58 ± 12.41*
Ischemia / reperfusion amlodipine (G5)	33.47 ± 10.19

$P < 0.05$ was considered significant

* Significant from the control group ($p < 0.05$).

** Highly significant from the control ($p < 0.001$)



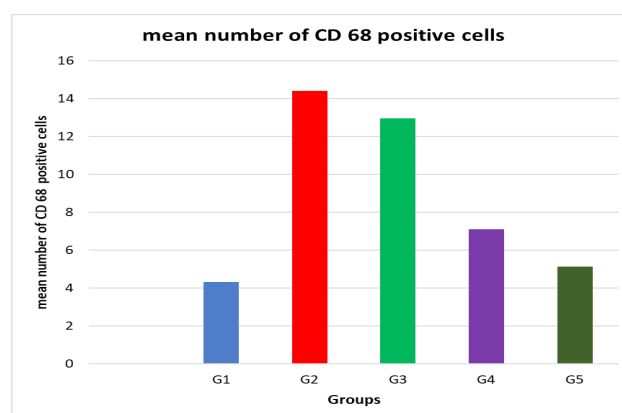
Graph 1b : Effect of ischemia , ischemia and reperfusion , ischemia amlodipine and ischemia reperfusion amlodipine on mean number of estrogen receptor beta immunopositive cells in the rat ovary.

Table 1c: Mean number of CD68 immunopositive cells (±SD) in the ovary of all studied groups.

Group	Mean number of CD68 positive cells
Control group (G1)	4.31 ± 0.7
Ischemia group (G2)	14.4 ± 1.68*
Ischemia / reperfusion (G3)	12.95 ± 1.56*
Ischemia + amlodipine (G4)	7.11 ± 1.2
Ischemia /reperfusion amlodipine (G5)	5.12 ± 0.9

P < 0.05 was considered significant

* Significant from the control group (*p* < 0.05).



Graph 1c : Effect of ischemia , ischemia and reperfusion , ischemia amlodipine and ischemia reperfusion amlodipine on mean number of CD 68 immunopositive cells in rat ovary.

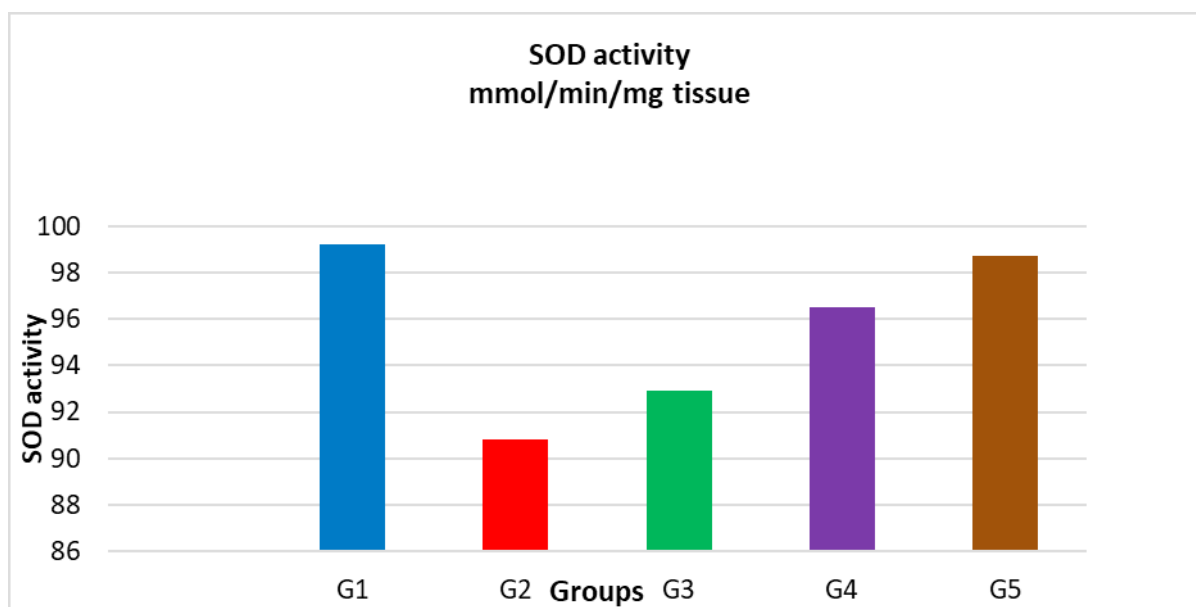
Table 2: Effect of ischemia , ischemia-reperfusion (I/R) and ischemia-reperfusion (I/R) plus amlodipine treatment on the change of superoxide dismutase (SOD) with glutathione (GSH) and malondialdehyde (MDA) in rat ovary.

Treatment	NO. of rats	SOD activity (mmol/min/mg tissue)	Amount of GSH (mg/ g tissue)	Amount of MDA (nmol/g tissue)
Control group (G1)	8	99.2 ± 0.2	69.15 ± 1.4	30.89 ± 0.66
Ischemia group (G2)	8	90.8 ± 0.2*	18.07 ± 1.6**	78.33 ± .12**
Ischemia / reperfusion (I/R) (G3)	8	92.9 ± 0.2*	22.01 ± 1.8**	70.15 ± .99**
Ischemia + amlodipine (G4)	8	96.5 ± 0.1	62.03 ± 1.7	42.43 ± 2.45
Ischemia /reperfusion + amlodipine(G5)	8	98.7 ± 0.4	66.05 ± 2.03	39.98 ± 1.78

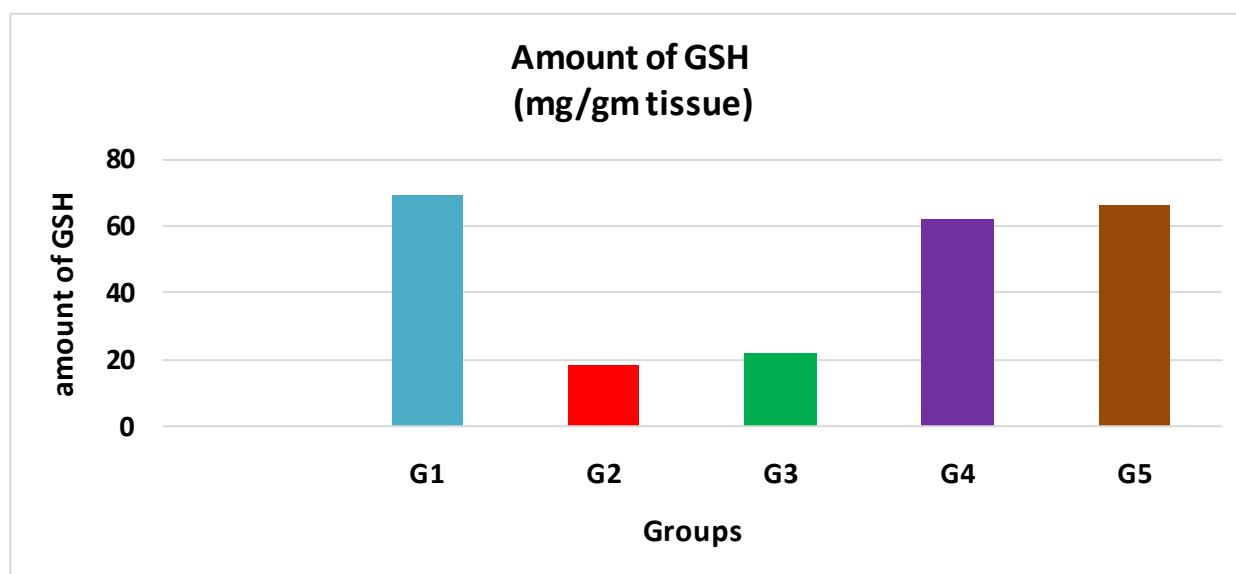
P < 0.05 was considered significant

* Significant from the control group (*p* < 0.05).

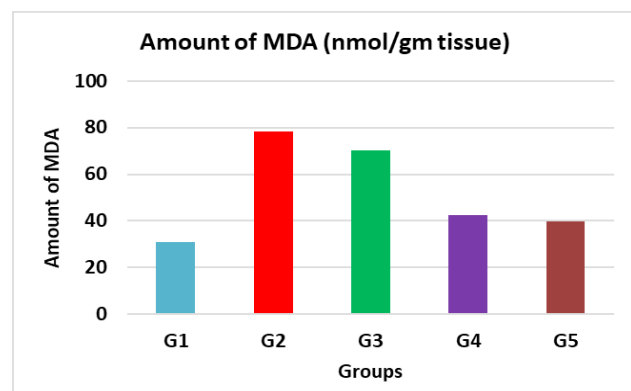
** Highly significant from the control (*p* < 0.001)



Graph 2 : Sodium dismutase activity (SOD) in ischemia , ischemia-reperfusion (I/R) , ischemia amlodipine and ischemia reperfusion amlodipine groups.



Graph 3: Amount of GSH (glutathione) in ischemia, ischemia-reperfusion (I/R) and ischemia amlodipine and ischemia reperfusion amlodipine groups.



Graph 4 : Amount of MDA (malondialdehyde) in ischemia, ischemia-reperfusion (I/R), ischemia amlodipine and ischemia reperfusion amlodipine groups.

DISCUSSION

The primary pathophysiological event in ovarian torsion is ischemia followed by reperfusion; thus, ovarian torsion detorsion is an ischemia-reperfusion (I/R) injury of the ovaries^[33-34]. It is not well known whether the ischemia due to torsion of the adnexa or the reperfusion injury due to detorsion of the adnexa are real threats to the vitality or to the functional capacity of ovarian tissue. Amlodipine is a long-acting calcium channel blocker with anti-inflammatory and antioxidant property which may protect the ovarian I/R injury^[35]. The present study was designed to demonstrate the modulating effect of amlodipine in preventing tissue damage induced by ischemia and ischemia reperfusion (I/R) in rat ovaries. This was proved by histological, immunohistochemical, morphometric, and biochemical studies.

In this research, Different histological changes were reported in the ovarian tissue of ischemia and I/R groups including various distorted and atretic follicles devoid of oocytes, secondary follicles with its antral cavity

containing desquamated follicular cells, corpus luteum having vacuolations and multiple dark nuclei. These findings were in agreement with the results of previous histological studies^[36]. The previous findings were suggested due to the formation of reactive oxygen species (ROS) during I/R^[37].

In the present study, ischemia and I/R groups revealed congested dilated blood vessels with extravasated RBCs. Ovarian torsion may impair its blood supply with resulting venous congestion and hemorrhage which can proceed to ovarian tissue necrosis^[38]. It was attributed to reaction induced by ROS with subsequent ovarian hemorrhage and localized acute inflammatory response at the injured site.

In our present study, PECAM-1 immunoreactivity was stronger in the endothelium of blood vessels, corpus luteum and in the stroma of ischemia and I/R groups than that in the control group. Macrophages are identified in tissues by their expression of cellular receptors and they can perform many functional activities, including phagocytosis, degradation of foreign antigens, matrix dissolution and production and secretion of cytokines, chemokines and growth factors. These effector functions allow macrophages to control the local immune and inflammatory responses^[18]. Macrophages are able to regulate cellular proliferation, differentiation and apoptosis, steroid production, vascularization and tissue remodelling during follicle growth, ovulation and luteinization. CD68 is a well established intracellular marker for macrophages. Its expression reveals specific information about the changing functional characteristics of the cells^[39].

Moreover, in ischemia and ischemia reperfusion groups, CD68 expression was strongly positive and the number of macrophages (immunopositive cells) were significantly increased. These findings were in agreement with^[18] who found that macrophages and CD68 expression were increased in both mouse and human ovaries in many

ovarian diseases. They explained that macrophages are involved in the removal of apoptotic cells or initiation of apoptosis and atresia. However,^[39] suggested that they have an active role in the production of factors promoting the follicular atresia. Addition of amlodipine to both ischemia and I/R groups causes reduction of CD68 expression and number of macrophages which reflect the effective amlodipine effect on reduction of the inflammatory and hemorrhagic effects. The previous findings were also in line with^[39-40] who found that rabbit luteolysis was associated with increased scavenger receptor positive macrophages with subsequent accumulation of CD68 positive macrophages.

The mean area% of caspase-3 immunoreaction was significantly high in ischemia and I/R groups compared with the other groups. These findings were in accordance to^[41] who demonstrated caspase-3 expression in the ovarian tissues in all the selected groups with a significant difference between the I/R group and all other groups. They explained their findings by caspase activation with subsequent apoptosis. Hence, Caspase-3 can be considered as a marker of apoptosis.

In this study, the level of ovarian MDA was increased by ischemia and ischemia-reperfusion injury. Previous researchers found a significant higher level of MDA concentration in ovarian tissue and plasma in the torsion and detorsion groups in comparison with the sham group^[42]. De-torsion operations for protection of the ovarian tissue result in reperfusion injury, which worsens the tissue damage^[43].

They concluded that, MDA is a toxic metabolite of the ROS that accumulates in I/R injury reflecting impaired cell wall permeability and integrity. It can be used as a marker of lipid peroxidation^[44].

In previous studies, it was reported that, in pregnant rats, external SOD injection during ovarian ischemia hindered the decline in ovarian hormonal function^[45]. They also stated that, associated with I/R injury there was inclination of lipid peroxide level and declination of SOD level. Amlodipine has a potent antioxidant activity during in vitro studies^[46]. It stimulates anti-inflammatory activity and SOD effect^[47]. In this research I/R injury induced declination in SOD level. Our results were in agreement with previous studies^[45] who attributed SOD declination to their utilization in oxidative stress process. Inversely, amlodipine administration prior to I/R injury result in increasing SOD level which could be explained by stimulatory effect of amlodipine on SOD activity.

Amlodipine creates its protective effect on ovarian tissue induced damage through its anti-inflammatory and antioxidant actions. This effect has been reported previously in long term administration of amlodipine in some experimental studies^[35]. In this study, amlodipine was administered for short term in experimental animal model of ovarian tissue ischemia and reperfusion. Amlodipine is a long-acting calcium channel blocker which decreased

the infarcted ovarian tissue upon its administration prior to reperfusion after ischemia-reperfusion. The results of the histological parameters in our study indicated that administration of amlodipine, had beneficial effects in the prevention of reperfusion injury of ovaries. In the histopathological findings of ischemia and I/R groups, normal ovarian architecture was not observed. But in the ischemic groups given amlodipine, partial improvement in ovarian architecture and cellular structures was observed. All histological and biochemical results indicate that early administration of amlodipine, before reperfusion, is required for mitigation of I/R injury, and the ovary-protective effect of amlodipine is mediated closely through its effect on the activity of SOD, which is an antioxidant enzyme. The mechanisms of ovarian injury after I/R and cell death still are not understood fully. However, one of the most important pathophysiological mechanisms for I/R injury is oxidative excessive stress.

In this research, less damage was noticed in ovarian tissue of the amlodipine-treated group. It showed nearly normal follicles accompanied with few degenerated follicles. Vacuolations, congested vessels and hyaline degeneration were still observed in the stroma. A probable explanation for these changes is that amlodipine enhanced follicular development as a result of ROS inhibition. Additionally, The mean area% of caspase-3 immunoreaction was significantly reduced with amlodipine administration prior to I/R when compared to sham control group. It was proved that, amlodipine improved follicular cells development through improving proliferation of ovarian granulosa cells, decreasing LPO and oxidative stress. So, the apoptosis pathway inhibition was obtained through decreasing the expressions of caspase-3^[48].

The mechanisms of cellular injury remain incompletely understood. Ovarian torsion leads to reduction in arterial and venous blood flow. Toxic metabolites such as MDA and ROS are increased due to ovarian torsion. This ischemic process is dangerous for cells because of increasing toxic molecules^[8]. In this study, amlodipine administration significantly decreased MDA levels and significantly increased SOD and Glutathione activities compared to the other groups. MDA level was significantly higher in the ischemia and I/R groups than in the amlodipine and ischemia reperfusion (I/R) group. SOD and Glutathione levels were significantly lower in ischemia and I/R group than in the amlodipine and I/R group. It was reported that amlodipine preserved the ovary from injury due to I/R^[35]. In previous studies, edaravone as antioxidant was useful protection in the early treatment of ovarian I/R injuries^[49]. They reported that, reperfusion of the hypoxic tissues may lead to a new physiopathologic process involving further tissue damage. Toxic metabolites such as MDA and ROS (reactive oxygen species) are increased due to ovarian torsion. This ischemic process is dangerous for cells because of increasing toxic molecules^[50]. These radicals harm tissue via lipid peroxidation. By this way, they can cause cellular damage^[51].

In previous studies, it was stated that decrease in the blood flow causes hypoxia, which in turn increases levels of ROS including hydrogen peroxide and other chemical forms^[52]. It has been demonstrated that oxygen free radical generation is a critical mechanism causing injury in post-ischemic cells and tissues. Reperfusion of the hypoxic tissues that may lead to further tissue damage are associated with the overgeneration of ROS and reactive nitrogen species. When the blood reflows to the hypoxic tissues, reoxygenation of the tissues causes the conversion of a large amount of free oxygen radicals to be produced. This sequence of events is known as reperfusion injury^[53]. Post-ischemic lipid peroxidation can be blocked by antioxidants^[54]. To protect against I/R damage caused by oxidative stress, cells have a number of antioxidant enzymes and repair activities, most of which are expressed at a low level. Reactive oxygen species produced after I/R injury normally are scavenged by a variety of antioxidant enzymes and compounds that include SOD, catalase, and glutathione peroxidase. The SODs convert superoxide anion into hydrogen peroxide (H₂O₂), which is a key component of the antioxidant defense system^[55].

CONCLUSION

Our data strongly suggest that, conservative treatment with amlodipine is effective in reducing tissue damage induced in ovaries by ischemia and I/R. Administration of amlodipine reduced ovarian damage as proved by histological, immunohistochemical, morphometric and biochemical studies in ischemia and I/R-induced injury in rat model. Also we indicated that, the protective effect of amlodipine is mediated primarily through an antioxidant action. Consequently amlodipine would be helpful in protection of ovaries from torsion-detorsion-induced damage in humans presumably via antioxidant down-regulation properties. This protective effect of amlodipine on ovaries will be beneficial not only in ovarian torsion but also in the treatment and prophylaxis of other ovary-related diseases in which oxidative stress has direct or indirect involvement. Further studies are recommended to determine optimal timing and dosage of amlodipine. Antioxidant compounds as amlodipine may contribute to salvage surgically untwisted ovaries, if future successful studies are performed.

CONFLICTS OF INTEREST

There is no conflict of interest to declare.

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الملخص العربي

الاصابة الحادثة في مبيض الفأر نتيجة وقف تدفق الدم وإعادة الارواء و التأثير الوقائي المحتمل لعقار الاملوديبين: دراسة هستولوجية وهستوكيميائية مناعية وكيميائية حيوية

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المقدمة: التواء المبيض يمثل حالة من حالات الطوارئ الخاصة بالنساء. ولكن التشخيص المبكر للتواء يمكن أن يساعد في اصلاح الالتواء للمبيض المصاب والمحافظة على إعادة الخصوبة. سلوك النهج المحافظ في العلاج يمكن ان يؤدي الى تأثير موضعى وعام على بقاء و حيوية المبيض الذى تم اصلاح التواءه.

الهدف من العمل: تم عمل الدراسة الحالية لتقييم التأثير الوقائي لعقار الاملوديبين على التغيرات الهستولوجية والهستوكيميائية المناعية والكيميائية الحيوية وتم عمل دراسات القياسات العددية (المورفومتري) الناتجة عن وقف تدفق الدم و إعادة الحقن في المبايض في نموذج الجرذان.

المواد و الطرق: تم استخدام أربعون من اناث الجرذان حيث تم تقسيمهم الى خمس مجموعات متساوية: المجموعة الأولى: (الضابطة)، المجموعة الثانية: (وقف تدفق الدم) حيث يتم وقف تدفق الدم لمدة ثلاث ساعات. المجموعة الثالثة: (وقف توقف الدم و إعادة الارواء): حيث يتم وقف تدفق الدم كالمجموعة الثانية و إعادة الارواء لمدة ثلاث ساعات. المجموعة الرابعة: (وقف تدفق الدم و عقار الاملوديبين) حيث يتم تناول عقار الاملوديبين بالفم بجرعة 5مجم ساعة واحدة قبل وقف تدفق الدم ثم وقف تدفق الدم لمدة ثلاث ساعات. المجموعة الخامسة: (وقف تدفق الدم و إعادة الارواء و عقار الاملوديبين) حيث يتم وقف تدفق الدم الى المبيض لمدة ثلاث ساعات ثم تناول عقار الاملوديبين بنفس الجرعة السابقة ثم ثلاث ساعات من إعادة الارواء. وقد تم استئصال المبيض للفحص الهستولوجي والهستوكيميائي المناعي والكيميائي الحيوي مع عمل دراسات القياسات العددية الناتجة عن وقف تدفق الدم و إعادة الارواء في المبايض في نموذج الجرذان.

النتائج: في مجموعتي (وقف تدفق الدم) و(وقف توقف الدم و إعادة الارواء) أظهرت المبايض تقشير و تناثر في الخلايا الحبيبية في التجويف المسامى للحويصلات المتأكلة مع وجود انحلال و تمزق بنواة البويضة مع وجود اتساع و احتقان بالاوعية الدموية و تآكل زجاجى ووجود فراغات في المناطق البيينية و غزو ضخخ للخلايا ووجود ترسب لصبغة الهيموسيدرين و نزيف ضخخ في منطقة السدى الداخلية للمبيض. وقد ظهر تفاعل ايجابي لصبغة البيكام I وسى دى 68 المناعيتين و تفاعل سلبي لصبغة مستقبلات الاستروجين المناعية. وقد ظهر انخفاض في تركيز سوبراوكسيد ديسميوتاز و الجلوتاثيون بينما زاد تركيز المألوندهيد في مجموعتي (وقف تدفق الدم) و(وقف توقف الدم و إعادة الارواء). إضافة الاملوديبين حسن النتائج الهستولوجية والقيم الكيميائية.

الاستنتاج: استخدام الاملوديبين يخفف من التأثير الضار لوقف تدفق الدم و إعادة الارواء.