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The Effects of Different Intraabdominal Pressure Protocols in Laparoscopic Procedures on Oxidative Stress Markers and Morphology in Rat Ovaries

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. To determine the effects of different intraabdominal pressure (IAP) on the ovaries in a laparoscopic rat model.
Objectives. The aim of the study was to determine the effects on the ovaries of different intraabdominal pressures (IAP) in laparoscopic surgery in a rat model.
Material and Methods. Thirty-two post-pubertal nonpregnant Sprague-Dawley rats were divided randomly into four groups. In the control group, no intraabdominal pressure (IAP) was applied. In Group Pp10 and Group Pp15, an IAP of 10 and 15 mm Hg, respectively, were applied by carbon dioxide insufflation for 60 min, and a 30-min desufflation was carried out. In Group IPp15, a 15 mm Hg IAP was applied for 10 min, and then CO_2 was desufflated for 10 min. After this ischemic preconditioning, IAP was established at 15 mm Hg for 60 min, after which CO_2 was desufflated for 30 min. Erythrocyte and ovarian tissue malondialdehyde (MDA) and histopathologic damage scores were evaluated.
Results. In Groups Pp10 and Pp15, ovarian tissue MDA values were significantly increased compared to the control group. In Groups Pp10 and Pp15, erythrocyte MDA values were significantly increased when compared to Group IPp15 and the control group. Ovarian histopatological assesment scores were significantly higher in Group Pp15 than in Groups Pp10 and IPp15.
Conclusions. Pneumoperitoneum causes injuries to abdominal organ such as the ovaries. The ischemic preconditioning method is more effective in reducing oxidative stress due to laparoscopic pneumoperitoneum than low-pressure pneumoperitoneum methods (**Adv Clin Exp Med 2014, 23, 6, 885–892**).

Key words: ischemic preconditioning, pneumoperitoneum, oxidative stress, reperfusion, laparoscopy.

As skills and knowledge in diagnostic laparoscopy have improved and more advanced surgical instruments have been developed, the use of laparoscopic techniques has become much more common and has earned the admiration of the majority of surgeons. More complex procedures are being performed *via* laparoscopy, including traditional abdominal hysterectomy. As laparoscopic surgery has developed and surgeons have gained more experience, the procedure has been extended to the pregnant population. The most common reported laparoscopic operations during pregnancy are cholecystectomy, adnexial surgery, appendectomy and management of heterotopic pregnancy [1].

In laparoscopic techniques, a working space is established by insuffling a gas into the abdominal cavity. However, pneumoperitoneum (PN) is

not a harmless procedure. In some laparoscopic procedures, an intraabdominal pressure (IAP) of 18–25 mm Hg is needed [2, 3], but these pressure levels cannot be accessed in routine laparoscopy, as most clinical procedures are performed at 10–15 mm Hg [4]. This pressure range, although it is higher than the normal portal systemic pressure (7–10 mm Hg), maintains a balance between establishing working space with adequate visualization and the undesireable effects of increased IAP [5–9].

The increasing experience of large number of trials has revealed the side effects of PN [10]. These trials have mostly focused on blood flow changes in the intraabdominal organs. PN leads to a 10–80% reduction in the rate of blood flow to the intraabominal organs, but it has been reported to return to the normal range after desufflation [10]. In terms of the hypoperfusion and subsequent reperfusion periods, both clinical and experimental studies show that laparoscopic procedures result in a typical model of ischemia and reperfusion (I/R) injury in the organs [11–13]. After desufflation, visceral perfusion returns to normal, but oxidative stress remains in the tissues. The use of minimal pressure to get adequate visualization is more recommended than the use of constant pressure in laparoscopic procedures [9].

The ischemic preconditioning method is another way to reduce I/R injury. The ischemic preconditioning method is defined as a brief insufflation followed by brief desufflation at the beginning of the procedure. During the ischemic preconditioning method, some cellular proteins are secreted in order to increase tissue resistance to I/R injury [14, 15].

The aim of this study was to determine the effects of different intraabdominal pressure models and the ischemic preconditioning model on oxidative stress markers and morphology in laparoscopic rat ovary surgery.

Material and Methods

The Animals and Experimental Design

The study protocol complies with the European Community guidelines for the use of experimental animals. All the experiments were approved by the local Animal Care Ethics Committee at Ege University, İzmir, Turkey. The study involved 32 nonpregnant Sprague-Dawley rats weighing 264 ± 28 g, housed in suitable environmental conditions (12 h daylight at 25 ± 2ºC temperature with sequential dark and light cycles) and fed *ad libitum* with rat chow and tap water. The rats were randomly divided into 4 groups: Group Pp10 (n : 8), in which pneumoperitoneum (PN) was achived at 10 mm Hg; Group Pp15 (n:8), in which PN was achived at 15 mm Hg; Group IPp15, in which the ischemic preconditioning model was used; and the control group, which only underwent general anesthesia.

Briefly, each rat was anesthetized with an intramuscular injection of a mixture of Ketamine (60 mg/kg, Alfamine®, Ege Vet, Izmir, Turkey) and Xylazine (10 mg/kg, Alfazyne®, Ege Vet, Izmir, Turkey). Using sterile techniques, an 18-gauge angiocatheter was placed in the abdominal cavity from the caudal of the sternum. Possible gas escape from the abdomen was blocked with sutures close to the angiocatheter entry point. A CO_2 insufflator (Nortech, Model No:3-315-00) was attached to the angiocatheter with the help of cannula. In the control group, the angiocatheter was inserted into the abdominal cavity but there was no gas insufflation. In Group Pp10 and Group Pp15, PN was performed at 10 mm Hg and 15 mm Hg, respectively, by an automatic insufflator for 60 min, and then desufflation was performed for 30 min. In Group IPp15, after the ischemic preconditioning procedure, PN was achived at 15 mm Hg by an automatic insufflator for 60 min and desufflation was carried out for 30 min. The ischemic preconditioning procedure consisted of performing PN at 15 mm Hg for a duration of 10 min and desufflation for 10 min, as decribed above. In the control group the angiocatheters were removed after 90 min and midline laparatomies were performed; in the other 3 groups this was done after a 30-min desufflation period. Both ovaries were removed from the subjects immediately after the laparatomy. Afterwards, an intracardiac blood sample was drawn into tubes with K-EDTA using 22-gauge × 1 needles. These laparotomy procedures took approximately 2 min. After the sampling, all the subjects were sacrified with intracardiac potassium injections.

After the fat and connecting tissue were cleaned from the samples, half of the samples were washed with Ringer's lactate solution at ice temperature and stored at –80ºC for the biochemical assesments. The other half of the samples were fixed within a formaldehyde (10%) solution and stored at room tempature for 24 h for the histological assesments. Hemolysates were prepared from the blood samples and stored at –80ºC for the biochemical studies.

Biochemical Analysis

The development of oxidative injury was estimated by the levels of malondialdehyde (MDA) and thiobarbituric acid reactive substances (TBARS), which constitute biochemical evidence of lipid peroxidation, in tissue homogenates of the ovarian tissues and hemolysates of the blood samples.

Preparation of Tissue Homogenates and Analytical Methods

The samples were homogenized at a proportion of 1 : 10 (w/v) in cold phosphate-buffer (0.5 M, pH = 7.0). The homogenates were centrifuged for 5 min at 700 × g at 4°C to sediment cellular debris. The TBARS contents in the supernatants were determined immediately.

Quantification of MDA

MDA levels were determined by colorimetric assay. After dilution in the required amount of tissue homogenates, TBARS levels were determined according to the protocol described by Sozmen et al [16], by incubation with MDA solution (0.12 M TBA in 15% TCA and 1% HCl) for 30 min at 95°C, and were calculated as nmol/mg protein using a 1,1,3,3,tetramethoxypropane standard curve while the hemolysate MDA levels were expressed as nmol/g Hb.

Histological Procedures

The excised ovarian tissues were fixed by overnight immersion in 10% neutral buffered formaldehyde (0.2M; pH = 7; Merck) for 24 h at 4°C, then dehydrated, embedded in parafin wax and sectioned by microtome (Leica RM 2145). For the histological analyses, 3 μm thick serial sections were dewaxed, and rehydrated tissue slides were stained with hematoxylin eosin (HE) and mounted with entellan. All the sections were examined and photographed using an Olympus C-5050 digital camera with Olympus BX51 microscope. The histological procedures applied in this study are conventional and well-established methods [17]. The chemicals were purchased from Sigma (St Louis, MO, USA) unless noted otherwise. The slides were immersed in the stain/buffer solutions at all times during the incubations.

Two investigators, blinded to the group distinctions of the specimens, obtained 5 images from random fields under various objectives in 10 different sections for determination and evaluation of the cellular components. No morphological evaluation was done on cells in areas with necrosis or with poor morphology, or on cells from the slide margins. In all cases the areas of interest were selected randomly.

In order to evaluate histological morphological alterations *via* light microscopy, the ovarian samples were analyzed according to a scoring system specifically developed for this study by the authors (UA, YU and OYD)

The criteria for ovarian injury were changes in the gerninative epithelium, increases in stromal connective tissue (cellular components and tissue skeleton) or medulla (increases in vascular structures, increases in *tunica-media* vessel thickness), alterations in the morphology of primordial follicles, preanthral follicles, anthral follicles and mature follicles, splitting of the granulosa cells and increases in interstitial spaces, vacuolisation of the oocytes, cellular alignment of the theca cells and increases in the vascularity and thickening of the *zona pellucid*. Alterations in these structures were evaluated and graded semiquantitatively as follows: 0 for no difference, 1 for minor alterations, 2 for moderate alterations and 3 for severe alterations.

Statistical Analysis

The statistical analysis of the data was performed using the SPSS 11.0 software package for Windows (Statistical Package for Social Sciences, SPSS Inc., Chicago IL, USA). All the values were expressed as mean ± S.E.M. The Kruksal-Wallis analysis of variance and the Mann-Whitney *U* test were used for statistical comparison of the groups. A p-value of less than 0.05 was accepted as statistically significant.

Results

A biochemical analysis of erythrocyte and tissue MDA levels for the ovaries was carried out.

Erythrocyte MDA Levels

Eythrocyte MDA values in Group Pp10 and Group Pp15 were significantly increased when compared to the control group ($p = 0.004$ and $p = 0.004$ respectively). There was no statistically significant difference between Group IPp15 and the control group ($p = 0.148$). Eythrocyte MDA values in Group Pp10 and Group Pp15 were significantly increased when compared to Group IPp15 ($p = 0.006$ and $p = 0.004$); but no statistically significant difference was noted between Group Pp10 and Group P15 ($p = 0.873$) (Fig. 1).

Ovarian MDA Levels

Ovarian MDA values for Group Pp10 and Group Pp15 were significantly increased when compared to the control group ($p = 0.016$ and $p = 0.025$ respectively). Ovarian MDA values in Group IPp15 were higher than in the control group, but the difference between the two groups was not statistically significant. No statistically

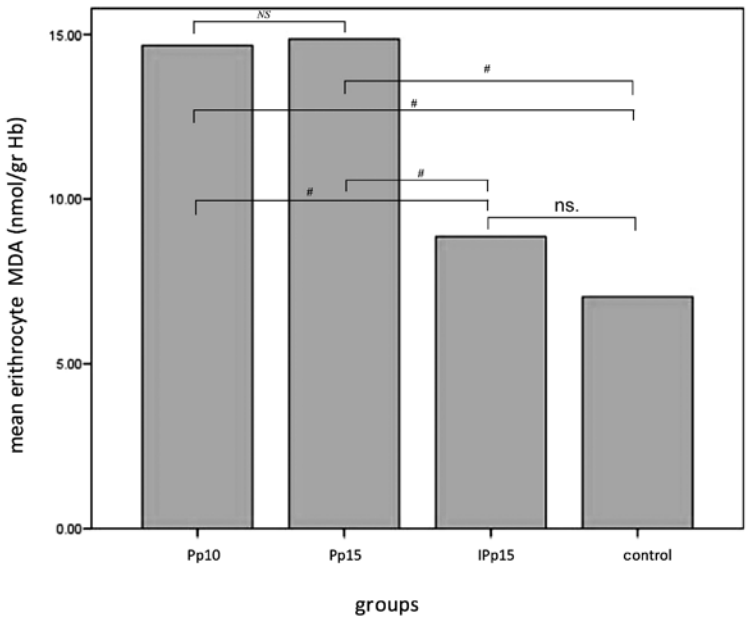

Fig. 1. Statistical analysis of erythrocyte MDA levels for all groups,
# $p < 0.05$; ns. – non-significant

significant differences were found when comparing ovarian MDA values of Group Pp10, Group Pp15 and Group IPp15 (Fig. 2).

Histological Findings for the Ovaries

The histological examination scores and the results of the statistical analysis for the ovaries are shown in Fig. 3. The histological examination of the ovaries revealed normal ovarian morphology in the control group (Fig. 4); but two of the subjects displayed an increase in vascular structures and vessel formation in the stroma and there was also a lack of thecal cell arrangement. In the ovaries of Group IPp15, dispersed lymphatic dilatation and varicose enlargements of the vessels were observed; there was no evidence of capillary congestion. Most of the sections displayed many reggressed follicles. *Zona pellucida* formation was normal in most of the follicles. Additionally, a loss of integrity between the *tunica albuginea* and the mesothelium was noted (Fig. 4). The histological evaluation scores of Group IPp15 were significantly higher than the control group's score ($p = 0.003$).

In Group Pp10, *zona pellucida* formation was found to be normal in all samples except one. In

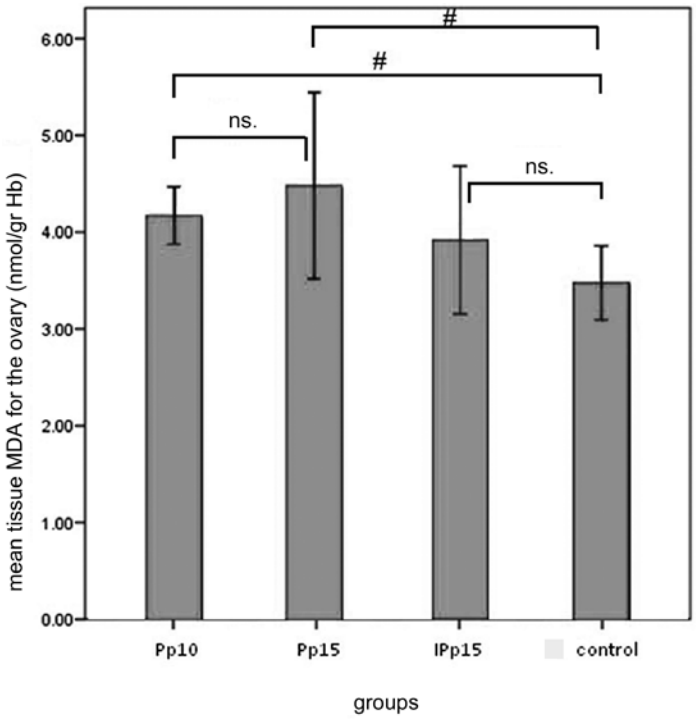

Fig. 2. Statistical analysis of tissue MDA levels for the ovaries from all groups,
# $p < 0.05$; ns. – non-significant

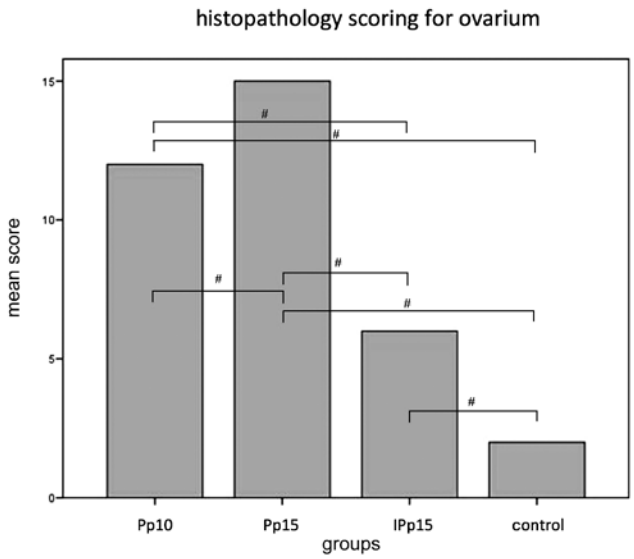

Fig. 3. Histological examination scores for the ovaries and the results of the statistical analysis, # $p < 0.05$

addition to medullar lymphatic dilatation findings in the stroma, medullar congestion and hemorrhage were noted. However, there were no findings of oocyte maturation defects in the follicles (Fig. 4). The histological scores of Group Pp10 were significantly higher than the control group score and

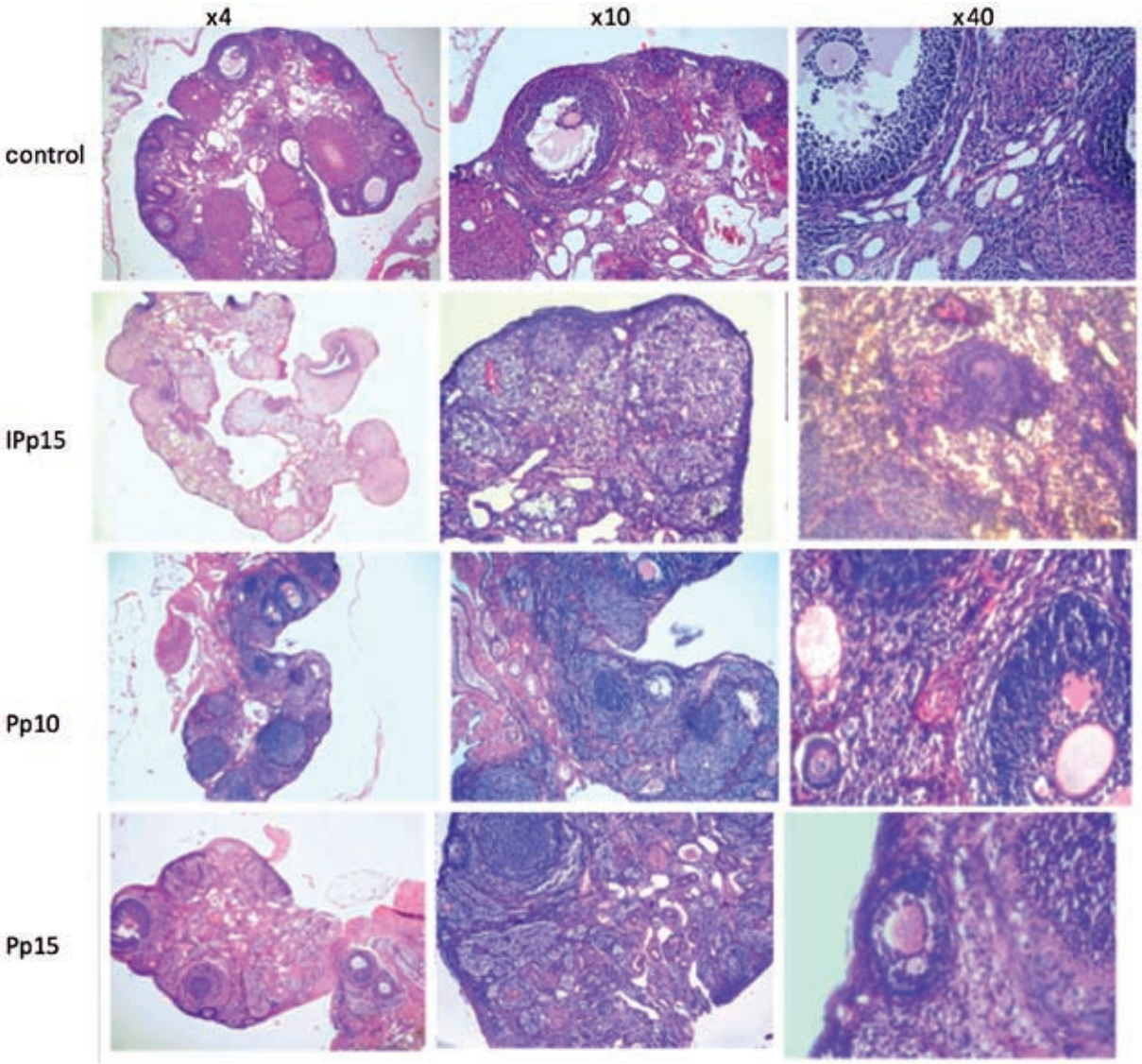

Fig. 4. Ovarian histology, HE staining. Various degrees of magnification

the Group IPp15 score ($p = 0.003$ and $p = 0.004$, respectively).

In Group Pp15, there were no findings of follicular cycle arrest, but evident dilatation and congestion of medullar blood vessels were noted, as well as stromal lymphocytic infiltration. Oocyte maturation defects were noted within the follicles. Due to vessel congestion in close proximity with the follicles, deformation of the oocytes was determined (Fig. 4). The histological scores of Group Pp15 were significantly higher than those of the control group, Group Pp10 and Group IPp15 ($p = 0.003$, $p = 0.004$ and $p = 0.004$, respectively).

Discussion

Laparoscopy has a both advantages and disadvantages [2, 10]. After the development of minimally invasive laparoscopic surgical techniques, many trials were conducted to determine the side effects of PN. PN is performed for visualization, but it can cause ischemia and reperfusion (I/R) injury. The nature and severity of I/R injury depends on the time and level of IAP and the properties of the intraabdominal organs exposed to IAP.

Previously, the clinically permitted IAP level was 25 mm Hg at the beginnig of laparoscopy, and IAP levels not exceeding 18 mm Hg during the laparoscopic procedure [2]. Recently, this range has been reduced to 10–15 mm Hg [18]. But in some gynecological laparoscopic procedures, 20 mm Hg IAP is required. Abu-Rafea at el. indicated that a higher CO_2 volume is required to estabilish appropriate pneumoperitoneum in tall, overweight and parous women at IAPs of 20–30 mm Hg at the beginning of laparoscopy [19]. Madl and Druml [20] previously reported that an IAP of no more than 10 mm Hg would not cause any major hazardous effects, but an IAP exceeding 12 mm Hg could result in inflamation of the tissues, including processes like leukocyte migration, a release of cytokines, increased formation of reactive oxygen intermediates, etc. An IAP between 18 and 20 mm Hg is described as intraabdominal hypertansion. An IAP over 20 mm Hg is an emergency situation, called abdominal compartment syndrome. Intraabdominal organ oxidative stress injuries occur due to changes in systemic circulation and the direct effects of IAP on abdominal vessels [13]. There is usually a physiological interval when reactive oxygen radicals and inflammatory cytokines overlay the tissue after surgical trauma, until the organism succesfully eradicates them *via* multiple signalling cascades. But uncontrolled production and elimination defects of these compounds may result in tissue necrosis and apoptosis [21]. Therefore, optimal care must be taken to limit the production of any post-inflammatory cytokines after surgery. Eleftheriadis et al. [22] defined PN-related hepatic oxidative stress for the first time in literature [23, 24], while Yılmaz et al. [25] firstly demostrated PN-related renal oxidative stress in rat models.

In the available literature many human and animal studies have investigated the consequences of high IAP-related injuries and the benefits of low IAP. Giraudo and et al. [26] performed a laparoscopic cholecystectomy using a gasless method, a 10 mm Hg method and a 14 mm Hg method. Their study revealed higher transaminase values for the 14 mm Hg group, while the gasless group and 10 mm Hg group displayed almost the same values. Yılmaz et al. [25] reported statistically different values of oxidative stress responses for 10 mm Hg and 15 mm Hg groups in a rat model; and Polat et al. [13] validated these results in their study with human subjects. Moreover, Guven and et al. [27] reported that pneumoperitoneum, even at normal IAP levels, leads to significant oxidative-stress-induced biochemical and histologic damage to the ovaries.

The ischemic preconditioning method is another way to decrease ischemic injury related to PN. Different time periods for the preconditioning method have been reported in previous publications. Five- or ten-minute-ischemia followed by a reperfusion of 5–10 min is most commonly used [28–32]. Cevrioglu and et al. [33] described the preconditioning method as PN at 15 mm Hg IAP for 10 min followed by immediate desufflation for 10 min.

Increased IAP forms free oxygen radicals and ischemia in the peritoneum in relation to the time and pressure applied [14]. After reperfusion, the amount of free oxygen radicals initially increases, then the amount of antioxidant substances that help the elimination of free oxygen radicals decreases [14]. Meanwhile, several proteins, such as heat shock protein 70 (HSP70) from coronary endotelial cells, are secreted in order to protect cells from the hazardous effects of the free radicals and ischemic injury in the course of the ischemic preconditioning method.

In the current study, there were no differences between the ischemic preconditioning group and the control group for the ovary and erythrocyte MDA levels. However, erythrocyte MDA values in Groups Pp10 and Pp15 were significantly increased when compared to the ischemic preconditioning group. The results of this study clearly demonstrate that resistance to prolonged ischemic stimulation was increased for the rat subjects after the use of the ischemic preconditioning method.

In this experimental study, statistically significant differences in the oxidative effects of PN on erythrocyte and ovary tissues were demonstrated biochemically. Although there was no difference between Groups Pp10 and Pp15, light microscopic analyses of the tissue histology for the ovaries revealed morphologically significant differences between the control group and the other three groups. Group Pp15 showed the highest histological scores, and Group Pp10 had higher scores than Group IPp15.

This study has clearly demonstrated that the ischemic preconditioning method should be used to reduce I/R injuries, rather than other low-pressure models. However, further detailed studies are needed to prove likewise effects of ischemic preconditioning on human beings. The analysis of PN *via* histological data contributes to the available scientific literature on the repair process in I/R injury. The method used is elegant, but the mechanisms governing the repair process of I/R injury in rats are different from those governing human oxidative stress mechanisms. Thus it is possible to report that the repair processes after I/R injury are regulated differently in rat subjects. In conclusion, the authors believe that this is still a challenging area of research, and that it would be helpful to explain basic cellular processes *via* novel molecular approaches.

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