

Protective effects of postconditioning in transvaginally created pneumoperitoneum

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Abstract. There are reports of ischemic complications in clinical practice after laparoscopy using pneumoperitoneum. Conditioning has a beneficial effect for various ischemic diseases. This experimental study was designed to evaluate the effects of postconditioning in transvaginally created pneumoperitoneum. Sixty adult female rats, weighing 300±50 g were divided into four equal groups. Pneumoperitoneum was created by CO₂ insufflation under a pressure of 10 mmHg. Rats in the first group (sham) were subjected to only sham-operation or gas insufflation. The second group (TV/PP) was subjected to pneumoperitoneum for 60 min followed by 30 min of desufflation. The third group (post-5) was subjected to pneumoperitoneum for 60 min followed by 5 min of desufflation, 5 min of insufflation and again followed by 30 min of desufflation. The fourth group (post-2.5) was subjected to pneumoperitoneum for 60 min followed by 2.5 min of desufflation and 2.5 min of insufflation-repeated in two cycles- and then followed by 30 min of desufflation. The rats were sacrificed, and blood was collected after 30 min, 2 and 6 h from the last desufflation. Levels of oxidative stress markers, malondialdehyde (MDA), superoxide dismutase (SOD), reduced glutathione (GSH), sulfhydryl groups (SH) and inflammatory cytokine TNF- α , were analyzed. Levels of MDA in the post-5 group were significantly reduced compared to the TV/PP and post-2.5 groups. The level of GSH in TV/PP animals was markedly reduced compared to the Sham, Post-5 and Post-2.5 groups. In addition, levels of SH were increased in the Post-5 group in comparison to the Sham, TV/PP and Post-2.5 groups. No difference in the activity of SOD between the groups was found, and the concentration of

TNF- α in TV/PP animals was significantly higher than that in the Sham and postconditioning groups. Overall, the results of the present study indicate that postconditioning can reduce pneumoperitoneum-induced oxidative injury.

Introduction

Laparoscopy was first described in the beginning of the 20th century as a valuable adjunct to the diagnosis of diseases of the abdominal cavity. At present, laparoscopic surgery is reputed as one of the most effective diagnostic and therapeutic tools in general surgery. The benefits of laparoscopy versus open surgery include several advantages such as less post-operative pain, shorter recovery time and less post-operative scarring (1). However, it must also be pointed out, that there are a number of complications, which may occur after laparoscopy. It is known that pneumoperitoneum, which is created for adequate visualization of the organs during laparoscopy, increases intra-abdominal pressure. The latter reduces organ perfusion and this is associated with splanchnic ischemia/reperfusion (I/R) injury and oxidative stress (2-6). The level of injury is dependent on the magnitude of the pressure and application time (7). It has been confirmed that increasing intra-abdominal pressure from 10 to 15 mmHg, decreases blood flow in the stomach by 40%, the duodenum by 11%, the jejunum by 32%, the colon by 44%, the liver by 39% and the parietal peritoneum by 60% (2).

Numerous reports are available in the literature concerning the formation of excessive amounts of oxygen-derived free radicals in ischemic reperfused tissues. Many vital organs including the brain, lung, heart, liver, kidney, intestine, gastric mucosa and stomach can be subjected to I/R injury; and it seems that oxygen free radicals play a major role in the pathogenesis of the cellular injury (8,9).

Several strategies for the prevention of pneumoperitoneum-induced oxidative stress, such as preconditioning and postconditioning, Trendelenburg positioning and insufflation with helium, have been introduced in recent years, showing promising results (5,7,10-12). Postconditioning, which comprises short ischemia periods applied at the time of reperfusion was first described in a canine model (13) and

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demonstrated protective effect against reperfusion injury. Despite the positive scientific reports regarding the effects of postconditioning, there is still limited understanding of the application and different regimens. In addition, prevention of the negative effects of pneumoperitoneum remains a problematic issue in laparoscopic surgery. Recently authors reported the effect of a 10-min ischemia postconditioning regimen (with 5 min of each-desufflation and insufflation), after pneumoperitoneum (7). The effect of postconditioning was evaluated by blood level analysis of oxidative stress markers and assessment of tissue morphology in animal models, which were initially established by our group (14). In the present study, we used a similar animal model and comparatively assessed two 10-min postconditioning regimens: a) Desufflation/insufflation for 5 min each; b) desufflation/insufflation for 2.5 min each-repeated twice (2 cycles). Furthermore, we analyzed levels of blood oxidative stress markers at three different time points after pneumoperitoneum (30 min, 2 and 6 h).

The aim of the present experimental study was to characterize the effects of two different postconditioning regimens in transvaginally created pneumoperitoneum in a rat animal model.

Materials and methods

Animals and study design

Animals. Sixty adult female Wistar rats each weighing 300 ± 50 g were acquired from the Ilia State University animal house and housed in cages (under normal atmosphere, standard room temperature and 12-h light/dark cycle conditions) where standard rodent chow and water were available. The rats went without food for 12 h before any procedures. Animals were anesthetized by intraperitoneal (i.p.) injection of ketamine (37.5 mg/kg) and seduxen (3.75 mg/kg). All animal experiments were in compliance with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines. These studies were approved by the Local Institutional Committee on Animal Research of Pecs University, Hungary.

Study design. The animals were divided into four equal groups-each consisting of 15 rats, which also were divided into three subgroups according to the time of blood taken ($n=5$) (explained below). In all animals other than the sham group, the pneumoperitoneum was created by CO_2 insufflation under a pressure of 10 mmHg; because of this, the Veress needle was placed into the abdominal cavity from the vaginal orifice as previously described (14), which was connected to an insufflator (Karl Storz GmbH & Co. KG). In two cases bleeding was observed from the outer part of the vaginal orifice after the placement of the Veress needle. No other complications were noted during the surgical procedures.

Study groups. Group 1: Sham-operation group. A Veress needle was placed into the vaginal orifice without any other surgical procedure or gas insufflation. Group 2: Transvaginal pneumoperitoneum (TV/PP) group was subjected to pneumoperitoneum for 60 min followed by 30 min of desufflation. Group 3: Postconditioning with 5 min. (Post-5) was subjected

to pneumoperitoneum for 60 min, plus 5 min of desufflation and 5 min of insufflation followed by 30 min of desufflation. Group 4: Post-conditioning with cycle (Post-2.5) was subjected to pneumoperitoneum for 60 min plus 2.5 min of desufflation and 2.5 min of insufflation repeated in two cycles and followed by 30 min of desufflation.

Animals were sacrificed after 30 min, 2 and 6 h from the last desufflation. The blood samples were collected in each group at the aforementioned times from all animals by cardiac puncture. Oxidative stress markers such as malondialdehyde (MDA), superoxide dismutase (SOD), reduced glutathione (GSH) and sulfhydryl group (SH) levels as well as inflammatory cytokine $\text{TNF-}\alpha$ concentrations were measured in all samples.

Biochemical tests. For detection of MDA concentration, 4.5 ml thiobarbituric acid (TBA) and trichloroacetic acid (TCA) mixture was added to 0.5 ml diluted blood sample or its plasma. The sample was incubated at 100°C (in boiling water) for 20 min and cooled in icy water. Afterwards, the sample was centrifuged for 15 min at $1,400 \times g$, and measurement with a spectrophotometer was performed at 532 nm. The MDA concentration was calculated in nmol/ml (15).

For detection of reduced GSH and SH concentrations, 1 ml quintuple diluted blood sample and 4 ml trichloroacetic acid (TCA) mixture were used for determination. The mixture was centrifuged for 15 min at $18 \times g$. Two milliliters of the supernatant was added to 4 ml Tris buffer (0.4 M, pH 8.7), and 100 μl of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was added to the mixture immediately before the measurement was taken. Measurement with a spectrophotometer was made at 412 nm with concentrations calculated in nmol/ml (16).

For detection of SOD activity, 1 ml blood mixed with EDTA was used. Nine milliliters of Hartman's solution was added to the blood sample and centrifuged for 5 min at $380 \times g$. This washing procedure was repeated after the discarding of the supernatant. One milliliter of a chloroform and ethanol (2:1) mixture was added to 1 ml of hemolyzed erythrocytes and centrifuged for 4 min at $12,000 \times g$. The supernatant was then separated, and adrenalin (16.488 mg adrenalin diluted in 10 ml 0.1 N hydrochloric acid) was added to the supernatant with concentrations measured at 480 nm by a spectrophotometer. Concentrations were calculated in U/ml (17).

For the $\text{TNF-}\alpha$ concentration, detection was carried out using an ELISA kit (Quantikine® Rat $\text{TNF-}\alpha/\text{TNFSF1A}$ Immunoassay, R&D Systems; cat. no. RTA00) according to the manufacturer's instructions. Concentration was calculated in pg/ml.

Statistical analysis. All data are presented as mean values \pm standard error of mean (SEM), which were compared within the various groups using one-way analysis of variance followed by Bonferroni post hoc tests and the non-parametric Mann-Whitney U test as appropriate. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

After successful modeling of pneumoperitoneum in the experimental animals (all rats survived until the end of the

experiment), we measured blood oxidative stress markers upon different postconditioning settings.

Analysis of MDA levels after 30 min showed significant upregulation in all experimental groups compared to sham group (Fig. S1A). At 2 and 6 h, the levels of MDA in the post-5 group were significantly decreased (72.11 ± 2.85 nmol/ml after 2 h and 74.43 ± 1.24 nmol/ml after 6 h) compared with the TV/PP group at both 2 and 6 h, and post-2.5 group at 6 h (Fig. 1A and B; $P < 0.05$). In the TV/PP group, the level of MDA was significantly increased compared to the Sham and Post-5 groups (Fig. 1A and B; $P < 0.05$), and maximum level of 89.58 ± 7.87 nmol/ml was shown after 6 h (Fig. 1C; $P < 0.05$).

Next, we sought to analyze levels of GSH in the studied animals. Our experiment demonstrated that GSH blood levels were decreased in the TV/PP group (847.25 ± 86.17 nmol/ml) compared to the Sham ($1,075.86 \pm 48.92$ nmol/ml), Post-5 ($1,051.13 \pm 92.14$ nmol/ml) and Post-2.5 ($1,008.32 \pm 94.67$ nmol/ml) groups after 30 min (Fig. 2A; $P < 0.05$ for TV/PP vs. Sham or Post-5). After 2 and 6 h there were no marked differences in GSH level between the groups (Fig. S1B and C). Additionally, analysis of the levels of SH demonstrated that levels of blood SH were markedly increased in Post-5 groups (47.30 ± 4.81 nmol/ml after 30 min and 54.22 ± 5.33 nmol/ml after 2 h) in comparison to the Sham, TV/PP and Post-2.5 groups (Fig. 2B-D). Animals in the Post-2.5 group demonstrated significant reduction of blood SH levels after 30 min (36.75 ± 6.5), 2 h (38.21 ± 2.7) and 6 h (44.52 ± 4.1) compared to the TV/PP and Post-5 groups (Fig. 2B-D; $P < 0.05$).

We also analyzed inflammatory cytokine TNF- α concentrations in the blood of the experimental animals. The postconditioning groups (Post-5/Post-2.5) displayed significantly reduced level of TNF- α (13.11 ± 4.49 and 17.08 ± 2.15 pg/ml, respectively) compared to the TV/PP animals (29.89 ± 1.78 pg/ml) after pneumoperitoneum (Fig. 3A; $P < 0.05$). Remarkably, no differences between the groups regarding SOD activity were found at any time point (Figs. 3B and S2A and B).

Discussion

It is known that during pneumoperitoneum there is a cascade of chemical release occurring after peritoneal tissue stress, insult and damage (18). The proceeding hypoperfusion caused by insufflation of the abdominal cavity (under pressure of 10 mmHg) may induce ischemia/reperfusion (I/R) injury.

Authors have reported experimental results demonstrating diverse stress conditions of different gases and high/low-pressure settings for the establishment of pneumoperitoneum (19,20). The conditioning of pneumoperitoneum has been suggested to be valuable and is an I/R protective tool (10). There are few reports concerning the use of the conditioning model to prevent the negative effects related to the pneumoperitoneum associated with respiratory, homeostatic and physiologic consequences (5,7,11,12,21). It was concluded that conditioning is more effective than pre-ischemic administration of erythropoietin in reducing the negative effects of oxidative injury (22).

Ischemic postconditioning is a new, simple manipulation that can protect organs from I/R injury. It has been shown

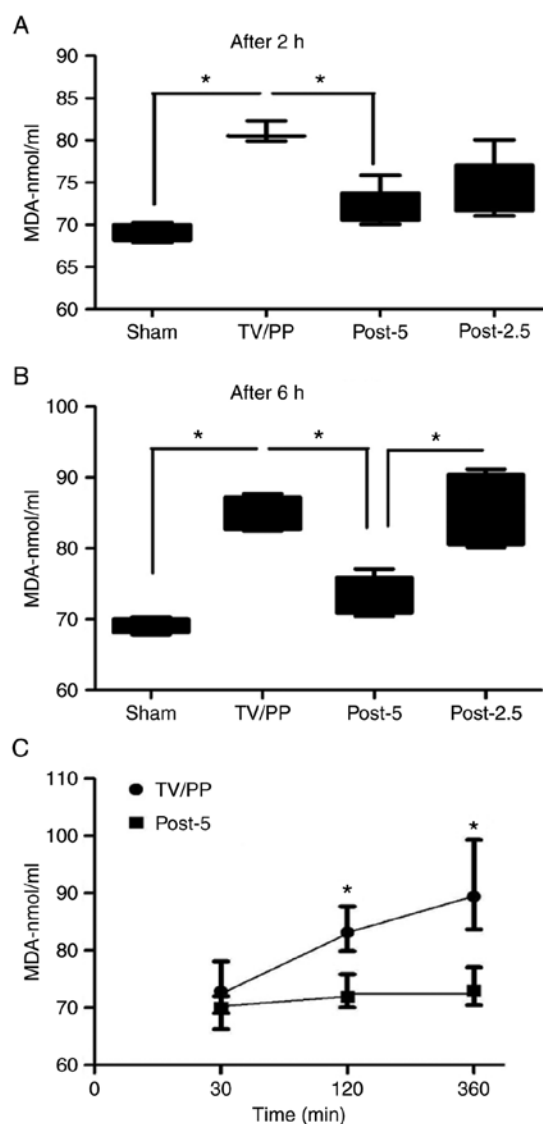


Figure 1. Blood levels of MDA in the Sham, TV/PP, Post-5 and Post-2.5 groups after (A) 2 and (B) 6 h of pneumoperitoneum. (C) Dynamics of the blood MDA levels in the TV/PP and Post-5 animal groups. * $P < 0.05$. MDA, malondialdehyde; TV/PP, transvaginal pneumoperitoneum.

that much of the post-ischemic injury occurs during the early moments of reperfusion, and that manipulation of this early reperfusion phase reduces I/R injury (10). We may assume that repetitive blood flow interruptions of tissues and organs applied immediately after a period of ischemia make tissues more resistant/tolerant to subsequent ischemic injury.

In our experiments, we created pneumoperitoneum conditions using a transvaginal approach due to the advantages of NOTES (Natural Orifice Transluminal Endoscopic Surgery) procedures, which are associated with less traumatic stress (14,23). Simultaneously, however, we wanted to achieve substantial oxidative stress, and therefore stressful levels of pneumoperitoneum pressure were applied (10 mmHg) (24,25). Indeed, this approach resulted in extensive elevation of oxidative stress parameters (TV/PP animal group). In order to effectively reduce negative signs of pneumoperitoneum, we decisively chose to use two different regimens of postconditioning in this transvaginally created pneumoperitoneum model.

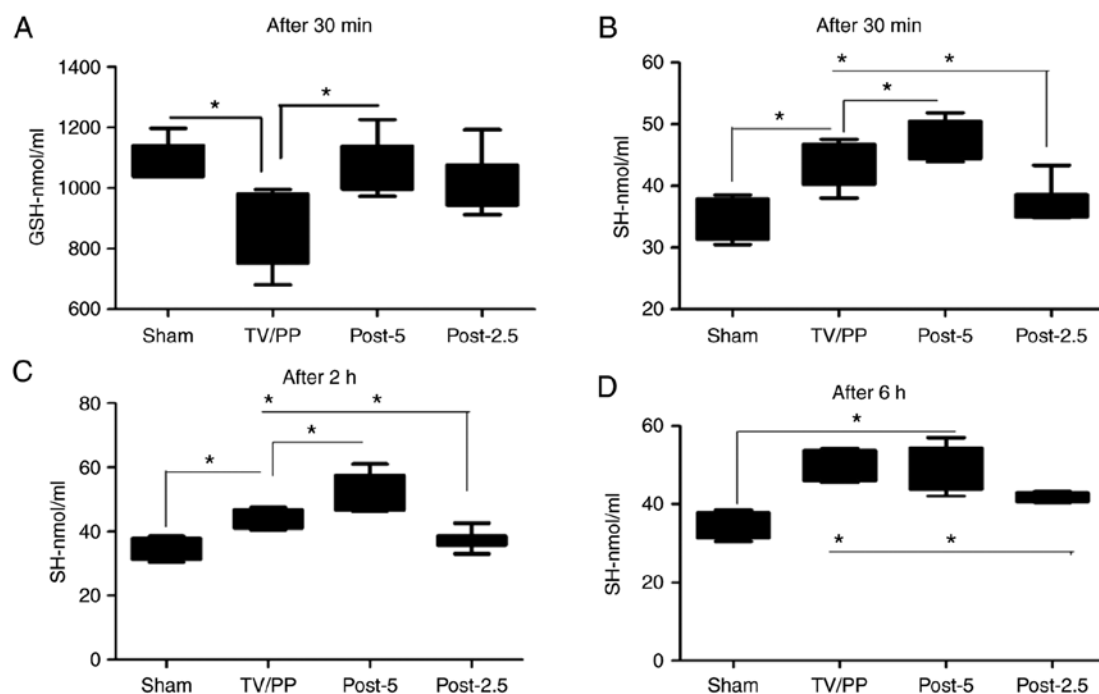


Figure 2. (A) Blood levels of GSH in the Sham, TV/PP, Post-5 and Post-2.5 groups after 30 min of pneumoperitoneum. (B-D) Levels of SH groups after 30 min (B), 2 h (C) and 6 h (D) of pneumoperitoneum. *P<0.05. GSH, reduced glutathione; SH, sulfhydryl groups; TV/PP, transvaginal pneumoperitoneum.

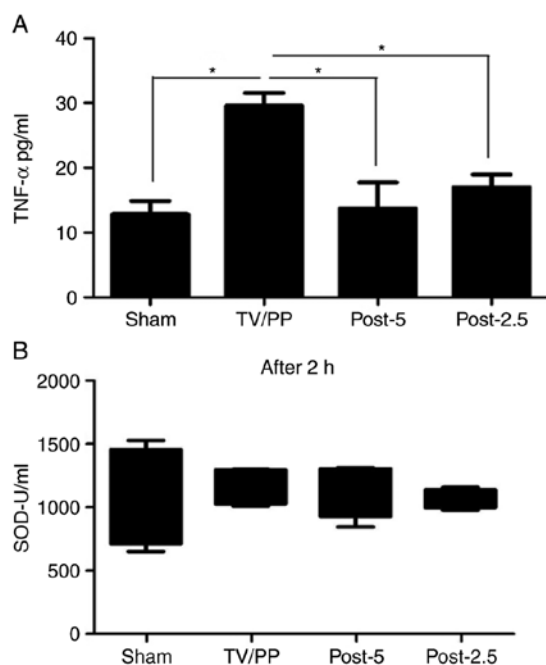


Figure 3. (A) Levels of TNF-α in the Sham, TV/PP, Post-5 and Post-2.5 groups after pneumoperitoneum. (B) Activity of superoxide dismutase (SOD) after 2 h of pneumoperitoneum. *P<0.05. TNF, tumor necrosis factor; TV/PP, transvaginal pneumoperitoneum.

Recently, the effects of a one cycle 5-min ischemic postconditioning has been reported in an animal model, which included 5 min of desufflation and 5 min of insufflation after pneumoperitoneum (7). In the present study, a similar animal model was used and we performed comparative analysis of the above mentioned regimen (Post-5) with two shorter cycled 2.5-min desufflation/insufflation regimen (Post-2.5). Duration

of total postconditioning time was the same in both groups (10 min).

To ascertain the extent of oxidative stress, levels of several markers, malondialdehyde (MDA), superoxide dismutase (SOD), reduced glutathione (GSH) and sulfhydryl group (SH) levels, as well as inflammatory cytokine TNF-α were determined. Free radicals have been seen to generate the lipid peroxidation process in organisms. MDA is one of the final products of lipid peroxidation in the cells, and an increase in free radicals causes overproduction of MDA (26). It is also known that GSH is a major endogenous antioxidant produced by cells, and that it participates directly in the neutralization of free radicals and reactive oxygen compounds. Reactive oxygen species overproduced as a result of oxidative stress are potent oxidizing and reducing agents that can directly damage cellular membranes by lipid peroxidation (8). SH is considered a major plasma antioxidant *in vivo*; and most of the SH-groups are present over albumin and are major reducing groups present in body fluids (27). Cytokines, such as TNF-α, play a key role in mediating the host inflammatory response. TNF-α is considered to be an initial mediator of the cytokine cascade and appears to be the first cytokine into the blood stream. The release of TNF-α is inhibited following hypoxia and re-oxygenation periods (28).

As expected, pneumoperitoneum caused upregulation of blood MDA and TNF-α levels. Introduction of the postconditioning method was found to significantly reduce lipid peroxidation process and cytokine release in the body of experimental animals, as well as increased levels of antioxidant factors such as GSH and SH. Notably, based on blood MDA level results, we can assume, that Post-5 regimen of postconditioning can more effectively reduce lipid peroxidation compared to the Post-2.5 regimen. Moreover,

based on analysis of the SH results we can suggest that the Post-2.5 postconditioning setup markedly reduced release of antioxidant factors. Assessment of GSH, TNF- α and SOD showed more or less similar effect among these postconditioning groups. Based on the results it can be suggested that both a single cycle postconditioning regimen (Post-5) as well as the cycled postconditioning (Post-2.5) method can reduce oxidative stress and subsequently may decrease pneumoperitoneum-caused complications. However, according to our experiments it is also suggested that the single cycle postconditioning regimen (Post-5) can be more effective in this regard. Further basic and clinical investigations are necessary to better characterize the postconditioning regimens and their effects although any of these approaches may already provide important clinical implications.

To conclude, pneumoperitoneum used during laparoscopic surgery induces elevation of oxidative stress markers. Postconditioning can normalize levels of oxidative stress markers after pneumoperitoneum. Furthermore, the method of postconditioning without cycles seems to be rather effective compared with postconditioning with cycles.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

KS, SJ and VK designed and performed the animal studies. KS and SJ performed and analyzed the biochemical experiments. GW, SJ and IA supervised and aided the experimental research, and participated in the data collection and analysis. KS, SJ, IA and GW drafted the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experiments were approved by the local ethics committee of the University of Pecs, Hungary.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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