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What Should Be the Ideal Time for Ischemic Preconditioning in a Laparoscopic Rat Model?

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Abstract

Background: Pneumoperitoneum (Pp) induces an ischemia and reperfusion (I/R) injury as a result of released oxidative stress markers. Ischemic preconditioning (IP) is one of the used methods to reduce the harmful effects of Pp, which is a mechanism for reducing organ I/R injury by a brief period of organ ischemia. The aim of this study was to investigate the ideal time for IP in the laparoscopic model.
Methods: Thirty-two rats were assigned into four groups: group 1 (control, $n = 8$) was subjected to a sham operation. Group 2 (5-minutes IP, $n = 8$) was subjected to 5 minutes of Pp with 15 mm Hg of pressure followed immediately by 5 minutes of deflation, and after that, 60 minutes of Pp with 15 mm Hg, followed by 60 minutes of deflation. Group 3 (10-minutes IP, $n = 8$) was subjected to 10 minutes of Pp and 10 minutes of deflation. Group 4 (Pp only, $n = 8$) was subjected to 60 minutes of Pp with 15 mm Hg of pressure, followed by 60 minutes of deflation. At the end of the experiment, plasma malondialdehyde (MDA) values, the oxidative stress marker, and plasma-reduced glutathione (GSH) levels, the marker showing antioxidant activity, were determined.
Results: Highest plasma MDA values were in group 4 (Pp only), followed by groups 2 and 3 and group 1 ($P = 0.181$). In addition, IP groups had almost the same values for MDA. Plasma GSH levels in the control group were significantly higher than those in the IP groups and the Pp-only group ($P < 0.001$). Similarly, as in MDA levels, no difference was found between plasma GSH levels of the IP 5-minutes and IP 10-minutes groups.
Conclusions: Five minutes of the IP model may be as reliable as 10 minutes of the IP model. In that case, 5 minutes of IP can be more suitable in reducing I/R injury in laparoscopy.

Introduction

During the last 30 years, laparoscopic surgery has evolved from a limited surgical procedure to a major surgical tool used to treat a multitude of indications. Today, laparoscopy is one of the most common surgical procedures performed by many surgeons.[1] Compared with laparotomy, multiple studies have shown laparoscopy to be safer, to be less expensive, and to have a shorter recovery time.[2,3] Although it has advantages, its adverse effects and hemodynamic results have not been adequately clarified.[4]

It was known that the pneumoperitoneum (Pp) is used for required visualization of the operation field during laparoscopy. But, gas insufflation produces significant splanchnic organ ischemia, followed by increased reperfusion injury after desufflation.[5] Further, ischemia and reperfusion injury (I/R), and then emerging oxidative stress induced by free oxygen radicals, is one of the most important mechanisms of organ dysfunction, and as is known, reactive oxygen species (ROS) and related molecules have toxic effects.[6,7]

For that reason, there are several methods that have been used to reduce or remove the harmful effects of Pp. One of the methods recommended for decreasing I/R injury is ischemic preconditioning (IP), which is a mechanism for reducing organ I/R injury by a brief period of organ ischemia. IP, first described in heart muscle, is an endogenous protective mechanism for short periods of I/R cycles.[8] It was shown, in various of animal and human studies, that IP decreased I/R injury.[9–11] To date, regarding IP, there were several studies reported of models with different pressure levels[10,11] or

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different pressure modalities[12]; however, any studies focusing on the question of what should be the ideal time for IP have not yet been investigated. The aim of this experimental animal study was to evaluate the minimum ideal time for IP and, by this means, reduction of the tissue injury caused by laparoscopic Pp under clinical applications.

Materials and Methods

Animals

Thirty-two female, nonpregnant Sprague-Dawley rats, weighing 250–280 g, were used. They were bred in isolation from male rats. They were kept at temperatures between 20 and 25°C, with relative humidity between 40 and 70%, and 12-hour light and dark cycles, with standard rat food and water *ad libitum*. Adequate hydration was ensured for all animals before the experiment. At the beginning of the experiment, each rat was given a number, from 1 to 32. Then, the rats were chosen randomly and put into separate cages for each group. The numbers of the rats in each cage were recorded by the principle author.

Experimental design

The design for this study was a randomized, controlled trial with blind outcome assessment, employing a protocole compatible with the 1996 revised form of the *Guide for the Care and Use of Laboratory Animals,* published by the National Institutes of Health (NIH, Bethesda, MD) and approved by the university ethics committee. Unnecessary suffering was avoided throughout the study.

Anesthesia

All rats were anesthetized at the beginning of the experimental procedures with an intramuscular (i.m.) injection of ketamine (5 mg/kg, Ketalar®; Eczacibasi-Werner Lambert, Istanbul, Turkey). Additional anesthetic doses were given, when required, during the course of the procedure (1 mg/kg, i.m.).

Surgical procedures

After the fulfilling of anesthesia, the animals were placed in a supine position with the limbs secured to the table. The rats were randomized to four groups, each consisting of 8 rats: group 1 (control) was subjected to a sham operation at the end of the 120-minutes anesthesia period without Pp; group 2 (5-minutes IP) was subjected to 5 minutes of Pp with 15 mm Hg of pressure, followed immediately by 5 minutes of deflation and, after that, 60 minutes of Pp with 15 mm Hg, followed by 60 minutes of deflation; group 3 (10-minutes IP) was subjected to 10 minutes of Pp with 15 mm Hg of pressure, followed immediately by 10 minutes of deflation and, after that, the same Pp and deflation period as group 2; and group 4 (Pp only) was subjected to 60 minutes of Pp with 15 mm Hg of pressure followed by 60 minutes of deflation.

Group 2 remained under anesthesia for a total of 130 minutes, and group 3 remained under anesthesia for a total of 140 minutes. The total anesthesia period in other groups was 120 minutes. At the beginning of the experiment, 1 of the rats in the 5-minutes IP group and 1 of the rats in the 10-minutes IP group died. Therefore, the experiment was concluded with 7 rats each in the IP groups and 8 rats each in the other two groups.

Creation of pneumoperitoneum

After shaving the abdomen with a safety razor and disinfecting with polyvidone iodine solution (Batticon; Trommsdorff-Adeka Ilac Sanayi, Samsun, Turkey) a 1-cm midline incision was made beneath the umblicus to reach the peritoneum. One of the ends of a 25-cm-long plastic tube was inserted into the peritoneal cavity as the vehicle for creating the pneumoperitoneum, after which the incision was closed with a tight purse-string suture to prevent leakage of CO_2 from the abdomen. The other end of the tube was connected to the CO_2 insufflator (Model No: 3-315-00; Nortech, Friborg, Switzerland). The pressure of the CO_2 insufflator was maintained constant at 15 mm Hg. An automatic insufflator provided CO_2 insufflation for the required intra-abdominal pressure. In the event of the intra-abdominal pressure decreasing due to transperitoneal CO_2 absorption or a possible gas leakage from the trocar entry site, the insufflator was automatically activated and pumped CO_2 into the abdominal cavities of the animals to keep the intra-abdominal pressure at the constant level. The system used to obtain Pp enabled simultaneous CO_2 insufflation into 8 rats under the same pressure.[11,13] For the purpose of making sure that there was no leakage from the cannulae, the ends of all cannullae were clamped before insufflation.

The sham operation

In the sham operation group, after the 120-minutes anesthesia period, after shaving the abdomen with a safety razor and disinfecting with polyvidone iodine solution, a 1-cm midline laparotomy incision was performed below the umblicus to reach the peritoneum and, after that, abdominal incisions were opened and extended, and approximately 1×1 cm squares of parietal peritoneum examples were excised with scissors.

Tissue and blood preparations

After finishing of the procedure, the Pp catheters were removed, abdominal incisions were opened and extended, and approximately 1×1 cm squares of parietal peritoneum examples were excised with scissors. Blood samples were taken from the abdominal aorta with a 22-G needle attached to a 10-cc syringe. Blood samples were drawn into heparinized tubes. The rats were then killed with an intracardiac potassium injection while still under anesthesia. Peritoneal samples were immersed in 10% formaldehyde for later examination under light microscope. Each tissue and blood sample was assigned a code number by the principal investigator and referred to the biochemists and pathologist participating in the study, who were blinded to the procedures applied to specific groups. All results were reported in relation to sample code numbers.

Biochemical analysis

Blood samples were drawn into heparinized tubes during the experiment. Plasma was separated by centrifugation at 800*g* and 4°C over 10 minutes. Erythrocytes were washed three times with ice-cold physiologic saline. The buffy coat,

together with part of the upper erythrocyte layer, was removed and discarded after each washing step. After the washing procedure, the packed cells and plasma were stored at −20°C until analysis. The packed erythrocyte hemoglobin concentration was determined spectrophotometrically in lysed cells by the cyanomethaemoglobin method.

Malondialdehyde (MDA) and reduced glutathione (GSH)

Plasma levels were determined by the thiobarbituric acid method of Okhawa et al.[14] MDA is an indicator of oxidative stress because it results from the breakdown of lipid peroxyl radicals. Although these are advantages, MDA reflects changes in numerous other biochemical systems as well as ROS, in particular, the prostaglandins. Therefore, in the evaluation of oxidative stress, we examined not only MDA, but also markers showing antioxidant status, called GSH. GSH was determined as an indicator of erythrocyte or tissue antioxidant capacity.[7,15,16] Blood GSH and MDA were purchased from the Sigma Chemical Co. (St. Louis, MO). Results were expressed as μmol/g hemoglobin for GSH and μmol/L for plasma MDA.

Histopathological analysis

Peritoneal samples were placed into buffered formalin solution. After 24 hours, samples were embedded in parafin, cut into sections 3 μm thick, and stained with hematoxylin eosin and assessed under a light microscope. All sections were evaluated by the same pathologist blinded to the groups. Each section was evaluated for intracellular edema, congestion, hemorrhage, and interstitial inflammatory cell infiltration, using the semiquantitative scale described by Hauet et al.[17]

Statistical analysis

All parametric results were expressed as the mean ± standard deviation for each group. The Shapiro-Wilks test was performed to check the normality of the data before running tests. Clinical parameters of groups were compared by using the analysis of one-way analysis of variance (ANOVA) and the Kruskall-Wallis test. The Levene test was performed to check the homogeneity of variances for the multiple comparisons. Comparisons of the between groups subjects were performed by using the Tamhane test. A *P*-value less than 0.05 was considered as significant.

Results

The plasma MDA and GSH levels are shown in Tables 1 and 2. These values are also displayed in Figures 1 and 2. Although there was no statistical difference between the groups, the highest plasma MDA values, the oxidative stress marker, in the group 4 (Pp only), followed by groups 2 and 3 (5-minutes and 10-min IP groups) and group 1 (control) ($P = 0.181$), respectively. In addition, the 5-min and 10-min IP groups had almost the same values for MDA (17.47 ± 6.55 vs. 17.62 ± 5.50 nmol/mL). Plasma GSH levels, the marker showing antioxidant activity, in the control group were significantly higher than those in the IP groups and Pp only group ($P < 0.001$).

Although there was a statistical difference between group 1 and the IP groups regarding GSH levels, preconditioning in IP groups led to GSH levels close to group 1 (4.80 ± 0.81 vs. 4.22 ± 0.44 mg/gHb and 4.23 ± 0.49 mg/gHb, respectively; $P < 0.001$). In addition, group 4 (Pp only) revealed a more significantly decreasing level of GSH than group 1 (4.80 ± 0.81 vs. 2.15 ± 0.36 mg/gHb, respectively; $P < 0.001$). Similarly, as in MDA levels, no difference was found between plasma GSH levels of the IP 5-min and IP 10-min groups (4.22 ± 0.44 vs. 4.23 ± 0.49 mg/gHb; $P = 1$). Histopathologic findings of light microscopy demonstrated no evidence of clear injury in peritoneal tissue samples. As is shown in Table 2 and Figure 2, there was no statistical difference among the groups ($P = 0.116$).

Discussion

Laparoscopic surgery requires adequate Pp for the visualization of intra-abdominal structures. Generally, Pp is established through the continuous insufflation of carbon dioxide (CO_2), which is the most commonly used gas due to its rapid rate of absorption and excretion, nonflammability, well tolerability, and lower risk of gas embolism.[18,19] But, it must not be forgotten that CO_2 may cause hypercapnia and acidosis that develops as a result of transperitoneal absorption of the gas used in Pp, and it could bring about vasoconstriction and increase vascular resistance and hypoperfusion.[5] Additionally, it has been demonstrated in various studies that intra-abdominal pressure for Pp above the normal portal circulation is associated with numerous adverse effects involving cardiovascular,[20–22] respiratory,[23] and renal circulatory changes.[6,24] These adverse effects mainly result from reduced venous return and increased systemic vascular resistance, causing significant reduction in blood flow to visceral structures. As a result, Pp followed by desufflation may lead to an I/R change. I/R injury causes the cumulation of different free oxygen radicals, also named as ROS. In normal conditions, ROS are formed continuously at low concentrations as a result of internal reactions, as well as exter-

Table 1. Plasma MDA and GSH Levels in Control, Pp Only, and IP Groups

Variables	*Group 1 (control,* n = *8)*	*Group 2 (IP 5 min,* n = *7)*	*Group 3 (IP 10 min,* n = *7)*	*Group 4 (Pp only,* n = *8)*	P-*value*
MDA (nmol/mL)	14.65 ± 1.43	17.47 ± 6.55	17.62 ± 5.50	21.11 ± 7.34	0.181
GSH (mg/gHb)	4.80 ± 0.81	4.22 ± 0.44[a]	4.23 ± 0.49[a,b]	2.15 ± 0.36[a,c,d]	<0.001

MDA, malondialdehyde; GSH, reduced glutathione; IP, ischemic preconditioning.
[a]Compared with control ($P < 0.001$).
[b]Compared with IP 5-minute group ($P = 1$).
[c]Compared with IP 5-minute group ($P < 0.001$).
[d]Compared with IP 10-minute group ($P < 0.001$).

Table 2. Histopathologic Results in Control, Pp Only, and IP Groups

	Group 1 (control, n = 8)	*Group 2 (IP 5 min, n = 7)*	*Group 3 (IP 10 min, n = 7)*	*Group 4 (Pp only, n = 8)*	P-*value*
Histopathologic scores	5.25 ± 0.46	4.71 ± 0.49	4.71 ± 0.49	4.75 ± 0.51	0.116

IP, ischemic preconditioning.

nal factors. It was well documanted that when there is an excessive amount of ROS, oxidative stress may cause damage to tissues. Eleftheriadis et al. demonstrated, in a rat model, that elevated intraabdominal pressure leads to oxygen free radical production[6]; similarly, Glantzounis et al. reported that free radicals are generated at the end of a laparoscopic procedure, possibly as a result of I/R injury.[25] Further, a study of Dinckan et al. showed that pentoxifylline may reduce oxidative injury following laparoscopic procedures,[26] and Ypsilantis et al. clearly showed, in a rat model, that administration of antioxidant mesna prevented the occurence of oxidative stress in various organs.[27]

For reducing or removing the harmful effects of Pp on tissues and organs, to date, various methods have been attempted. One of these methods that aimed to decrease I/R injury is lower pressure Pp procedures. Studies by Giraudo et al. and Samel et al. clearly demonstrated that lower pressure models were effective in reducing oxidative stress damage,[28,29] but, on the other hand, as our group has previously shown, there was no statistical significant difference in oxidative stress response at lower and higher pressure levels.[9,30]

The other method of reducing ischemic injury regarding Pp is IP. As was initially depicted in a cardiac dog model by Murry et al., IP is an injury-limiting mechanism that attenuates cardiac damage due to a severe I/R insult by previous short I/R cycles.[8] Afterward, an IP model was created through miscellanous laparoscopic studies. In these studies, it was shown that laparoscopic preconditioning may reduce oxidative injury in various intraabdominal organs, peritoneum, and blood.[9–11] Experiments conducted to demonstrate the efficacy of IP in reducing I/R injury have shown various IP pressure levels, but IP was generally applied at 15 mm Hg.[10,11] In another study, which investigated the effectiveness of IP, a different pressure modality was applied, named "the stepwise rising CO_2 insufflation method." In this study, the researchers concluded that this method could be effective, to a degree, in reducing oxidative stress and inflammatory cytokine response; thus, it might be an alternative IP method that could lead to a reduction in I/R injury.[12]

Although IP applications in laparoscopic surgery are not being used clinically, as opposed to cardiac and transplant surgery, our last study on the subject supplied considerable improvements on the ideal time of IP that can be used in clinical applications. The ideal time of IP is still unknown in laparoscopic surgery and remains to be elucidated. This subject is very important in clinical applications, because IP adds extra anesthesia time and prolongs operation time. So, we have to know the minimum time of IP. Thus, the hazardous effects of the clinical use of IP could be eliminated from the

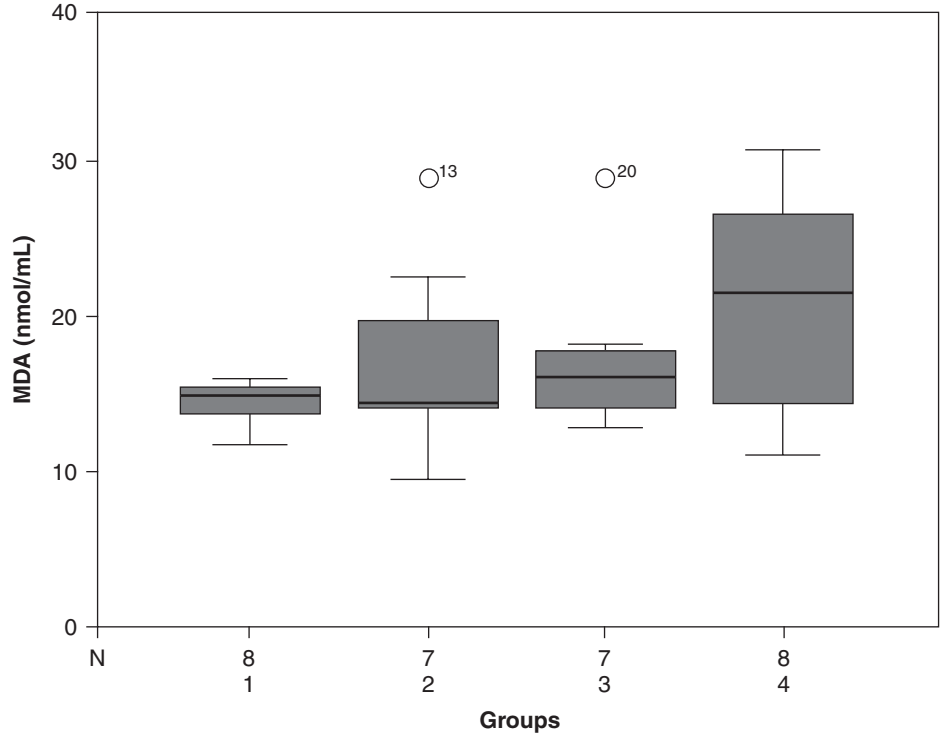

FIG. 1. Plasma levels of MDA in control, Pp only, and IP groups.

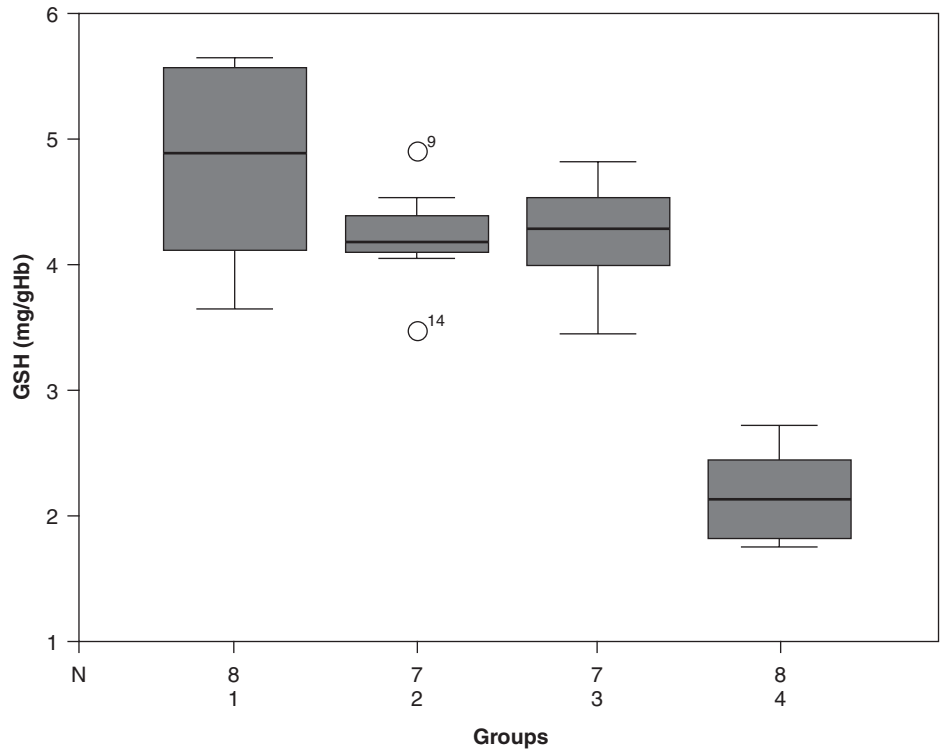

FIG. 2. Plasma levels of GSH in control, Pp only, and IP groups.

beginning. Finally, with this experiment, clinical studies in laparoscopic surgery can easily be designated.

To the best of our knowledge, this is the first report focused on the question of what should be the ideal time for IP in laparoscopy. In the literature, there were several studies using different IP application times, such 5–10 minutes of Pp, followed by 5–10 minutes of deflation, but none of them compare the different application times.[31–33] In the present study, a comparison of the IP 5-minutes and IP 10-minutes groups, in terms of oxidative stress response, demonstrated that there was no statistical difference between the IP groups. Consequently, our findings suggest that 5 minutes of an IP period is as effective as 10 minutes of an IP period in preventing the harmful effects of Pp in the laparoscopic rat model. In our study, we used MDA and GSH as oxidative stress markers, and we have shown that plasma GSH levels in the control group were significantly higher than those in the IP groups and Pp-only group ($P < 0.001$). We also demonstrated that although there was no statistical difference between the groups, the highest plasma MDA values were in the Pp-only (group 4) group, followed by the IP groups (groups 2 and 3) and control (group 1) group ($P = 0.181$). Oxidative stress markers have been investigated scarcely in laparoscopic studies.[6,25,34,35] In a study by Glantzounis et al., thiobarbituric acid-reactive substrates (TBARS), a marker of free radical production, plasma total antioxidant status (TAS), and uric acid were used as oxidative stress markers, and they suggested that free radicals are generated at the end of laparoscopy, possibly as a result of I/R injury created by Pp.[25] In another study, Bickel et al. validated the I/R mechanism following laparoscopic surgery by using lipid peroxide and total glutathione levels as oxidative stress markers.[35] It is well known that laparoscopic procedures cause I/R injury in the abdominal organs and tissues, and that I/R injury causes the release of many of oxidant and antioxidant markers. MDA is a low-molecular-weight aldehyde that is derived from the peroxidation of certain lipids. It is one of the most important oxidative stress markers.[27] GSH is an essential component of cellular defense mechanism and plays a role in decreasing the tissue injury induced by oxygen free radicals following I/R.[33] In various studies investigating the relationship between oxidative stress and laparoscopic preconditioning, MDA and/or GSH were used confidently and efficiently.[10,11,27] So, they are reliable markers for detection of oxidative stress following laparoscopic surgery.

In this study, we found that MDA levels were lower and GSH levels were higher in group 2 (5-minutes IP group) than group 1 (control group). Similarly, we found that MDA levels were lower and GSH levels were higher in group 3 (10-minutes IP group) than the control group. Although it is speculative that two parameters, MDA and GSH levels, are enough parameters to adapt this model to clinical applications, these findings may highlight human studies on this subject.

In our study, although there were differences among the groups in terms of oxidative stress markers in plasma, there were no evident signs of ischemic injury in the peritoneal tissue samples on light microscopy. Because of technical difficulties, in our study, to evaluate acute peritoneal injury, we used light, instead of electron, microscopy. In this respect, the literature has a limited knowledge. Only Sahin et al. demonstrated, in a stepwise insufflation IP model, significantly higher histopathologic scores in the liver.[12] It is possible that

light microscope is not able to show ischemic differences in peritoneal tissue as yet, and longer ischemic times are needed to see related results or whether using electron microscopy can be useful in early stages. Nevertheless, liver tissue can demonstrate different dynamics than peritoneal tissues.

Conclusions

As a result, in light of the findings of this study, we conclude that 5 minutes of inflation, followed by 5 minutes of deflation, seems to be as effective as 10 minutes of inflation, followed by 10 minutes of deflation, for preventing I/R injury, so it is advisable for clinical settings, but it should be emphasized that this was an experimental animal study, and finding an evident place in clinical practice human studies investigating minimum effective application time in IP are needed.

Disclosure Statement

No competing financial interests exist.

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