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RESEARCH ARTICLES

# Characterization of Intestinal Ischemia/Reperfusion-Induced Decrease of Intestinal Glutathione

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**ABSTRACT |** Intestinal ischemia followed by reperfusion has been widely studied, and oxidative stress generated by the production of reactive oxygen species is one major pathophysiological mechanism involved. The reduced form of glutathione (GSH) as part of the cellular antioxidant defense is affected during this process. The aim of this study was to perform a systematic study on the time course of intestinal GSH during intestinal ischemia, and ischemia followed by reperfusion (I/R) to clarify its role in I/R. Wistar rats were subject to laparotomy and ischemia achieved by clamping the intestinal vascular axis, and releasing the elastic band for the reperfusion period. Ischemia and I/R were monitored by surface oxygen and carbon dioxide electrodes. GSH was assayed by high pressure liquid chromatography. Our results showed that ischemia induced slight decreases in cecal GSH that could not be established as significant in the 120 min ischemia group. However, 5 and 30 min of reperfusion following different times of ischemia promoted a time-dependent decrease of GSH that reached 30% of control values. In conclusion, intestinal GSH may play a critical role during I/R in view of the correlation between its concentration after revascularization and the time of ischemia, which deserves further investigation.

**KEYWORDS |** Glutathione; Intestine; Ischemia; Oxidative stress; Reperfusion

**ABBREVIATIONS |** I/R, ischemia and reperfusion; GSH, reduced form of glutathione; ROS, reactive oxygen species

**CONTENTS**

## 1. INTRODUCTION

Intestinal ischemia is a clinical condition that emerges when blood flow to the mesenteric territory is insufficient to meet the requirements of the intestine. It is caused by circulatory failure, and tissue damage occurs as a result of the reduced supply of oxygen and nutrients to the affected area [1, 2]. Intestinal ischemia followed by reperfusion is a frequent phenomenon during intestinal stricture, gangrene, fulminant universal colitis, and mesenteric secondary low flow [3]. It is also caused by other illnesses such as neonatal necrotizing enterocolitis [4], cardiopulmonary bypass, strangulated hernias, intestinal transplantation, abdominal aortic aneurysm surgery, and in shock and sepsis [5] that compromise motor and secretory intestinal functions [4]. The above pathophysiological conditions result in bacterial translocation, endotoxemia, acute respiratory stress syndrome, and acute hepatic injury, which may eventually lead to the development of a multiple organ dysfunction syndrome [5], causing high morbidity and mortality [6]. Intestinal ischemia and reperfusion (I/R) injury is a complex and multifactorial pathophysiological process that involves the action of reactive oxygen and nitrogen species (ROS/RNS), inflammatory cytokines, and polymorphonuclear lymphocytes. Of the potential ROS sources described to date, xanthine oxidase, NADPH oxidase (NOX), mitochondria, and uncoupled nitric oxide synthase have been demonstrated to be the most likely contributors to reperfusion-induced oxidative stress and reperfusion-induced organ dysfunction and tissue damage [7]. There are a variety of antioxidant defense systems that coordinate cooperatively and protect the body from oxidative stress injury in aerobic organisms. These include many enzymatic antioxidants (e.g., superoxide dismutase, glutathione peroxidase, and catalase) and non-enzymatic antioxidants such as the reduced form of glutathione (GSH), which are subject to transcriptional regulation under various conditions, including ischemia and reperfusion injury [8]. It is noteworthy that GSH is a highly effective antioxidant which acts as an intracellular cysteine deposit, preventing the action of free radicals [9].

Gastrointestinal diseases have been associated with oxidative stress as an essential factor in their pathogenesis [10]. As an example of this, our group demonstrated that administration of the cysteine derivative *N*-acetyl-cysteine increases intracellular glutathione levels in experimental acute colitis and protects against the disease pathophysiology [11]. A number of studies have tried to demonstrate the effects of I/R on intestinal GSH, but no systematic study has characterized the response of this tissue to different ischemia periods and its response after a standard period of reperfusion. The aim of the present study was to determine the changes in GSH content in rat intestine after different periods of ischemia and those same periods of ischemia followed by a 30 min period of reperfusion.

## 2. MATERIALS AND METHODS

### 2.1. Surgical Procedures

Animals (male Wistar rats 250–350 g, Harlan Laboratories®) were fasted for 12 h and then anesthetized with ketamine plus xylazine (80/10 mg/kg) intraperitoneally. Medial laparotomy was performed to access the abdominal cavity. Ischemia was performed in the ileum area by a loop with elastic band (Vesseloops™), placed on the intestinal vascular axis. At one edge of the abdominal incision, 1 cm below the xiphoid cartilage, separation of subcutaneous muscle tissue was performed to determine trans-mural partial pressure values of $O_2$ and $CO_2$. These same measurements were made on the cecum surface in order to check hemodynamic stability. Reperfusion phase was started by removing the vascular compression of the intestinal vascular axis. After ischemia or ischemia and reperfusion (I/R) periods, intestinal samples were removed, washed in saline serum, and frozen for biochemical analysis.

### 2.2. Experimental Group

Fifty male Wistar rats (250–300 g) were kept in a standard animal facility, with access to food and wa-

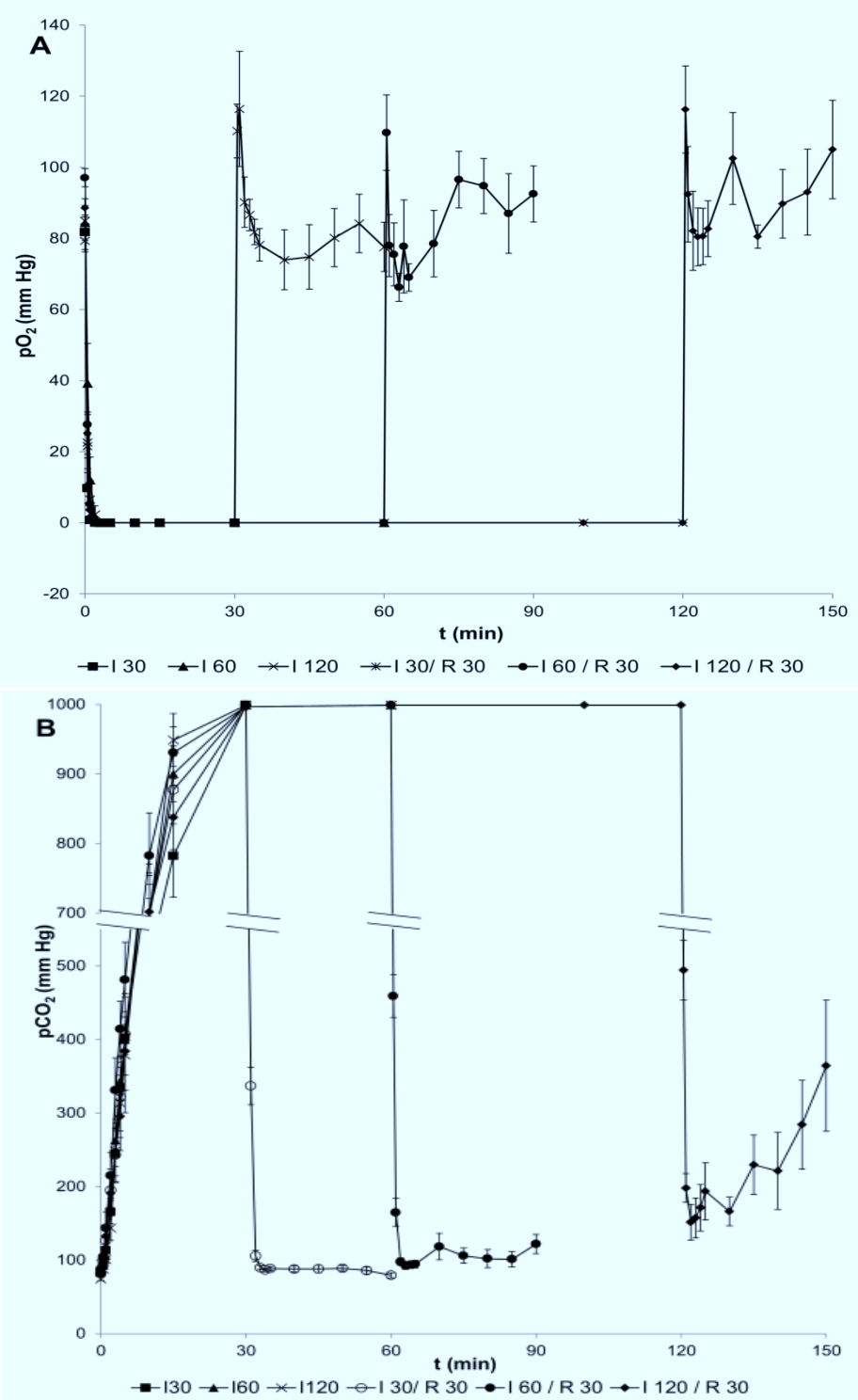


**FIGURE 1. Surface pO2 (A) and pCO2 (B) of the rat cecum in all experimental groups described.** Results are means ± SEM (n = 5 for each group).

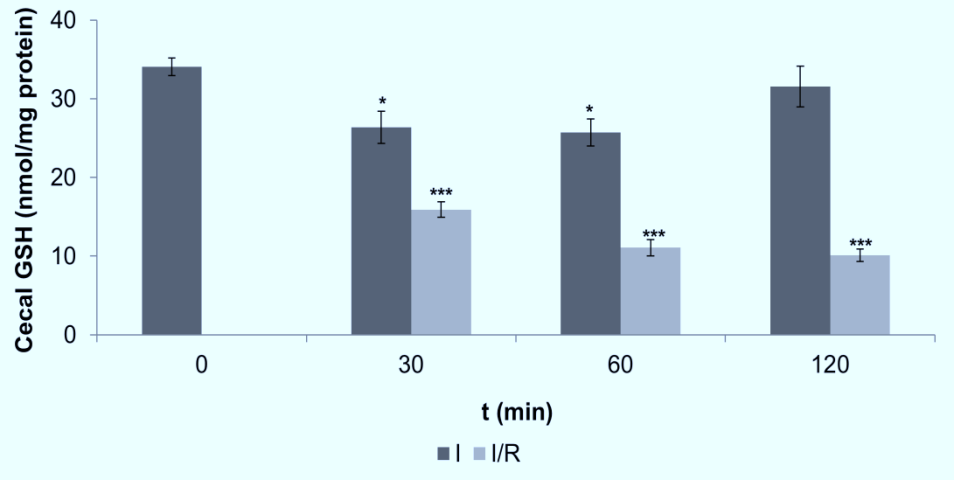


**FIGURE 2. GSH content of the cecum at different ischemia times, compared with the respective groups after 30 min of reperfusion.** Results are means ± SEM (n = 5 for each group). *, $p < 0.05$; **, $p < 0.0001$.

ter both preoperatively and postoperatively, in keeping with the principles of Good Laboratory Practice. The experimental design and animal welfare procedures were approved by the Animal Welfare Committee of the Catholic University of Valencia, in compliance with applicable legislation (Royal Decree 53/2013), and the U.S. Food and Drug Administration (FDA) recommendations on animal welfare in experimentation.

For the experimental study, Wistar rats were randomly divided into 4 experimental groups, as outlined below.

(1) Control (3 groups, n = 5): laparotomy without surgical intervention. The animals remained alive for periods of 30, 120, and 150 min.
(2) Basal (n = 5): for obtaining basal values of GSH.
(3) Ischemia: 3 groups (n = 5) with intestinal ischemia of 30, 60, and 120 min, respectively.
(4) Ischemia-reperfusion (6 groups, n = 5): I 30 min/R 5 min; I 30 min/R 30 min; I 60 min/R 5 min; I 60 min/R 30 min; I 120 min/R 5 min; I 120 min/R 30 min.

The parameters evaluated were: (1) analytical studies of oximetry/capnometry ($PtO_2/PtCO_2$) to confirm ischemia and reperfusion, and (2) biochemical analysis of intestinal GSH in all groups and subgroups described above.

### 2.3. Oximetry/Capnometry

Oxygen and $CO_2$ partial pressures were determined on the serous cover of the tissues (abdominal muscle rectus abdominis and frontal cecum surfaces, respectively) with a combined $PtO_2/PtCO_2$ sensor (Novametrix™) that provided continuos $PtO_2/PtCO_2$ monitoring [12, 13].

### 2.4. Assay for GSH Content

GSH concentration in the different samples was determined by the method described by Reed et al. [14]. The adduct GSH-CDNB (glutathione-fluorodinitrobenzene) was separated and quantified by high performance liquid chromatography (HPLC). Briefly, we used a gradient of methanol:water (4:1, v/v) and sodium acetate buffer (50 mM, pH 5.4). Detection was performed with a UV/Vis detector at 365 nm.

### 2.5. Assay for Protein Content

Protein content was measured using the Lowry method [15].

### 2.6. Statistical Analyses

All result values were expressed as the mean ± SEM. To analyze the quantitative variables of the experi-

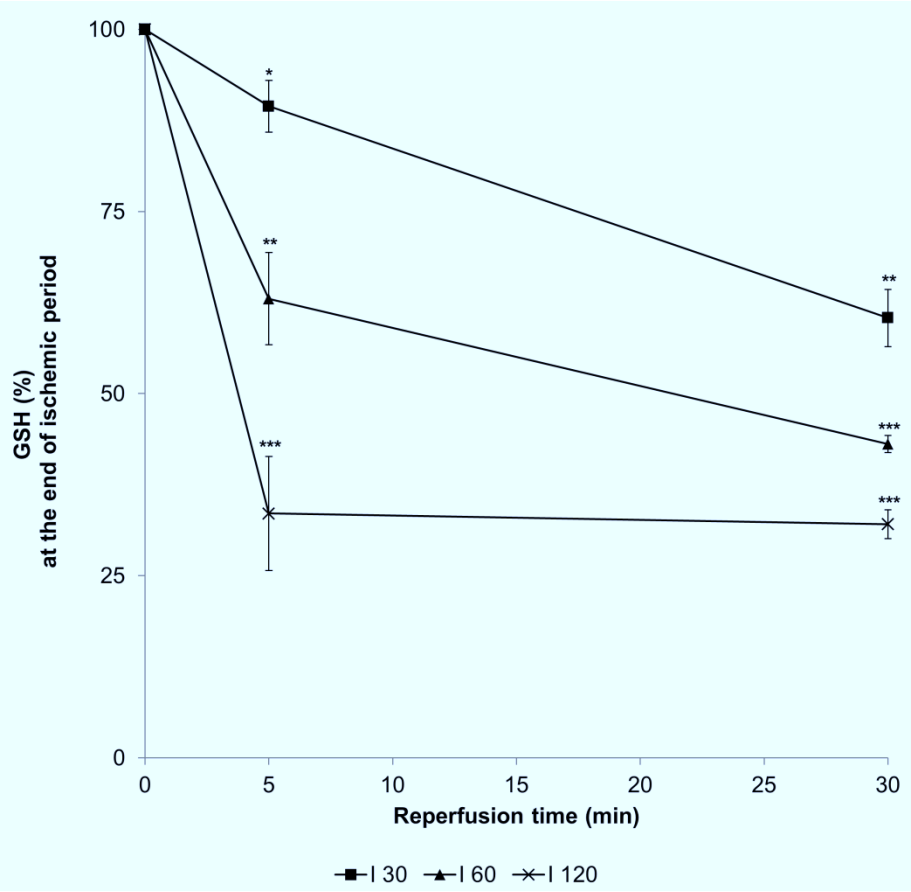


**FIGURE 3. Time course of the percentage of intestinal GSH content during reperfusion after different periods of ischemia.** Results are means ± SEM (n = 5 for each group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$, compared with GSH content at the end of ischemia.

mental groups, an ANOVA test was used to determine the existence of differences between them. To determine significance levels in each case, a Fisher's least significant difference test was performed. Nonnumeric variables were evaluated with the chi square test. A p value of less than 0.05 was considered to be statistically significant. The data were analyzed using the statistical software package SPSS 22.1.2 (IBM, USA).

## 3. RESULTS

**Figure 1** shows the recordings of pO2 and pCO2 on the cecum surface during the surgical procedure to confirm that the surgical procedure and the arterial occlusion were effective. $pO_2$ returned to physiological values after the ischemic period and stabilized during the subsequent 30 min reperfusion period, except in the "I 120 min/R 30 min" group, where much greater variations were observed at the end of the experiment. $pCO_2$ increased to values that reached the maximum detectable limit by the device during the different ischemic periods. Reperfusion clearly affected surface $pCO_2$ with higher deviations as ischemia period increased. **Figure 2** represents the content of intestinal GSH in the different groups studied. Ischemia slightly decreased GSH content in the cecum after 30 and 60 min of ischemia, but no significance could be established after 120 min of ischemia, when

compared with the control values. However, when compared with the animals that suffered ischemia and subsequently a standard reperfusion time of 30 min, the longer the ischemic period was, the more severe the GSH decrease was at the end of the reperfusion period. The GSH values 5 min after initiating reperfusion decreased also proportionally to the ischemic period (**Figure 3**).

## 4. DISCUSSION

The surgical procedure applied in this study achieved the goal of maintaining ischemia during the different periods chosen. Interestingly, $pCO_2$ recovered to control values (prior to ischemia) and stabilized only in the 30 min ischemia and reperfusion. All other groups showed big variations of $pCO_2$ after reperfusion, most probably related to the duration of ischemia. This finding deserves further research.

A recent review by Bhattacharyya et al. [10] situates GSH as the first non-enzymatic antioxidant in the pathogenesis of gastrointestinal mucosal diseases, as we had previously also demonstrated in a model of experimental acute colitis [11]. In fact, the tight correlation between the decreases of intestinal GSH content and the duration of ischemia only 5 min after the initiation of reperfusion (**Figure 3**) provides a very interesting clue to the role of this cellular antioxidant during intestinal I/R. Other authors have reported significant decreases in intestinal GSH after 30 min ischemia and 45 min reperfusion of only 18% of the control value [17], or a 30 % reduction in total antioxidant capacity (GSH is part of this capacity) after 1 h of ischemia followed by 2 h of reperfusion [18], or have only measured the blood GSH concentration without caring about the concentration in cecum [19]. As can be interpreted by the data herein, the modifications of intestinal GSH content occurred immediately after initiating reperfusion (as soon as 5 min) and were proportional to the duration of ischemia. The protective role of mucosal GSH in a model of rat hemorrhagic shock (1 h hypotension followed by 1 h reperfusion), defined by the authors as a prolonged "ischemia"/reperfusion condition, was questioned by the experiment that depleted it with phorone (diisopropylideneacetone) and showed that allopurinol, an inhibitor of xanthine oxidase (supposed to be the major source of ROS in the intestine under I/R conditions [7]), did not protect against intestinal lesion formation and that phorone-induced GSH depletion exerted a protective effect against hemorrhagic shock-induced gastrointestinal lesion [20]. Phorone is an interesting compound that was shown to deplete cytosolic GSH but not the mitochondrial pool when injected to animals [21] and in cells in vitro [16, 22], helping to understand the major role of mitochondrial GSH in cell survival. This might be an explanation for its protective effect, i.e., decreasing the cytosolic thiyl radical-dependent ROS generation [23]. In the previous report, we demonstrated that thiols react differentially with myoglobin and that the formation of thiyl radical upon an oxidative challenge to myoglobin may be modified by these sulfur-containing compounds. Further mechanistic aspects of this process need to be clarified. Interestingly, different reports show the lung [24] or other organ damage after intestinal ischemia/reperfusion. Research is underway in our laboratory to try to elucidate the mechanisms involved in this remote damage. The findings of the present study herein confirmed that the ischemic period is critical for the reperfusion-induced GSH decrease in the cecum, as well as probably other metabolites related to oxidative stress. Further and systematic research is needed to provide a consistent time course of events under ischemia and reperfusion conditions.

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## REFERENCES

1. Menke J. Diagnostic accuracy of multidetector CT in acute mesenteric ischemia: systematic review and meta-analysis. *Radiology* 2010; 256(1):93–101. doi: 10.1148/radiol.10091938.
2. Belkin O, Whittemore AD, Donaldson MC, Conte MS, Gravereaux E. Peripheral arterial

occlusive disease. In: *Sabiston Textbook of Surgery* (C Townsend, D Beauchamp, M Evers, K Mattox). 19th ed. Saunders Elsevier, Philadelphia, USA. 2012.
3. Wang F, Li Q, Wang C, Tang C, Li J. Dynamic alteration of the colonic microbiota in intestinal ischemia-reperfusion injury. *PLoS One* 2012; 7(7):e42027. doi: 10.1371/journal.pone.0042027.
4. Campos VF, Miranda-Ferreira R, Taha NS, Teixeira GD, Souza WT, Carmo CE, et al. Atenolol to treat intestinal ischemia and reperfusion in rats. *Transplant Proc* 2012; 44(8):2313–6. doi: 10.1016/j.transproceed.2012.07.058.
5. San Norberto Garcia EM, Taylor JH, Cenizo N, Vaquero C. Beneficial effects of intra-arterial and intravenous prostaglandin E1 in intestinal ischaemia-reperfusion injury. *Interact Cardiovasc Thorac Surg* 2014; 18(4):466–74. doi: 10.1093/icvts/ivt552.
6. Chen Y, Lui VC, Rooijen NV, Tam PK. Depletion of intestinal resident macrophages prevents ischaemia reperfusion injury in gut. *Gut* 2004; 53(12):1772–80. doi: 10.1136/gut.2003.034868.
7. Granger DN, Kvietys PR. Reperfusion injury and reactive oxygen species: The evolution of a concept. *Redox Biol* 2015; 6:524–51. doi: 10.1016/j.redox.2015.08.020.
8. Espinosa-Diez C, Miguel V, Mennerich D, Kietzmann T, Sanchez-Perez P, Cadenas S, et al. Antioxidant responses and cellular adjustments to oxidative stress. *Redox Biol* 2015; 6:183–97. doi: 10.1016/j.redox.2015.07.008.
9. Meister A. Glutathione metabolism. *Methods Enzymol* 1995; 251:3–7.
10. Bhattacharyya A, Chattopadhyay R, Mitra S, Crowe SE. Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiol Rev* 2014; 94(2):329–54. doi: 10.1152/physrev.00040.2012.
11. Ardite E, Sans M, Panes J, Romero FJ, Pique JM, Fernandez-Checa JC. Replenishment of glutathione levels improves mucosal function in experimental acute colitis. *Lab Invest* 2000; 80(5):735–44.
12. Prieto-Moure B, Caraben-Redano A, Aliena-Valero A, Cejalvo D, Toledo AH, Flores-Bellver M, et al. Allopurinol in renal ischemia. *J Invest Surg* 2014; 27(5):304–16. doi: 10.3109/08941939.2014.911395.
13. Benjamin E, Oropello JM. Does gastric tonometry work? No. *Crit Care Clin* 1996; 12(3):587–601.
14. Reed DJ, Babson JR, Beatty PW, Brodie AE, Ellis WW, Potter DW. High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. *Anal Biochem* 1980; 106(1):55–62.
15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193(1):265–75.
16. Romero FJ, Sies H. Subcellular glutathione contents in isolated hepatocytes treated with L-buthionine sulfoximine. *Biochem Biophys Res Commun* 1984; 123(3):1116–21.
17. Akinrinmade JF, Akinrinde SA, Odejobi A, Oyagbemi AA. Evidence of attenuation of intestinal ischemia-reperfusion injury following pre-treatment with methanolic extracts from Chromolena odorata in rats. *J Complement Integr Med* 2015; 12(1):23–32. doi: 10.1515/jcim-2014-0034.
18. Onal O, Yetisir F, Sarer AE, Zeybek ND, Onal CO, Yurekli B, et al. Prophylactic Ozone Administration Reduces Intestinal Mucosa Injury Induced by Intestinal Ischemia-Reperfusion in the Rat. *Mediators Inflamm* 2015; 2015:792016. doi: 10.1155/2015/792016.
19. Duran A, Otiuk H, Terzi EH, Tosun M, Oziiu H, Ocak T, et al. Protective effect of montelukast, a cysteinyl leukotriene receptor-1 antagonist, against intestinal ischemia-reperfusion injury in the rat. *Acta Chir Belg* 2013; 113(6):401–5.
20. Yasue N, Chan ET, Kaplowitz N, Guth PH. Effect of phorone and allopurinol on ischemia-reperfusion injury in gastrointestinal mucosa of the rat. *Pharmacology* 1992; 44(6):334–43.
21. Romero FJ, Roma J. Careful consideration of the effects induced by glutathione depletion in rat liver and heart. The involvement of cytosolic and mitochondrial glutathione pools. *Chem Biol Interact* 1989; 70(1–2):29–37.
22. Romero FJ, Soboll S, Sies H. Mitochondrial and cytosolic glutathione after depletion by phorone in isolated hepatocytes. *Experientia* 1984; 40(4):365–7.
23. Romero FJ, Ordonez I, Arduini A, Cadenas E.

The reactivity of thiols and disulfides with different redox states of myoglobin. Redox and addition reactions and formation of thiyl radical intermediates. *J Biol Chem* 1992; 267(3):1680–8.

24. Wang GZ, Yao JH, Jing HR, Zhang F, Lin MS, Shi L, et al. Suppression of the p66shc adapter protein by protocatechuic acid prevents the development of lung injury induced by intestinal ischemia reperfusion in mice. *J Trauma Acute Care Surg* 2012; 73(5):1130–7. doi: 10.1097/TA.0b013e318265d069.