Journal of BUON **17:** 377-382, 2012

ORIGINAL ARTICLE

Hydroxyzine, cimetidine and vitamin C in reducing skin flap necrosis in ischemia-reperfusion injury in rats. A comparative study

Sp. Georgopoulos[1], D. Mastorakos[2], A. Kondi-Pafiti[3], K. Katsenis[4], N. Arkadopoulos[2], D. Kannas[2], M. Archontaki[1], N. Vestarchis[5], G. Kokkalis[1]

[1]Department of Plastic and Reconstructive Surgery, Greek Anticancer Institute, St. Savvas Hospital, Athens; [2]Department of Surgery, Aretaieion Hospital, Medical School, University of Athens, Athens; [3]Pathology Laboratory, Aretaieion Hospital, Medical School, University of Athens, Athens; [4]Department of Vascular Surgery, Aretaieion Hospital, Medical School, University of Athens, Athens; [5]School of Applied Mathematical and Clinical Science, National Technical University of Athens, Athens, Greece

Summary

Purpose: *The purpose of the current experimental research was to investigate whether hydroxyzine can reduce the necrotic area in ischemia-reperfusion injury in epigastric rat skin flaps and to compare its role with cimetidine and vitamin C.*

Methods: *From a total of 77 ischemic rat skin flaps, 18 were treated with normal saline, 18 with vitamin C, 18 with cimetidine and 18 with hydroxyzine before reperfusion. Flap necrotic area, neutrophils and mast cells were measured on the 7th day. Analysis of variance for multiple comparisons and post hoc Dunnett's test were used for statistical analyses.*

Results: *The sham group of animals (n=5) showed 0% flap necrosis. The saline-treated group demonstrated 75±15.3% of necrosis. The vitamin C, cimetidine and hydroxyzine groups had 56.2 ± 24.4%, 25.8 ± 19.3%, and 33.6 ± 27.8% of flap necrosis, respectively. In addition, the number of neutrophils and mast cells were decreased in the pharmacologically treated groups compared with flaps perfused with normal saline ($p<0.05$).*

Conclusion: *Our data suggest that administering hydroxyzine in rat epigastric skin flaps before reperfusion may attenuate necrosis, neutrophils and mast cell counts. The beneficial effect of cimetidine was the same as hydroxyzine's but the use of vitamin C was less effective.*

Key words: cimetidine, hydroxyzine, injury, ischemia, reperfusion, vitamin C

Introduction

Reconstructive microsurgery comprises a surgical technique utilizing revascularized tissue transfer to repair tissue defects after trauma or malignant tumor removal. It is well known that the most difficult part of plastic surgery contribution in surgical oncology is not the excision of a tumor itself but the coverage of the defect, especially when free flaps are to be used. Vascular occlusion is the reason of partial or total necrosis of free flaps. Reperfusion [1] induce a complex chain reactions caused by free radicals [2-4] and neutrophils [1,5,6]. The term ischemia-reperfusion injury describes the experimentally and clinically prevalent finding that tissue ischemia with inadequate oxygen supply followed by successful reperfusion initiates a wide and complex array of inflammatory responses that may both aggravate local injury and also induce impairment of remote organ function [7] causing further harassment in cancer patients. The specific goal of our investigation was to investigate: a) the role of hydroxyzine in reducing the necrotic area in ischemia-reperfusion injury in rat skin flaps (which has not been determined in any other study) and b) to compare the hydroxyzine's effect with vitamin C and cimetidine effect.

Methods

After institutional protocol approval, a total of 77 female Sprague-Dawley rats, each weighing about 250 g, were allowed to

Correspondence to: Spyros Georgopoulos, MD, PhD. Kythnou 4, Glyfada 166 74, Athens, Greece. Tel: +30 6936903403, E-mail: sgeorgopgr@yahoo.gr

Received 15-09-2011; Accepted 02-10-2011

acclimatize and then anesthetized with intraperitoneal injection of xylazine (Sedaxylan/Eurovet Hellas: inj. Sol. 20 mg/ml) at a dose of 5 ml/kg and ketamine (Imalgene 1000/P.N. Gerolymatos: inj. Sol. 100 mg/ml) at a dose of 90 ml/kg [8]. The skin over the chest and the abdomen was depilated using a depilatory cream (Veet, Reckitt Benckiser Group plc, USA) and an island skin flap measuring 3×6 cm (18 cm^2) was raised with 3.5× magnification loupes [9] (Figure 1). The flaps were incised and elevated completely and the inferior epigastric vessels were skeletonized and left as the only vascular pedicle. The epigastric fat pad was raised with all flaps and the epigastric nerve was incised in order to denervate the flap. Then the femoral artery and vein were isolated proximal and distal to the epigastric pedicle, and all muscle perforators were incised and coagulated. The distal femoral artery was then ligated with a 7-0 silk suture to prevent backflow perfusion. Ischemia was induced by clamping the femoral artery with a microvascular Acland 2V near the pedicle flap. Flaps were resutured with a 4-0 silk continuous suture. Animals were returned to their cages to recover and then reanesthetized, the incision opened partially and the clamps were removed after 10 h of ischemia. Sham animals underwent flap elevation, vessel ligation, and resuturing without an ischemic insult to the epigastric flaps. A rodent restraint cone was then placed to prevent auto cannibalization of the flaps.

After completing the operative procedures, 10 h of ischemia were applied. Thirty min before reperfusion the rats were randomized to receive the following medication intraperitoneally:
Group I: Ischemia/reperfusion flaps treated with saline (1 ml) n=18.
Group II: Ischemia/reperfusion flaps treated with vitamin C (Vitamin C/L'aguettant: inj. Sol. Iv 500 mg/5 ml) (60 mg/kg) n=18.
Group III: Ischemia/reperfusion flaps treated with cimetidine (Tagamet/Vianex, Greece: inj. Sol. Iv 200 mg/2 ml) (250 mg/kg) n=18.
Group IV: Ischemia/reperfusion flaps treated with hydroxyzine (Atarax/UCB Pharma, Greece: inj. Sol. Iv 100 mg/2 ml) (50 mg/kg) n=18.
Group V: Sham flaps (non-ischemic) n=5.

All animals were kept to their cages for 7 days in the animal facilities of the institution under controlled temperature, humidity, and photoperiod conditions and were then euthanized under anesthesia. The study protocol was approved by the Research Ethics Committee of the National and Kapodistrian University of Athens and conducted under the supervision of the department of Veterinary and Animal Welfare Applications, in accordance with the Declaration of Helsinki.

The flaps were followed for 7 days at which time the rats were euthanized. After euthanasia 0.5×0.5 cm skin biopsies were taken from the middle of the distal third of the epigastric flap.

After fixation skin samples were evaluated for mast cell count and neutrophil count to determine neutrophil and mast cell sequestration in the flap. All biopsies were fixed in 10% neutral formalin, sectioned into 3-μm-thick slices and stained with toluidine blue for mast cell evaluation or hematoxylin and eosin for the basic pathological investigation and neutrophil evaluation. Mast cells and neutrophils were counted by an optical Carl Zeiss Standard light microscope at a × 40 magnification per 20 random high power fields (HPF) in a random, blinded fashion.

Estimation of necrotic/surviving area

After euthanasia the necrotic and surviving area of the flaps were assessed by a paper template placed on the abdominal area of the animal and traced by the experimenter after which the area was calculated by computer software (AutoCAD 2009 - AutoDesk, USA). The percentage of flap necrosis was expressed as the ratio of necrotic flap area at 7 days over whole flap area times 100.

Statistical considerations

Mean values for skin necrosis, mast cell counts and neutrophil counts in the flaps for each one of the pharmacologically treated groups and the sham group were compared using analysis of variance for multiple comparisons and the *post hoc* Dunnett's test. A p value < 0.05 was considered statistically significant. All the calculations were made using TexaSoft, WINKS SDA Software, 6th Edition (Cedar Hill, Texas, 2007).

Results

Flap viability/necrosis

The sham (non ischemic) group showed no necrosis. The control (saline treated) group, subjected to 10 h of ischemia, demonstrated a 75.1±15.3% (mean ±standard deviation) necrosis (Figure 2). Animals treated with cimetidine and hydroxyzine before reperfusion showed significantly less flap necrosis (25.8 ± 19.3% and 33.6 ± 27.8% respectively), than the saline-treated group ($p<0.05$) and the vitamin C-treated group ($p<0.05$). The protective effect of vitamin C was less (56.2 ±24.4%) compared with the animals in the control group, reaching nevertheless statistical significance ($p<0.05$). The difference between the cimetidine and hydroxyzine groups was not statistically significant (Figure 3).

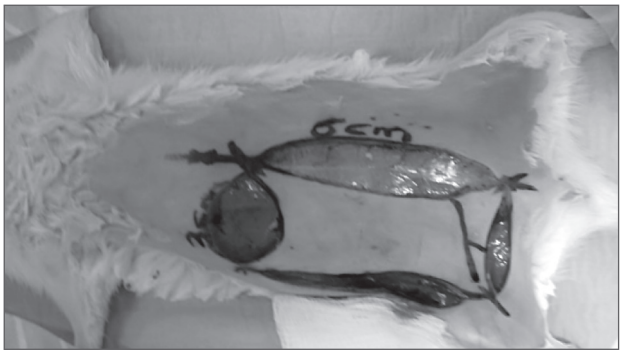

Figure 1. Flap incision.

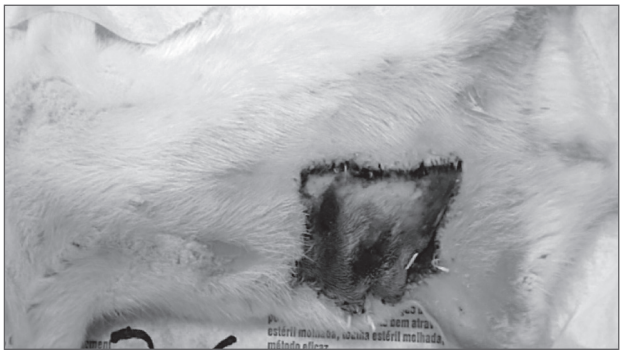

Figure 2. Seventh post-op day (Group I) showing flap necrosis.

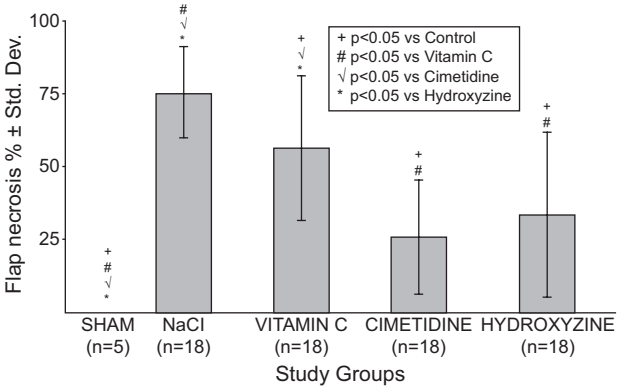

Figure 3. Percent skin flap necrosis at the end of the 7th day.

Neutrophil counts

Sham flaps had very low neutrophil counts (6.2±0.8 per 20 HPF) against all the other groups ($p<0.05$). The neutrophil counts of the control group were 23.1±5.8 per 20 HPF. Animals treated with vitamin C had less neutrophil counts (19.3±5.0 per 20 HPF), than the control group ($p<0.05$). Animals treated with cimetidine or hydroxyzine had significantly decreased neutrophil counts (15.5±4.1 per HPF and 14.2±4.3 per 20 HPF, respectively) compared with the control group ($p<0.05$) as well as with the vitamin C group ($p<0.05$). The difference between the cimetidine and hydroxyzine groups was not statistically significant (Figures 4,5).

Mast cell counts

Mast cell counts in the sham animals (16.2±1.3 per 20 HPF) were significantly lower than those of the other groups ($p<0.05$). They were elevated in the control animals (41.2 ± 5.7 per 20 HPF), and were decreased a little in animals treated with vitamin C (35.6 ± 6.1 per 20 HPF) compared with the control group ($p<0.05$). There was a more pronounced decrease in the groups treated with cimetidine (29.7±57 per 20 HPF) and hydroxyzine (27.4±5.8 per 20 HPF) compared with the control animals ($p<0.05$) as well as the vitamin C group ($p<0.05$). The difference between the cimetidine and hydroxyzine groups was not statistically significant (Figures 6,7).

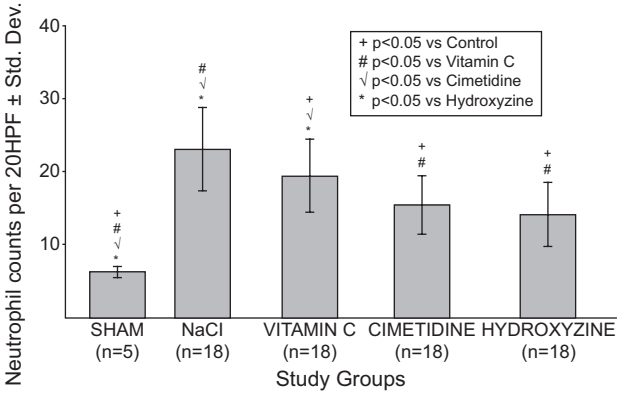

Figure 4. Flap neutrophil counts after 7 days.

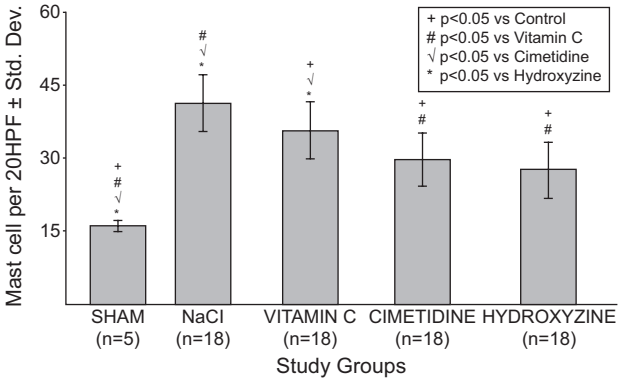

Figure 6. Mast cell counts after 7 days.

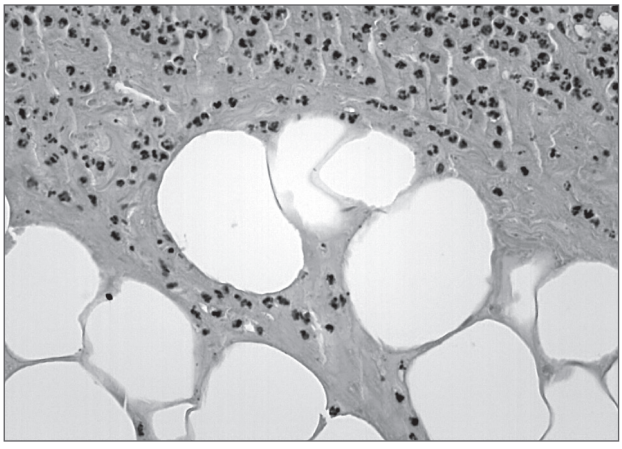

Figure 5. Histological section of subcutis showing extensive infiltration of leukocytes (Hematoxylin-Eosin ×200).

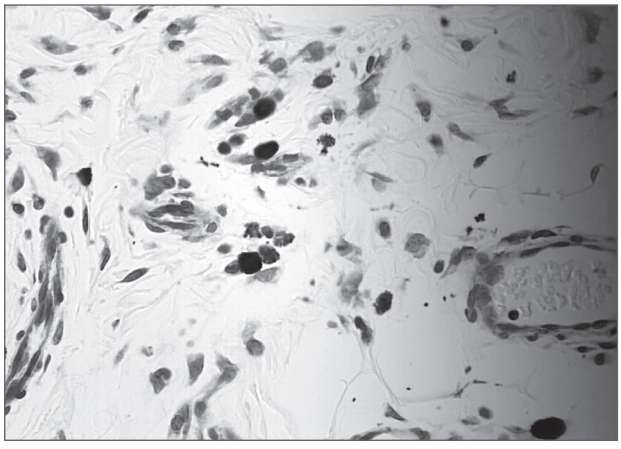

Figure 7. Histological section of subcutis showing extensive infiltration of mast cells (Toluidine blue ×200).

Discussion

After wide tumor removal follows the coverage of the deficit with autologous tissue, the circulation of which may be interrupted for a while.

Reperfusion injury is a phenomenon that occurs when tissue is subjected to ischemia for a variable period of time, after which it is reperfused [1]. Perfusion alone cannot reverse the damage already present in the flap as a result of ischemia, nor can it prevent further injury resulting from the introduction of oxygen into the ischemic flap when blood flow is re-established [10]. Many factors have been implicated in the cause of reperfusion injury, and free radicals, which occur after an ischemic event have enjoyed increasing popularity [3,4].

In our study, perfusion of ischemic flaps with a vitamin C solution significantly increased survival in an epigastric island flap model compared with flaps perfused with normal saline. In addition, the number of neutrophils and mast cells decreased compared with flaps perfused with normal saline. Although there are a few references concerning the use of vitamin C in ischemia reperfusion injury, we considered that our comparison among the substances we used would be more reliable if the experimental conditions were the same.

The therapeutic actions of vitamin C in reducing flap necrosis may be due to a combination of its antioxidant activity on the various free radicals which are produced during the reperfusion phase. Vitamin C terminates lipid peroxidation in the cell membrane and scavenges hydroxyl radicals as well as superoxide radicals which produce hydroxyl radicals [10]. Matsuba et al. demonstrated the salutary effects of high-dose vitamin C (14 mg/kg/h) in diminishing early post burn lipid peroxidation and reducing microvascular leakage of fluid and protein from the intravascular space to the interstitial space in mongrel dogs [11]. In addition to these findings some experimental and clinical studies have shown that vitamin C can prevent oxygen free radical production in ischemic conditions in various organs [12,13] but also in elderly patients [14].

Yet, in the current study the number of neutrophils was lower compared with the control group. This indicates that although reperfusion of the ischemic tissue worsens the cellular inflammation and interstitial edema, the accession of vitamin C may attenuate the whole process. However, ongoing studies have shown that the activation and migration of neutrophils to tissues is not a process independent from oxygen radical formation [15]. More importantly, the radicals and their products lead to the up-regulation of neutrophil adhesion molecules, which are capable of chemotaxis of the circulating neutrophils, activation of rolling, and adhesion molecules, and their stimulation to produce their own oxidants [13]. So the overall effect is that vitamin C, as free radical scavenger, can reduce the inflammation-mediated tissue injury and increase the tissue perfusion.

Turning our attention to histamine blocking substances, hydroxyzine is a potent histamine 1 (H1) receptor inverse agonist. This receptor, which is activated by the biogenic amine histamine, is expressed throughout the body, and is specific in smooth muscles, on vascular endothelial cells, in the heart, in the skin and in the central nervous system [16].

In our study, perfusion of ischemic flap with a hydroxyzine solution significantly increased survival in an epigastric island flap model compared with flaps perfused with normal saline. Yet, the numbers of neutrophils and mast cells were significantly decreased compared with flaps perfused with normal saline.

Cimetidine is also a histamine 2 (H2)-receptor antagonist. H2 receptors are found principally in the parietal cells of the gastric mucosa [17]. Of the currently recognized 4 subtypes of G protein-coupled histamine receptors, only the H1 and H2 subtypes have been positively identified in human skin [18,19].

In our study, perfusion of an ischemic flap with a cimetidine solution also increased significantly the survival in an epigastric island flap model compared with flaps perfused with normal saline. In addition, the number of neutrophils and mast cells were significantly decreased compared with flaps perfused with normal saline.

Our results clearly indicate that histamine may probably mediate a component of tissue injury because antihistamines (both H1 and H2 blockers) significantly decreased the amount of skin flap necrosis after 10 h of ischemia. Some studies have shown that mast cells are a major source of mediators of necrosis in ischemia reperfusion injury to skeletal muscle [20]. Moreover, the incubation of xanthine oxidase and hypoxanthine with rat peritoneal mast cells may result in the release of histamine [21]. Mannaioni et al. also supported the same hypothesis, the release of histamine by free radicals [2]. Furthermore Yamaki et al. showed that the recruitment of leukocytes to areas of inflammation is a multistep process in which the initiation of slow rolling leukocytes by histamine stimulation is a precondition for subsequent firm adhesion and extravasation into the surrounding tissues [22]. So neutrophil rolling is dependent on histamine, and the administration of H1 and H2 blockers will abolish the neutrophil-leucocyte rolling and therefore, inhibit leucocyte recruitment in venules. Consequently, according to the previous studies, a possible mechanism that could define our results is that antihistamines would block the effect of histamine released by mast cells. The decrease of the activity of the available histamine at the

capillary level would diminish neutrophil rolling and accumulation at the injury site. The extension of tissue damage would then be reduced, because neutrophils, the principle mediators of injury, would be decreased.

Our results demonstrated that antihistamines may reduce ischemia/reperfusion injury in rat skin flaps. Recently, some studies have shown the beneficial effect of cimetidine on the warm hepatic ischemia-reperfusion injury in rats [23]. Weimer et al. [24] showed that histamine activates H1 receptors in epithelial cells to release cytokines that are inflammatory chemotactic factors, a fact that results in the recruitment of more mast cells. It has also been shown that treatment with antihistamines can significantly reduce systemic mastocytosis and the release of inflammatory chemotactic factors from epithelial cells [25]. Our results are in accordance with these findings.

One more thing we can conclude from our study is that, although there is no significant difference between the cimetidine and hydroxyzine groups as far as necrosis, the neutrophils and mast cells are concerned, there is a more pronounced difference between these two groups and the vitamin C group in the percentage of flap viability, and the number of neutrophils and mast cells. Antihistamines appear to be more effective than the effect of vitamin C in protecting from ischemia reperfusion injury in a skin flap model.

The capability of successfully administering a free radical scavenger after ischemic injury may have promising clinical implications, like tissue reconstruction with free flaps after an oncological operation (e.g. mastectomy). It is particularly significant that inexpensive, safe, commonly used medications such as vitamin C, cimetidine and hydroxyzine may play an important role in the prevention of ischemia/reperfusion injury. Further studies seem warranted to explore the clinical use of such agents.

Conclusions

1. Hydroxyzine, cimetidine and vitamin C can significantly reduce skin flap necrosis in ischemia reperfusion injury in a rat epigastric flap model.

2. Hydroxyzine, cimetidine and vitamin C attenuate the number of neutrophils and mast cells seen between the skin flaps.

3. Hydroxyzine appears to be more effective than vitamin C in protecting from ischemia reperfusion injury in the skin flap model.

4. The difference between the hydroxyzine and cimetidine groups was not statistically significant.

5. The fact that simple every day agents have such effects gives them another potential use in tissue reconstruction after tumor excision.

References

1. Khalil, Aziz FA, Hall JC. Reperfusion Injury. Plast Reconstr Surg 2006; 117: 1024-1033.
2. Mannaioni PF, Giannella E, Palmerani B et al. Plenary lecture: Free radicals as endogenous histamine releasers. Agents Actions 1988; 23: 129-142.
3. Brisson BA, Miller CW, Chen G, McCutcheon LJ, Janzen EG. Detection of free radicals in ischemic and reperfused canine gracilis muscle flaps by use of spin-trapping electron paramagnetic resonance spectroscopy. Am J Vet Res 2001; 62: 384-348.
4. Büttemeyer R, Philipp AW, Mall JW, Ge B, Scheller FW, Lisdat F. In vivo measurement of oxygen-derived free radicals during reperfusion injury. Microsurgery 2002; 22: 108-113.
5. Siemionow M, Arslan E. Ischemia/reperfusion injury: A review in relation to free tissue transfers. Microsurgery 2004; 24: 468-475.
6. Scholz T, Evans G. Impact of Hypertonic and Hyperoncotic Saline Solutions on Ischemia-Reperfusion Injury in Free Flaps. Plast Reconstr Surg 2008; 122: 85-94.
7. Dorweiler B, Pruefer D, Andras TB et al. Ischemia-reperfusion injury: Pathophysiology and clinical implications. Eur J Trauma Emergency Surg 2007; 33: 600-612.
8. Girling S (Ed). Veterinary Nursing of Exotic Pets. Blackwell Publishing, 2003.
9. Petry JJ, Wortham KA. The anatomy of the epigastric flap in the experimental rat. Plast Reconstr Surg 1984; 74: 410-413.
10. Zaccaria A, Weinzweig N, Yoshitake M, Matsuda T, Cohen M. Vitamin C reduces ischemia-reperfusion injury in a rat epigastric island skin flap model. Ann Plast Surg 1994; 33: 620-623.
11. Matsuda T, Tanaka H, Hamumadass M et al. Effects of high dose vitamin C on post burn microvascular fluid and protein flux. J Burn Care Rehabil 1992; 12: 525-532.
12. Korkmaz A, Kolankaya D. The protective effects of ascorbic acid against renal ischemia-reperfusion injury in male rats. Ren Fail 2009; 31: 36-43.
13. Okazaki T, Otani H, Shimazu T, Yoshioka K, Fujita M, Iwasaka T. Ascorbic acid and N-acetyl cysteine prevent uncoupling of nitric oxide synthase and increase tolerance to ischemia/reperfusion injury in diabetic rat heart. Free Radic Res 2011; 45: 1173-1183.
14. Lee JY, Kim CJ, Chung MY. Effect of high-dose vitamin C on oxygen free radical production and myocardial enzyme after tourniquet ischaemia-reperfusion injury during bilateral total knee replacement. J Int Med Res 2010; 38: 1519-1529.
15. Molter GP, Silomon M, Bauer C, Ziegeler S, Soltesz S. Effects of hyperoncotic or hypertonic-hyperoncotic solutions on polymorphonuclear neutrophil count, elastase- and superoxide-anion production: A randomized controlled clinical trial in patients undergoing elective coronary artery bypass grafting. Acta Anaesthesiol Scand 2007; 51: 914-921.
16. Jones SR, Carpin KM, Woodward SM et al. Hyperbaric oxygen inhibits ischemia-reperfusion-induced neutrophil CD18 polarization by a nitric oxide mechanism. Plast Reconstr Surg 2010; 126: 403-411.
17. Parsons ME, Ganellin CR. Histamine and its receptors. Br J

Pharmacol 2006; 147 (Suppl 1): S127-135.
18. Lippert U, Artuc M, Grützkau A et al. Human skin mast cells express H2 and H4, but not H3 receptors. J Invest Dermatol 2004; 123: 116-123.
19. Grossmann M, Jamieson MJ, Kirch W. Histamine response and local cooling in the human skin: involvement of H1- and H2-receptors. Br J Clin Pharmacol 1998; 48: 216-222.
20. Lazarus B, Messina A, Barker JE et al. The role of mast cells in ischaemia-reperfusion injury in murine skeletal muscle. J Pathol 2000; 191: 443-448.
21. Boros M, Kaszaki J, Bakó L, Nagy S. Studies on the relationship between xanthine oxidase and histamine release during intestinal ischemia-reperfusion. Circ Shock 1992; 38: 108-114.
22. Yamaki K, Thorlacius H, Xie X, Lindbom L, Hedqvist P, Raud J. Characteristics of histamine-induced leukocyte rolling in the undisturbed microcirculation of the rat mesentery. Br J Pharmacol 1998; 123: 390-399.
23. Shaik IH, Mehvar R. Effects of cytochrome p450 inhibition by cimetidine on the warm hepatic ischemia-reperfusion injury in rats. J Surg Res 2010; 159: 680-688.
24. Weimer LK, Gamache DA, Yanni JM. Histamine-stimulated cytokine secretion from human conjunctival epithelial cells: Inhibition by the histamine H1 antagonist emedastine. Int Arch Allergy Immunol 1998; 115: 288-293.
25. Kurosawa M, Amano H, Kanbe N et al. Heterogeneity of mast cells in mastocytosis and inhibitory effect of ketotifen and ranitidine on indolent systemic mastocytosis. Allergy Clin Immunol 1997; 100: S25-32.