Protective Effect of Heparin Against Ischemia–Reperfusion Injury in Rat Free Skin Flaps

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ABSTRACT

Background: Ischemia–reperfusion (I/R) injury is a major cause of partial or total flap necrosis in reconstructive surgery. Neutrophil infiltration, oxidative stress, and apoptosis contribute to tissue damage, Heparin may reduce I/R-induced injury by improving microvascular perfusion and limiting.inflammation.

Objective: To evaluate the protective effect of heparin against I/R injury in rat epigastric skin flaps.

Methods: sixteen adult Wistar albino rats were randomly assigned into two groups control group and heparin group (n=8 each). The heparin group received a single intravenous bolus of heparin (150 U/kg) 15 minutes before reperfusion. Flap viability, neutrophil infiltration, lipid peroxidation (MDA), superoxide dismutase (SOD) activity, and caspase-3 activity were assessed 48 hours after reperfusion. Statistical analyses were performed using ANOVA and Tukey's post hoc test.

Results: Heparin significantly improved flap survival (75–83%, mean 80%) compared to the saline control group (2–17%, mean 9%). Neutrophil infiltration was significantly reduced (distal mean 122.8/HPF; central mean 46.1/HPF). Heparin treatment decreased MDA levels, preserved SOD activity, and lowered caspase-3 activity compared to untreated I/R flaps ($P<0.001$).

Conclusion: Heparin confers substantial protection against I/R injury in rat skin flaps by reducing inflammation, oxidative stress, and apoptosis. These findings suggest its potential use as a prophylactic agent in flap surgeries.

KEYWORDS: Ischemia–reperfusion injury, Skin flap, Heparin, Neutrophils, Oxidative stress, Apoptosis, Malondialdehyde, Superoxide dismutase.
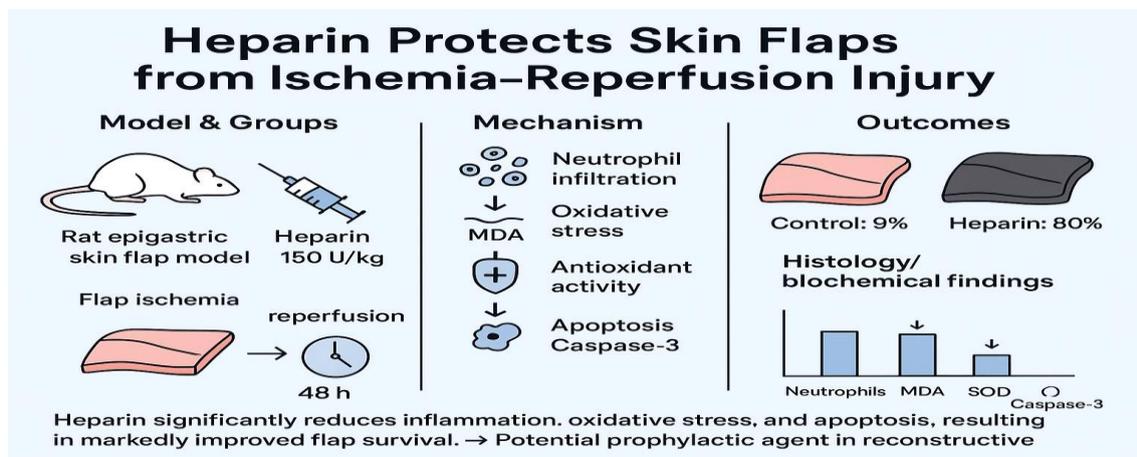
INTRODUCTION

Ischemia–reperfusion (I/R) injury represents a major challenge in reconstructive and plastic surgery, particularly in free and pedicled skin flaps. Temporary interruption of blood flow is often unavoidable during flap elevation and transfer, and the subsequent reperfusion paradoxically exacerbates tissue injury [1,2]. The pathogenesis of I/R injury is complex, involving oxidative stress, inflammatory cell infiltration, endothelial dysfunction, and apoptosis, all of which contribute to flap necrosis and surgical failure [3–5].

During ischemia, depletion of oxygen and nutrients leads to cellular energy deficits and accumulation of metabolic byproducts. Reperfusion, while restoring oxygen supply, triggers the generation of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, and hydroxyl radicals. These ROS attack lipids, proteins, and nucleic acids, causing membrane damage, enzyme inactivation, and DNA fragmentation [6,7]. Lipid peroxidation products such as malondialdehyde (MDA) are reliable biomarkers of oxidative stress and have been correlated with tissue injury severity in experimental models [8].

Neutrophils play a central role in mediating I/R injury. Reperfusion induces endothelial activation, which promotes neutrophil adhesion and migration into the ischemic tissue. Activated neutrophils release ROS and proteolytic enzymes, further damaging parenchymal and endothelial cells, and contributing to microvascular obstruction [9–11]. The resulting inflammatory cascade amplifies tissue injury, prolongs ischemia at the microvascular level, and triggers apoptotic pathways. Caspase-3, a key effector in apoptosis, is often upregulated in I/R injury and contributes to endothelial and parenchymal cell death [12].

Several pharmacological strategies have been investigated to attenuate I/R injury. Antioxidants such as vitamin C, vitamin E, and superoxide dismutase mimetics can neutralize ROS, while anti-inflammatory agents reduce neutrophil infiltration [13–15]. Anticoagulants, including heparin, are of particular interest because they not only prevent thrombosis in ischemic micro vessels but also exhibit anti-inflammatory properties by inhibiting leukocyte adhesion and reducing endothelial activation [16–18]. Heparin has demonstrated protective effects in myocardial, intestinal, and hepatic I/R models, reducing oxidative stress, neutrophil-mediated injury, and apoptosis [19–21].

Despite the promising data from other organ systems, the effects of heparin on skin flap survival after I/R injury remain underexplored. This study aimed to investigate the efficacy of heparin in protecting rat epigastric skin flaps from I/R-induced necrosis. We hypothesized that pre-reperfusion administration of heparin would enhance flap survival by mitigating oxidative stress, reducing neutrophil infiltration, and suppressing apoptosis. Understanding these mechanisms could have significant implications for clinical reconstructive surgery, particularly in high-risk patients with compromised microcirculation or prolonged ischemic times.

MATERIAL AND METHODS

Animal Model preparation

Sixteen adult Waster Albino rats of the same age (6 months), weighing between 250-300 g, were divided into two equal groups by a "simple random sampling method". The rats were housed in separate cages and fed with standard rat chow and water. The rats were anesthetized with intramuscular ketamine hydrochloride (65 mg/kg) plus xylazine hydrochloride (0.65 mg/kg), the abdomen was shaved by hair removal cream and prepared with povidone-iodine solution and placed on supine position.

Experiment design

The rates were divided into 2 groups:

Group I: Control group (n=8): 15 minutes before reperfusion, they were received intravenous 10 ml normal saline solution 0.9%.

Group II: Heparin (n=8): 15 minutes before reperfusion, they were received intravenous injection of a single bolus of heparin (150 units/kg body weight)

Surgical procedure

A flat template measuring 4x6 cm (24cm^2) was positioned over the skin of the abdomen and epigastric skin flap was marked **(fig.1).** The marked skin flap was elevated deep to the panniculus carnosus on the left abdominal region with vascularization and drainage coming from left superficial inferior epigastric vessels. The flap was subjected to eight hours of ischemia by clamping the inferior epigastric pedicle. The epigastric nerve was carefully dissected out and left intact to minimize auto cannibalization **(fig.1).** The flap was repositioned on its original bed and sutured with 4-0 monofilament continuous silk sutures, sparing the caudal border where simple stitches were used to make later removal of clamp easier. The rats were kept in cages with free access to water and food. Close to the end of the ischemia period, the rats were anesthetized again. Fifteen minutes before the release of the vascular clamp and reperfusion, the drugs were injected intravenously through the tail veins using a 30-gauge needle with syringe **(fig. 1).** The flaps in the sham group have not been subjected to ischemia and did not receive any drug injection.
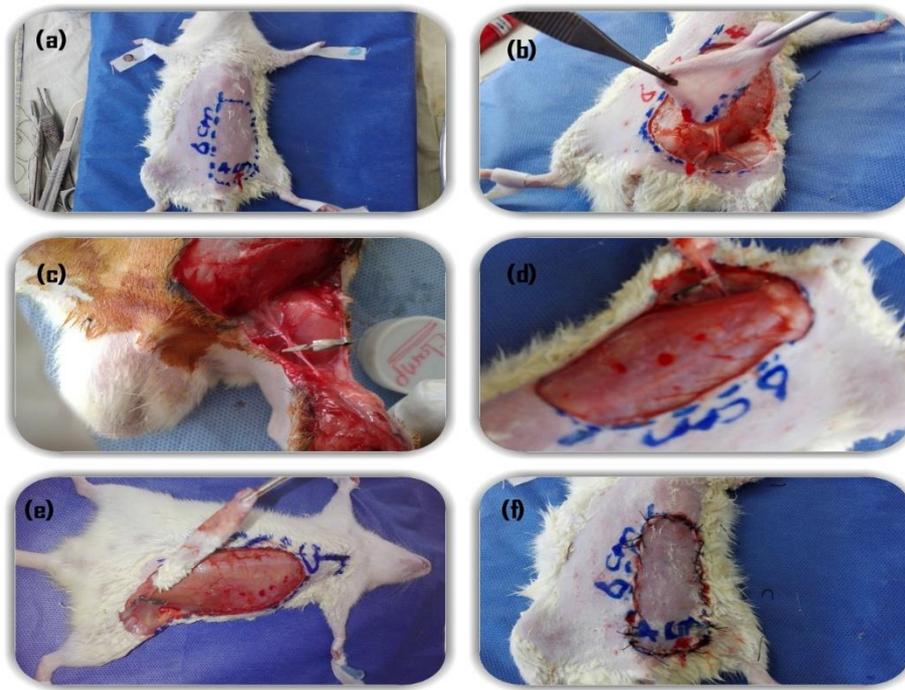
Fig.1: Steps of surgical procedure

A: Flap marking. B: Elevation of the flap based on inferior epigastric pedicle. C, D.E: Vascular clamping. F: skin suturing

Evaluation of the flap

Gross evaluation of flaps
After 48 hours of reperfusion, the animals were anesthetized and the percentages of flap viability versus area of skin necrosis were determined by *Sasaki's paper template method*.

Neutrophil count
Tissue samples were collected after 48 hours of reperfusion and fixed in 10% buffered formaldehyde, embedded in paraffin, and cut at 6 µm thickness. All microscopic section were stained with hematoxylin and eosin and examined immunohistochemically by LY-6G marker specific to neutrophils. Two skin specimens were taken from the flap measuring 1x1cm³, one specimen from the distal portion and the other from the central portion of the flap (3cm from the distal end). Neutrophil infiltration in the skin specimens was quantitatively evaluated by counting the neutrophils in non-overlapping high-power fields (x 400) using an image analyzer.

Tissue homogenates (Biochemical analysis):
Skin tissue was promptly excised randomly after decapitation, weighed, and rinsed in ice-cold isotonic saline (0.9% NaCl). After washing with 0.9% NaCl, tissue homogenates for Malondialdehyde enzyme (MDA) were minced and homogenized with a potter-Elvenhjem tissue homogenizer in a ratio of 1 g of wet tissue to 9 ml of ice-cold 1.15% KC1. However, (SOD) tissue homogenates were prepared in 5 ml cold 50 mm potassium phosphate buffer (pH 7.5, 1 mm EDTA) for each one-gram tissue. Then Centrifuge at 10,000 x g for 15 minutes at 4 °C, the supernatant was collected for assay and store on ice.

Malondialdehyde enzyme (MDA) assay
Malondialdehyde level in the tissue samples was measured with the thiobarbituric acid (TBA) test.The reaction mixture contained 0.1 ml of sample tissue homogenate, 0.2 ml of 8.1% sodium dodecyl sulfate (SDS), 1.5 ml of 20% acetic acid solution and 1.5 ml of 0.8% aqueous solution of TBA. The mixture pH was adjusted to 3.5 and final volume was made up to 4.0 ml with distilled water, and heated at 95°C for 60 min. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15: 1, v/v) were added, and the mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the absorbance of the upper layer was measured at 532 nm.

Superoxide dismutase enzyme (SOD) activity assay
Superoxide dismutase enzyme activity was measured by colorimetric method using commercial kit supplied by Bio-Diagnostic company (Egypt). This assay relied on the ability of the enzyme to inhibit the phenazine methosulphate mediated reaction of nitroblue tetrazolium dye. Purified SOD was shown to inhibit the initial rate of photo activated phenazine methosulphate mediated reaction of $O_2^{\cdot}$ to O_2 which then reduced nitroblue tetrazolium

Caspase-3 activity assay
It was detected Fluorometrically using Bivision Research Products Assay Kit (MountainView, CA, USA). 50 mg of tissue was homogenized in 2× reaction buffer and incubated at 37 °C with 1 mm caspase-3 substrate (DEVD-APC) for 1 h. Substrate

cleavage was measured with a spectrofluorometer at 400 nm.

Statistical Analysis

Continuous variables were presented as mean ± standard deviation and compared utilizing Student's t-test (for two independent groups). In contrast, categorical variables, such as puncture trial, the frequency of analgesia request, and PDPH, were presented as frequencies and percentages and compared between the two groups (utilizing the Chi-squared test). VAS was presented as interquartile ratio and median, as well as the Mann-Whitney U test comparisons, were made. The 26th version of the IBM SPSS software was utilized for the analysis. A p-value <0.05 was considered statistically significant.

RESULTS

Group I: Control group

The percentage of flaps survival ranged from 2% to 17% with a mean of 9% **(fig. 2, 3, 4).**

Fig. 2: Gross picture of saline group I showing severe necrosis involving most of the flap.

Fig. 3: Paper template of control group flaps showing the massive necrosis involving most of the flap.
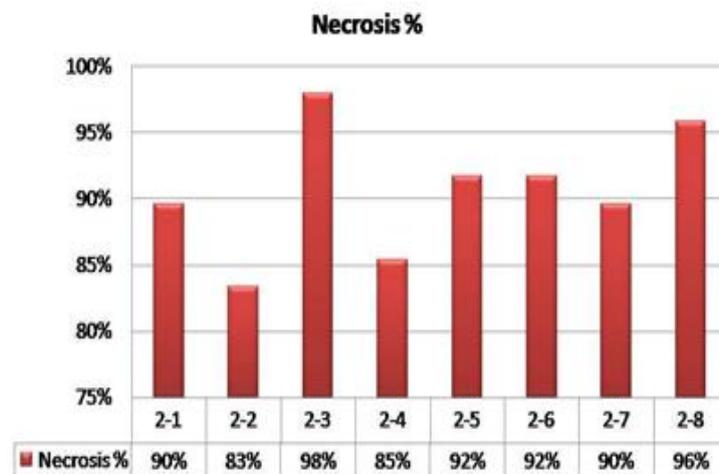

	2-1	2-2	2-3	2-4	2-5	2-6	2-7	2-8
Necrosis %	90%	83%	98%	85%	92%	92%	90%	96%

Fig. 4: Necrosis percentage per each flap area of control group I

Neutrophil count: It ranged from426/HPF to1050/HPF distally and from 289/HPF to 987/HPF centrally in the control group II with a mean of 816.50/HPF distally and 632.13/HPF centrally **(fig.5,6).**
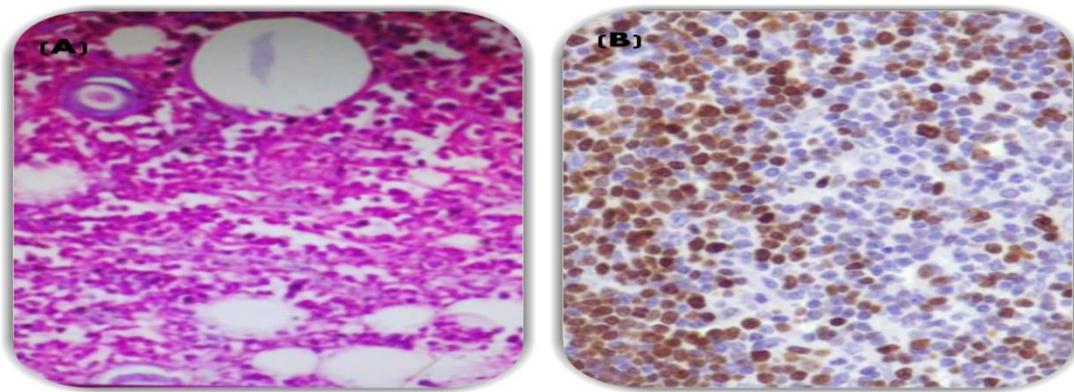
Fig. 5: Histological pictures of stained flap's specimens of control group I

A: severe neutrophil infiltrate is seen in the flaps of saline group (H&EX400).
B: severe neutrophil infiltrate is seen in the flaps of saline group
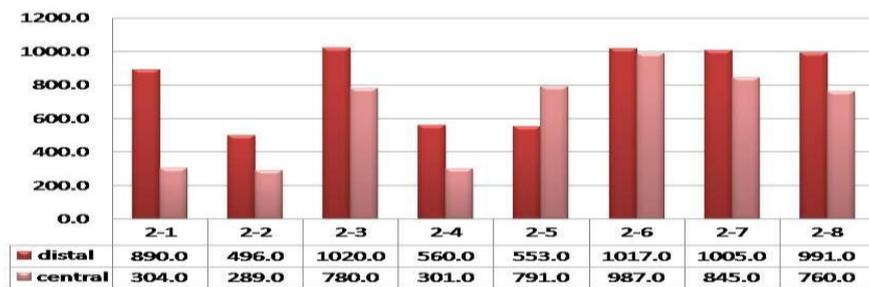 (ly6g markerX400).

	2-1	2-2	2-3	2-4	2-5	2-6	2-7	2-8
distal	890.0	496.0	1020.0	560.0	553.0	1017.0	1005.0	991.0
central	304.0	289.0	780.0	301.0	791.0	987.0	845.0	760.0

Fig. 6: Neutrophil count for both distal and central biopsies of each flap of control group and the relation between two biopsies.

Group II: Heparin
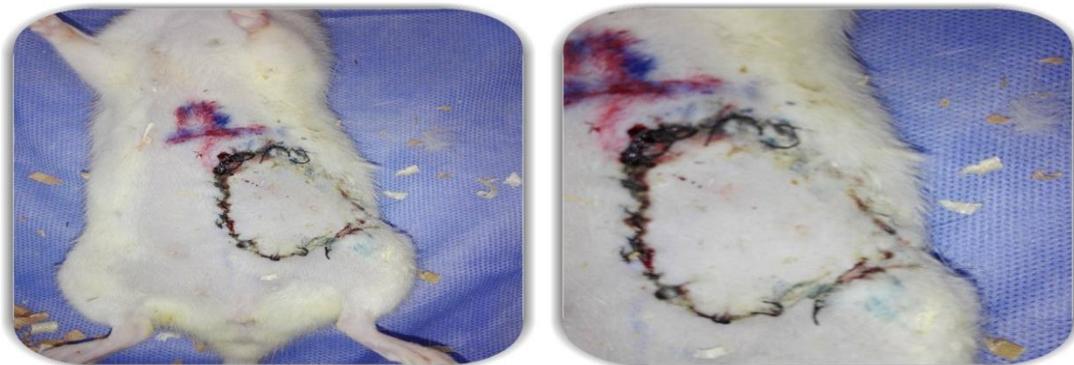The percentage ranged from 75%- 83% in the heparin group with a mean 80% **(Fig.7,8,9).**

Fig. 7: Gross picture of heparin group II showing minimal distal necrosis with considerable flap survival.

1   2   3   4   5   6   7   8

Fig. 8: Paper template of heparin group II flaps showing areas of flaps' necrosis relative to areas of survival.
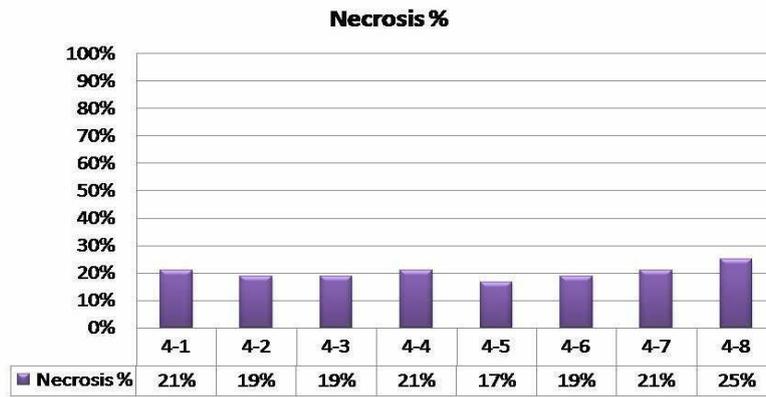
Fig. 9: Necrosis percentage per each flap area of heparin group II

Neutrophil count: It ranged from 103/HPF to150/ HPF distally and from 33/HPF to 63/HPF centrally with a mean 122.8/HPF distally and 46.1/HPF centrally **(fig.10, 11).**
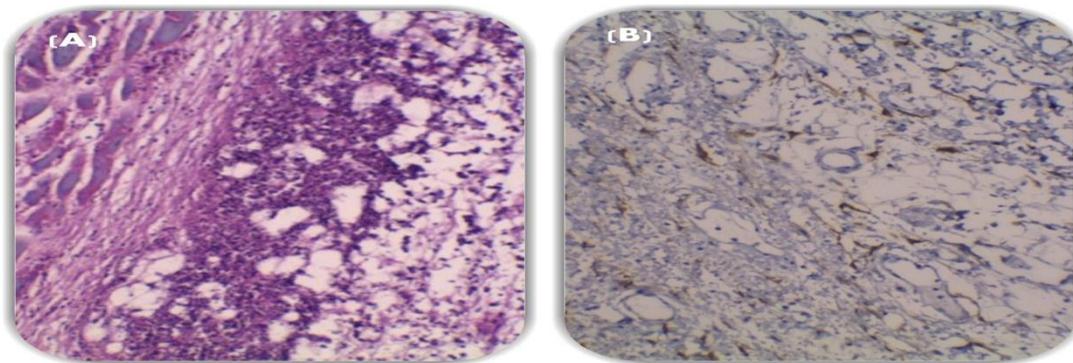

Fig. 10: Histological pictures of stained flaps of heparin group
A: Mild to moderate neutrophils infiltrate is seen in the flaps of heparin group (H&EX400)
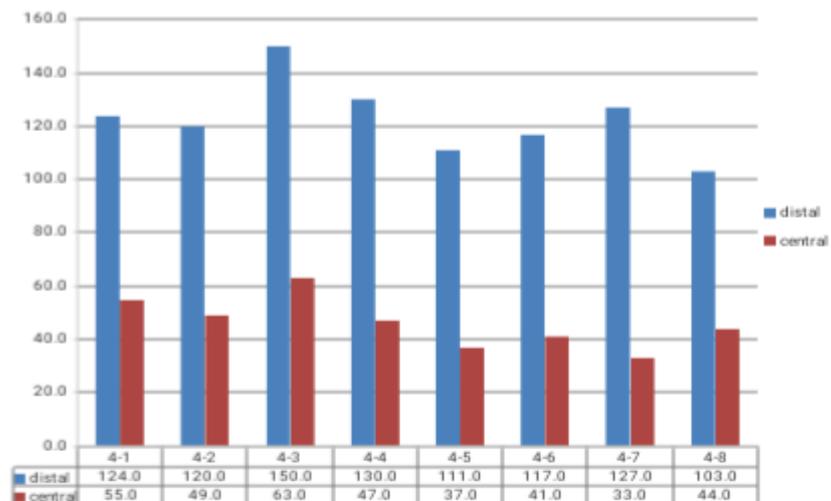B: Mild to moderate neutrophils infiltrate is seen in the flaps of heparin group (ly6g markerX400)

Fig. 11: Neutrophils count for both distal and central biopsies of each flap of heparin group and the relation between two biopsies

MDA

A comparison between the studied groups as regards the lipid peroxidation biomarker, MDA (nmol/g.wet tissue), there were statistically significant differences between of its values among all studied groups (F = 133.9, P< 0.001).

SOD Enzyme activity

A comparison between all studied groups as regards the enzymatic antioxidant biomarker, SOD Enzyme activity (U/ml), there were statistically significant differences among all the studied groups ($F = 19.76$, $P < 0.001$).

Caspase 3 activity

A comparison between the studied groups as regards the apoptotic marker, caspase 3 activity (U/mg.tissue), there were statistically significant differences between values of caspase 3 activity (U/mg.tissue) among all studied groups ($F = 99.04$, $P < 0.001$).

DISCUSSION

This study demonstrates that heparin significantly protects rat epigastric skin flaps against I/R injury. Flap survival in the heparin-treated group reached 75–83%, markedly higher than the saline-treated control group (2–17%), indicating a robust protective effect. The preservation of distal and central flap tissue highlights heparin's efficacy in maintaining microvascular perfusion even in regions most susceptible to ischemia.

Neutrophil infiltration was substantially reduced in the heparin group. Neutrophils are pivotal mediators of I/R injury, and their recruitment to ischemic tissue amplifies ROS production and enzymatic degradation of the extracellular matrix [9–11]. The observed reduction in neutrophil counts suggests that heparin attenuates the inflammatory cascade, likely through inhibition of endothelial activation and leukocyte adhesion. These findings are consistent with previous studies in myocardial and intestinal I/R models where heparin administration reduced neutrophil-mediated injury [14,18,22].

Oxidative stress analysis further supports heparin's protective role. MDA levels were significantly lower in heparin-treated flaps compared to controls, indicating reduced lipid peroxidation. Preservation of SOD activity demonstrates maintenance of endogenous antioxidant defenses, which is critical for neutralizing ROS during reperfusion [7,8,19]. These biochemical improvements correlate with improved histological outcomes and enhanced flap viability.

Caspase-3 activity, a marker of apoptosis, was significantly reduced in the heparin group, indicating decreased programmed cell death in both distal and central flap regions. Apoptosis is a key mechanism of cellular loss in I/R injury, and suppression of caspase-3 activity suggests that heparin can protect endothelial and parenchymal cells from ischemia-induced death [12,20]. Mechanistically, heparin appears to exert its protective effects via multiple pathways:

1. **Anticoagulant action**: prevents microthrombi formation and preserves perfusion.
2. **Anti-inflammatory effects**: reduces leukocyte adhesion and neutrophil-mediated damage.
3. **Antioxidant activity**: indirectly reduces ROS-induced lipid peroxidation.
4. **Anti-apoptotic effects**: inhibits caspase-3 activation and cellular apoptosis [16–21,23].

The findings have significant clinical implications. In reconstructive surgery, particularly in free or pedicled flaps with prolonged ischemic periods, pre-reperfusion heparin administration could improve flap survival and reduce postoperative complications. Additionally, heparin could be considered in combination with other pharmacological agents, such as antioxidants, to enhance tissue protection [13,15].

However, several limitations should be noted. This study evaluated a single bolus of heparin; dose-response relationships remain unclear. Only short-term outcomes (48 hours) were assessed, and long-term tissue remodeling or functional outcomes were not studied. Future studies should examine optimal dosing, timing, and combination therapies to maximize clinical utility.

In conclusion, heparin significantly mitigates I/R injury in rat skin flaps by reducing neutrophil infiltration, oxidative stress, and apoptosis. These findings provide a strong rationale for further translational research and potential clinical application in flap surgery, highlighting heparin as a promising pharmacological intervention for improving surgical outcomes.

Authors' contributions

All authors contributed to the conception, design, data analysis, and manuscript preparation. All authors read and approved the final manuscript.

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