ANKARA MEDICAL JOURNAL

Research Article

Ankara Med J, 2024;(1):84-95 // doi 10.5505/amj.2024.97455

EFFECTS OF PIRFENIDONE ON ISCHEMIA-REPERFUSION INJURY IN RAT EPIGASTRIC ISLAND FLAP MODEL: EXPERIMENTAL STUDY

Mehmet Sönmez[1], Canan Yılmaz[2], Nuran Sungu[3], Ebubekir Karakaş[4], İpek Allı[5]

[1]Ankara Yildirim Beyazit University, Department of Plastic, Reconstructive and Aesthetic Surgery, Ankara, Türkiye

[2]Gazi University, Department of Medical Biochemistry, Ankara, Türkiye

[3]Ankara Yildirim Beyazit University, Department of Medical Pathology, Ankara, Türkiye

[4]Ankara Bilkent City Hospital, Plastic, Reconstructive and Aesthetic Surgery Clinic, Ankara,Türkiye

[5] Health Sciences University Ankara Bilkent City Hospital, Department of Plastic,Reconstructive and Aesthetic Surgery, Ankara,Türkiye

Correspondence:
Mehmet Sönmez (e-mail: mehmet_snmz@hotmail.com)

Submitted: 22.01.2024 // Accepted: 27.02.2024

Ankara Yıldırım Beyazıt University Faculty of Medicine
Department of Family Medicine

ANKARA MEDICAL JOURNAL

Abstract

Objectives: Pirfenidone is a non-peptide synthetic low molecular weight substance with anti-inflammatory, antioxidant and antifibrotic effects. Its positive effects have been shown on ischemia-reperfusion injury in various tissues such as testis, kidney, liver, lung and small intestine. Our aim is to investigate the effects of Pirfenidone on ischemia-reperfusion injury in skin flaps.

Materials and Methods: Eighteen Wistar male rats were divided randomly into three groups: Sham, Ischemia-Reperfusion (IR) and Pirfenidone plus Ischemia-Reperfusion (IR+Pirf). The epigastric island flap was elevated and returned to its place in the Sham group. In the second group (IR), the flap was elevated, and flap perfusion was interrupted with an avascular clamp for eight hours. In the third group (IR+Pirf), 300 mg/kg Pirfenidone was given orally before ischemia. Tissue samples were taken to evaluate biochemical substances (1st day) and histopathologic examination (7th day). On the seventh day, standardized photographs were taken to calculate the viable areas of the flap and all animals were sacrificed.

Results: Tissue MPO levels were statistically higher in the IR+Pirf group than in the Sham and IR groups ($p=0.006$). Tissue MDA levels were statistically higher in the IR+Pirf group than in the IR group ($p=0.026$). The lymphocyte count was lower in the Sham group than in the IR and IR+Pirf groups ($p=0.002$). The reepithelization ratio was higher in the IR+Pirf group than in the IR and Sham groups $p=0.010$). Flap survival areas showed no significant difference between groups ($p=0.194$).

Conclusion: As a conclusion, single dose treatment of Pirfenidone in rat skin flap ischemia-reperfusion model enhanced significantly re-epithelization and has no significant effect on flap survival.

Keywords: Pirfenidone, epigastric, island flap, ischemia-reperfusion.

Introduction

The aim of reconstructive surgery is to eliminate tissue deficiencies caused by various reasons and to restore form and function together. For this purpose, tissue pieces called flaps are frequently used. In free flap operations, the flap remains ischemic during the transfer of the flap taken from the donor site to the recipient area. If this ischemia period is prolonged above a critical level, tissue damage continues to increase even if reperfusion develops. This phenomenon is called ischemia-reperfusion injury, which is one of the most important limitations that increase morbidity in flap surgery.[1]

Prolongation of ischemia causes the accumulation of hypoxanthine in the tissue, resulting in the formation of superoxide radicals and an increase in tissue damage. Additionally, endothelial damage causes activation of the inflammatory process and migration of neutrophils to the region. In this context, scientific studies mainly focused on either increasing antioxidant activity or suppressing the inflammatory process.[2]

Pirfenidone is a non-peptide synthetic low molecular weight substance with anti-inflammatory, antioxidant and antifibrotic effects.[3,4] Its antioxidant property is supported by the continuation of nitric oxide production, which plays an active role in the fight against free radicals.[3] Based on the above-mentioned effects of pirfenidone, its effects on ischemia-reperfusion injury in tissues such as testis, kidney, liver, lung and small intestine have been investigated in the literature, and it has been shown to have positive effects.[1,3,5-7]

There was no literature study about the effects of pirfenidone on the skin flap ischemia–reperfusion model. Therefore, our aim is to investigate the effects of Pirfenidone on the inflammatory response and oxidative tissue damage in ischemia-reperfusion injury.

Materials and Methods

All animals have received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals," and ethical consent was obtained from Gazi University Laboratory Animal Local Ethic Committee (Date:28/12/2023, Approval Number: E-66332047-604.01.02-546170). The epigastric island flap is a flap with a fixed anatomy in rats; the main vascular pedicle enters from the inguinal region as a superficial epigastric artery and vein. It can be obtained from both sides of the midline based on a single vascular pedicle. Therefore, we planned to elevate our flap with a size of 6x3 cm, with a single vascular pedicle, crossing the midline only up to random pattern feeding.[8] (Figure 1)

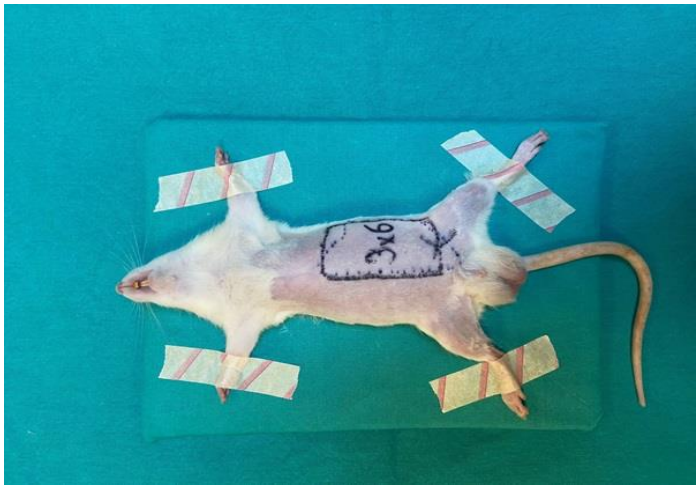

Figure 1. Flap planning.

Eighteen Wistar male rats were divided randomly into three groups. In the first group (Sham group), the epigastric island flap was elevated and returned to its place without any ischemia-reperfusion injury. The reason for the creation of this group was that surgical stress could also affect flap circulation.[9] In the second group (IR), surgery and ischemia-reperfusion injury were planned. In order to create the same stress in rats in this group, an equal volume of 0.5% carboxymethylcellulose solution that does not contain Pirfenidone was given by oral gavage before the surgical procedure. Then, the flap was elevated, and the flap circulation was interrupted for 8 hours with a microvascular atraumatic clamp (Figure 2).

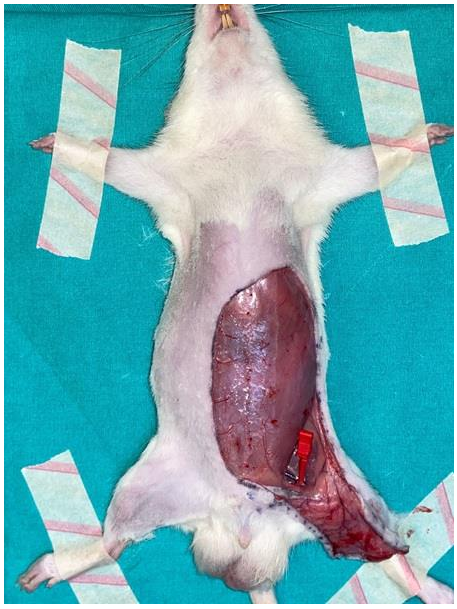

Figure 2. The atraumatic microvascular clamp was applied to the main vascular pedicle.

The flap was returned to its place; the rats were awakened. When the 8-hour ischemia period was completed, the clamp was removed with forceps under mild anesthesia, the circulation was checked, and if necessary, the area was repaired with suturing. In the third group (IR+Pirf), surgery and ischemia-reperfusion injury were planned.

Pirfenidone (supplied by Apex Bio, Houston, TX, USA) was administered orally to this group at a dose of 300 mg/kg by dissolving it fresh in 1 ml of 0.5% carboxymethylcellulose at the beginning of the experiment.[6]

All animals were monitored for seven days. On the 1st day of the experiment, 1 cm standardized tissue samples were taken from the distal part of the flap for biochemical analysis under short-term anesthesia from all groups.[1] On the 7th day, standardized photographs were taken to calculate the viable areas of the flap and tissue samples were taken for histopathological tests.[9,10] Flap survival areas were calculated with Image J software (National Institutes of Health and the Laboratory for Optical and Computational Instrumentation (LOCI), University of Wisconsin, USA). Following the completion of these procedures, the experimental animals were sacrificed by giving a high dose of anesthetic (150 mg/kg i.p. route) to stop the cardiac blood flow.[9]

Biochemical analysis

Malondialdehyde (MDA) levels (indicators of ischemic tissue damage), myeloperoxidase (MPO) activity (correlates with the amount of neutrophil migration), glutathione peroxidase (GPx) and superoxide dismutase (SOD) enzyme activity levels (indicators of tissue antioxidant capacity) were investigated.[9,11-15]

Tissue MDA levels were determined using the method described by Ohkawa et al. The principle of this method is based on the spectrophotometric measurement of the red color due to the complex formed by the MDA in the sample with Thio barbituric acid (TBA) at an ambient pH of 3.5 after the proteins in the homogenate are bound with sodium dodecyl sulfate (SDS).[11] Tissue MPO activity is based on the reduction of O-dianisidine by oxidation of H2O2 by tissue homogenate and measurement of reduced O-dianisidine at 410 nm.[12] GPx activity was determined by a modification of the double enzyme method of Paglia and Valentina. In this method, the rate of GSSG formation is determined at a wavelength of 340 nm by the decrease in the optical density of the mixture as a result of the conversion of NADPH to NADP.[13] A colorimetric commercial kit was provided for measurement. SOD activities are based on the principle defined by Yi-Sun, that xanthine forms $O_2^{\cdot-}$ with xanthine oxidase, and this creates a colored compound with NBT, and this color intensity is measured spectrophotometrically. The higher the SOD activity in the environment, the less intense the color will be, as it will remove $O_2^{\cdot}$.[14] A colorimetric commercial kit was provided for measurement. Tissue protein levels were studied with the Lowry method, and MDA, MPO, SOD and GPx results were given per mg protein.[15] The biochemistry specialist was blinded to group information.

Histopathological analysis

Tissue samples taken from the distal flap on the seventh day were subjected to sectioning after routine procedures and after they were stored in Formol. Inflammation (absent/mild/moderate/severe), re-epithelization (absent/<50%/>50%/keratin), collagen orientation (absent/vertical/mixt/horizontal), fibroblast (absent/mild/moderate/severe), neovascularization (absent/weak/moderate/marked), collagen (absent/mild/moderate/severe) and necrosis (absent/epidermal/epidermal and dermal/ dermal loss) were examined and scored in tissue sections with hematoxylin-eosin staining under light microscope and these parameters were compared between groups.[16] The histopathologist was blinded to group information.

Statistical Analysis

All data were expressed as mean ± standard deviation. SPSS version 26 programs were used in the analysis of the data. The $p<0.05$ value was considered statistically significant. After the normality check (Kolmogorov-Smirnov), 'One way ANOVA' or 'Kruskal Wallis' tests and post-hoc analysis with Bonferroni correction were used to detect statistically significant differences between the groups.

Results

Biochemical Results

Statistical analysis of biochemical substances showed a significant difference in tissue MPO levels and tissue MDA levels between groups. According to the post hoc test, tissue MPO levels were statistically higher in the IR+Pirf group than in the Sham and IR groups ($p=0.006$).

Tissue MDA levels were statistically higher in the IR+Pirf group than the IR group ($p=0.026$) but statistically the same in the Sham group. Although the mean GPx level was higher in the IR+Pirf group compared to other groups, this difference was not statistically significant. Table 1 summarizes these results (Table 1).

Histopathologic Examination

Lymphocyte and reepithelization scores showed a statistically significant difference between groups. The lymphocyte count was lower in the Sham group than in the IR and IR+Pirf groups ($p=0.002$). Additionally, the reepithelization ratio was higher in the IR+Pirf group than in the IR and Sham groups ($p=0.010$). Polymorphonuclear leukocyte and fibroblast count, collagen orientation, collagen count, necrosis and neovascularization did not show a significant difference between groups. Table 2 summarizes statistically significant; Table 3 summarizes statistically non-significant differences.

Table 1. Tissue biochemical substance statistical values.

	Groups	Mean±SD	Min-max	P value	Post-hoc p values
MPO (U/mg protein)	1	0.48±0.13	0.38-0.73	**0.003**	1-2:1.00
	2	0.48±0.34	0.11-0.91		**1-3:0.006**
	3	1.29±0.54	0.6-1.77		**2-3:0.006**
SOD (U/mg protein)	1	67.42±16.55	48.46-97.01	0.76	-
	2	60.7±23.52	26.92-96.4		
	3	60.9±11.93	46.97-77.15		
GPx (U/mg protein)	1	89.56±36.34	55.75-138.56		
	2	87.73±22.47	55.9-117.03		
	3	102.97±30.82	67.88-149.49		
MDA (nmol/mg protein)	1	11.94±3.02	7.98-15.18	**0.027**	1-2:0.913
	2	9.63±2.55	6.41-12.95		1-3:0.212
	3	16.16±5.17	11-21.12		**2-3:0.026**

P<0.05 is considered significant. (Group 1= Sham, Group 2=IR, Group 3= IR+Pirf)

Table 2. Lymphocyte and reepithelization scores.

	Groups	Mode, Min-max	P value	Post-hoc p values
Lymphocyte	1	0.0, 0.0-0.0	**.002**	**1-2:0.023**
	2	1.0, 0.0-2.0		**1-3:0.001**
	3	1.0, 1.0-3.0		2-3:0.25
Reepithelization	1	0.0, 0.0-3.0	**.010**	1-2:0.815
	2	0.0, 0.0-3.0		**1-3:0.012**
	3	3.0, 3.0-3.0		**2-3:0.006**

P <0.05 is considered significant. (Group 1=Sham, Group 2=IR, Group 3=IR+Pirf)

Table 3. Statistical results of other histopathologic criteria.

	P value
Polymorphonuclear leukocyte	0.066
Collagen Orientation	0.317
Collagen	0.191
Fibroblast	0.733
Necrosis	0.065
Neovascularization	0.138

P<0.05 is considered significant.

Epidermal necrosis, dermal inflammation and unorganized collagen fibers were seen in Sham group rats (Figure 3).

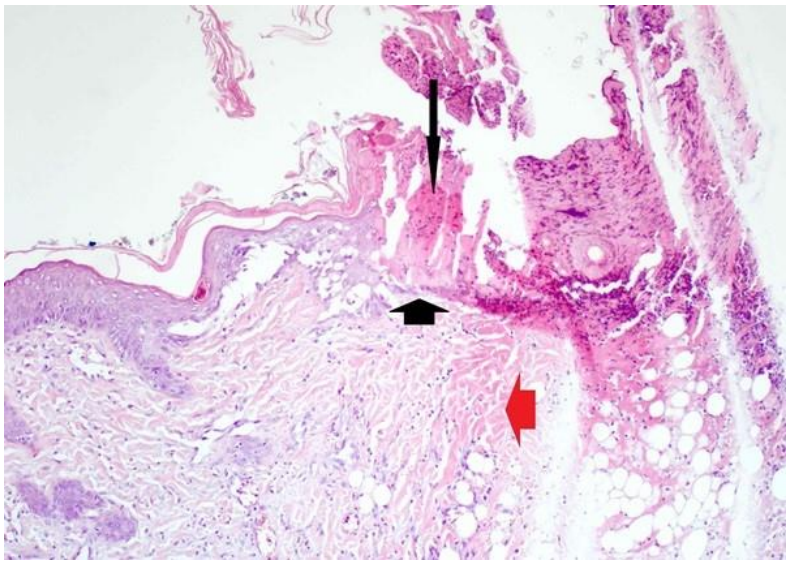

Figure 3. Sham group rat 7th-day histopathologic view (Hematoxylin-eosin staining, x 100 magnification, long black arrow: epidermal loss, short black arrow: slight epithelization, short red arrow: inflammation and unorganized collagen fibers).

Marked granulation tissue was seen in IR group rats (Figure 4).

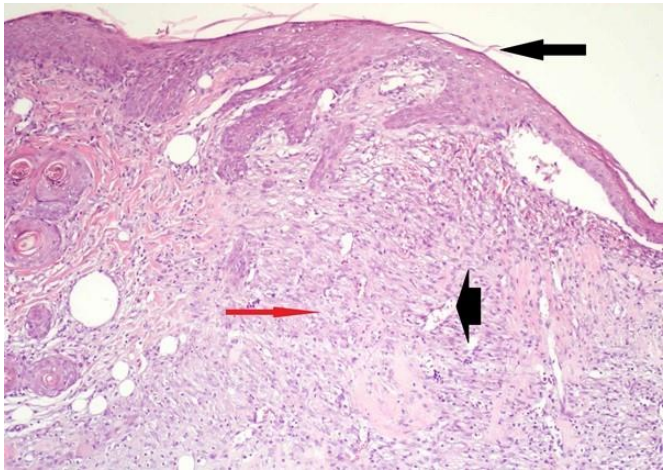

Figure 4. IR group rat 7th-day histopathologic view (Hematoxylin-eosin staining, x 100 magnification. Long black arrow: keratinized epidermis, short black arrow: vascular proliferation in the dermis, long red arrow: fibroblast proliferation).

On the other hand, keratinized epidermis and organized collagen fibers were seen in IR+Pirf group rats (Figure 5).

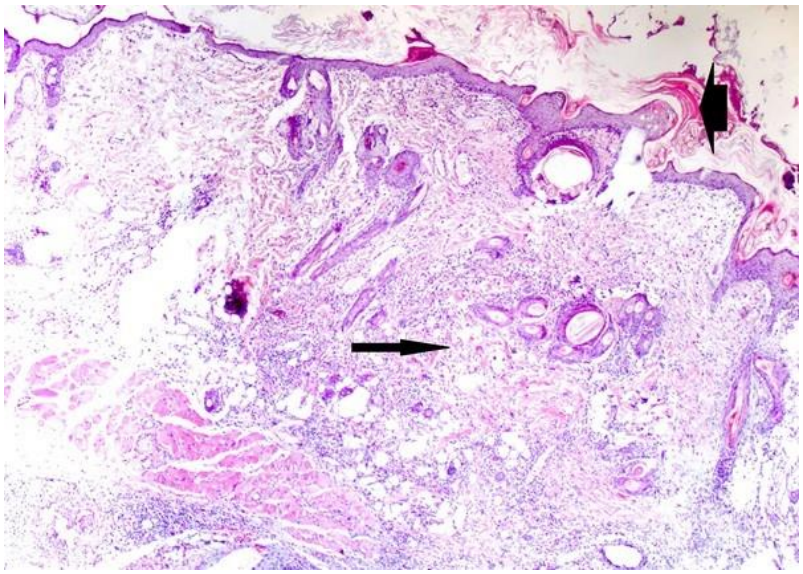

Figure 5. IR+Pirf group rat 7th-day histopathologic view (Hematoxylin-eosin staining, x 100 magnification. Long black arrow: organized collagen fibers, no granulation, continued inflammation, short black arrow: keratinized epidermis).

Flap Survival Ratios

Statistical analysis of flap survival areas showed no significant difference between groups (p=0.194). However, IR+Pirf group flaps survived completely. Table 4 summarizes these results.

Table 4. Flap survival results.

	Group	Mean±SD	Min-Max	P value
Flap Survival Ratio (%)	1	82.06±27.53	33.10-100.00	**0.194**
	2	75.41±41.54	0.00-100.00	
	3	100.00±0.00	100.00-100.00	

P<0.05 is considered significant. (Group 1=Sham, Group 2=IR, Group 3=IR+Pirf)

Discussion

We investigated the effects of Pirfenidone on rat epigastric island flap in an ischemia-reperfusion injury model. Our findings showed that the IR+Pirf group had higher MDA and MPO levels, higher reepithelization ratio and higher lymphocyte count. Although Pirfenidone group rats had no flap necrosis, this result was not statistically significant.

Many studies investigated the effects of pirfenidone on experimental ischemia-reperfusion models in different tissues. Kolukcu et al. investigated the effects of Pirfenidone on ischemia-reperfusion injury in testicular torsion-induced rat models.[1] They administered Pirfenidone 325 mg/kg via oral route immediately after ischemia and analyzed the biochemical levels in blood samples that were taken from the inferior vena cava. They found that SOD and GPx levels were higher, and MDA levels were lower in the Pirfenidone group than in the control group. Our results were not correlated with these results; we think that this can be caused by the sample obtaining difference or acting mechanism. They obtained vena cava blood for biochemical analysis; we obtained tissue specimens. We think that our results indicate the local effects of Pirfenidone in the skin flap. For the acting mechanism, one study pointed out that Pirfenidone alleviates the recovery of nitric oxide production by endothelial nitric oxide synthase; this effect could explain the protective effect in ischemic acute kidney injury in rats.[3] In addition, this study argued that Pirfenidone could have antioxidant activity that is responsible for its own chemical structure. Accordingly, they found liver tissue SOD and GPx levels were the same in IR and IR+Pirf groups. Thus, Pirfenidone could modulate ischemia-reperfusion injury in the skin flap via different mechanisms in our study.

Some studies focused on the anti-inflammatory effect of Pirfenidone in different animal models. Kaibori et al. investigated endotoxin-induced liver injury after hepatic ischemia in rats and found that the number of neutrophile counts was significantly lower in orally single-dose Pirfenidone (300mg/kg) treated rats.[5] Saito et al. carried out a study on the lung ischemia-reperfusion model in rats and found that a single dose of Pirfenidone (300 mg/kg) was effective in declining neutrophile infiltration significantly at two hours after reperfusion.[6] On the other hand, Arumugam et al. found that Pirfenidone treatment was ineffective in preventing circulating neutrophile count fall in rat small intestine ischemia-reperfusion model.[7] We did not see any anti-inflammatory effect of Pirfenidone on the 7th day. This could be caused by single-dose Pirfenidone treatment. Repeated doses can be effective in augmenting the long-standing anti-inflammatory effect of Pirfenidone.

In our study, we found that the re-epithelization rate and lymphocyte count were significantly higher in the IR+Pirf group. In addition, histopathologic slices showed organized collagen fibers and no granulation.

Accordingly, Mecott et al. conducted a pilot study in patients with second-degree burns, and they found that Pirfenidone treatment caused a decrease in wound healing time by enhancing wound re-epithelization.[17]

Our study has some limitations. First, a relatively small sample size could be obtained because of ethical issues. Second, single-dose treatment was planned. Different results can be obtained with a study with a larger sample size and repeated Pirfenidone doses.

In conclusion, single-dose treatment of Pirfenidone in rat skin flap ischemia-reperfusion model:

- Significantly enhanced re-epithelization
- Improves skin flap survival. However, this effect was not significant,
- Has no significant effect on inflammatory infiltration, collagen count/organization and fibroblast count on the 7th day.

Ethical Considerations: All animals have received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals," and ethical consent was obtained from Gazi University Laboratory Animal Local Ethic Committee (Date:28/12/2023, Approval Number: E-66332047-604.01.02-546170).

Conflict of Interest: The authors declare no conflict of interest.

References

1. Kölükçü E, Firat F, Deresoy FA, Katar M, Atılgan D. The effects of pirfenidone on ischaemia-reperfusion injury in testicular torsion-induced rat model. Andrologia. 2021 Mar;53(2):e13922 (doi: 10.1111/and.13922).
2. Percival TJ, Rasmussen TE. Reperfusion strategies in the management of extremity vascular injury with ischaemia. Br J Surg. 2012;99 Suppl 1:66-74 (doi: 10.1002/bjs.7790).
3. Lima-Posada I, Fontana F, Pérez-Villalva R, Berman-Parks N, Bobadilla NA. Pirfenidone prevents acute kidney injury in the rat. BMC Nephrol. 2019; ;20(1):158 (doi: 10.1186/s12882-019-1364-4).
4. Ji X, Naito Y, Weng H, Ma X, Endo K, Kito N et al. Renoprotective mechanisms of pirfenidone in hypertension-induced renal injury: through anti-fibrotic and anti-oxidative stress pathways. Biomed Res. 2013;34(6):309-19 (doi: 10.2220/biomedres.34.309).
5. Kaibori M, Yanagida H, Uchida Y, Yokoigawa N, Kwon AH, Okumura T et al. Pirfenidone protects endotoxin-induced liver injury after hepatic ischemia in rats. Transplant Proc. 2004;36(7):1973-4 (doi: 10.1016/j.transproceed.2004.08.061).
6. Saito M, Chen-Yoshikawa TF, Suetsugu K, Okabe R, Takahagi A, Masuda S, Date H. Pirfenidone alleviates lung ischemia-reperfusion injury in a rat model. J Thorac Cardiovasc Surg. 2019;158(1):289-96 (doi: 10.1016/j.jtcvs.2018.08.098).
7. Arumugam TV, Shiels IA, Margolin SB, Taylor SM, Brown L. Pirfenidone attenuates ischaemia-reperfusion injury in the rat small intestine. Clin Exp Pharmacol Physiol. 2002;29(11):996-1000 (doi: 10.1046/j.1440-1681.2002.03766.x).
8. Bayramicli M. Deneysel Mikrocerrahi Temel Arastirma, Doku ve Organ Nakil Modelleri. In: Bayramicli M, ed. *Karin Bolgesi Flepleri.* 1st ed. Istanbul: Argos; 2005:465-83
9. Kilic F, Eskitascioglu T, Aydin A, Cakici OU. Ameliorating Effects of β-Glucan on Epigastric Artery Island Flap Ischemia-Reperfusion Injury. J Surg Res. 2021;261:282-92 (doi: 10.1016/j.jss.2020).
10. Lee YK, Nata'atmaja BS, Kim BH, Pak CS, Heo CY. Protective effect of telomerase-based 16-mer peptide vaccine (GV1001) on inferior epigastric island skin flap survivability in ischaemia-reperfusion injury rat model. J Plast Surg Hand Surg. 2017;51(3):210-6 (doi: 10.1080/2000656X.2016.1235046).
11. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979;95(2):351-8 (doi: 10.1016/0003-2697(79)90738-3).
12. Zhang T, Zhang X, Shao Z, Ding R, Yang S, Ruan J et al. The prophylactic and therapeutic effects of cholinolytics on perfluoroisobutylene inhalation induced acute lung injury. J Occup Health. 2005;47(4):277-85 (doi: 10.1539/joh.47.277).
13. Paglia DE, Valentina WN. Studies on quantitative and qualitative characterization of erytrocte glutathione peroxidase. J. Lab. Clin. Med. 1967;70:158-69.

14. Yi-Sun S, Oberly LW, Li Y. A simple method for clinical assay of superoxide dismutase, Clin. Chem. 1988;34:497-500.
15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ Protein measurements with the folin phenol reagent, J. Biol. Chem. 1951;193:265-75.
16. Gal P, Kilik R, Mokry M, Vidinsky B, Vasilenko T, Mozes S et al. Simple method of open skin wound healing model in corticosteroid-treated and diabetic rats: standardization of semi-quantitative and quantitative histological assessments. Vet Med. 2008;53(12):652-9.
17. Mecott GA, González-Cantú I, Dorsey-Treviño EG, Matta-Yee-Chig D, Saucedo-Cárdenas O, Montes de Oca-Luna R, et al. Efficacy and Safety of Pirfenidone in Patients with Second-Degree Burns: A Proof-of-Concept Randomized Controlled Trial. Adv Skin Wound Care. 2020 Apr;33(4):1-7 (doi: 10.1097/01.ASW.0000655484.95155.f7).