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THE EFFECT OF NECROSTATIN -1 AND ENOXAPARIN MOLECULES ON RANDOM PATTERN FLAP VIABILITY

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Abstract: Distal flap necrosis is seen more often in random pattern flaps and is an important complication that shortens the flap length. There has been much research many drugs and molecules in an effort to prevent this complication. The aim of this study was to investigate the efficacy of necrostatin-1 and enoxaparin molecules in preventing distal flap necrosis and increasing flap viability in a random pattern flap model created in rats. A total of 32 Wistar albino female rats, each weighing 300-350 gr were separated into 4 groups. All the animals underwent an operation to create a 3×9 cm caudal-based Mcfarlane flap. The treatments defined for each group were applied. Full layer tisssue samples 1×1 cm^2 were taken from all the flaps and stored until histopathological and immunohistochemical examination, the parameters of inflammation, capillary proliferation, necrosis, fibroblast proliferation and fibrosis were compared histopathologically. In the necrostatin-1 group, the inflammation, necrosis and fibrosis scores were observed to be lower and the capillary proliferation and fibroblast proliferation scores were higher. In the enoxaparin group, the fibroblast proliferation and capillary proliferation scores were higher. The receptor interacting protein kinase-1 immunohistochemical staining results showed statistically significantly less staining in the necrostatin-1 group compared to the other groups. The results of this study suggest that necrostatin molecule has important therapeutic potential in increasing flap viability in the random pattern flap model, considering the percentage of flap necrosis, and the immunohistochemical and histopathological data. The flap necrosis percentage and histochemical parameters of the enoxaparin molecule demonstrate that the effects on flap viability are limited.

Keywords: Necrostatin-1, Enoxaparin, Rat, Flap necrosis, Flap viability

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1. Introduction

Tissue loss occurring after trauma or oncology surgery is a significant medical problem which results in severe morbidity and mortality (Gottlieb and Krieger, 1994). A flap can be basically defined as a piece of tissue transferred from one region to another while protecting the vascular structure (Janis, 2014). Flaps are classified in two groups according to the vascular structure, as axial pattern or random pattern flaps. As the application of random pattern flaps is simple, they are often used in reconstructive surgery for the repair of tissue defects (McGregor and Morgan, 1973). The most significant problem encountered after flap surgery is flap necrosis and partial or full flap loss. Distal flap necrosis seen especially in random pattern flaps is a significant problem (Janis, 2014). The need to increase flap length which can be used in this type of flap without distal flap necrosis developing has led to many studies having been conducted (Taylor et al., 1992).

Necroptosis is defined as programmed cell death, similar to the morphologically necrotic cell death that is seen in ischaemic tissues (Christofferson and Yuan, 2010). This pathway is regulated by tumour necrosis factor receptor (TNFR) and Fas ligand receptor activation, independently of the caspase system. Receptor interacting protein kinase-1 (RIPK-1) and receptor interacting protein kinase-3 (RIPK-3) activity is required for TNFR activation (Degterev et al., 2005; Xie et al., 2013). In recent studies, necroptotic cells have been shown in ischaemic-reperfusion created in rat skin flap models and it has been reported that necroptosis could have a role in flap necrosis (Liu et al., 2019a). Due to this role of RIPK-1 and RIPK-3 in necroptosis, it has been considered that inhibition of these enzymes could be a good strategy

against necroptosis. Degterev defined necrostatin-1 (nec-1) specific to RIPK-1 as a compound that blocks necrotic cell death in human and rat cells (Degterev et al., 2005).

The aim of this study was to examine and compare the effects on flap viability of necrostatin-1 and enoxaparin, which is a low-molecular weight heparin (LMWH), molecules in a rat model of random pattern skin flaps as described by McFarlane.

2. Materials and Methods

Approval for the study was granted by the Animal Experiments Local Ethics Committee of Kahramanmaras Sutcu Imam University (KSU) (protocol no: 18, session no:2021/07, decision no: 01, dated: 13.10.2021). The study was supported by the Scientific Research Projects Unit of KSU Experimental Animals Centre (project no: 2021/7-4D).

2.1. Formation of the study groups

The rats were separated into 4 groups of 8 animals. A caudal-based flap operation was performed to all groups in the McFarlane flap model.

- Group 1: No treatment was applied to this group.
- Group 2: Dimetil sülfoksit (DMSO)+isotonic mixture was administered intraperitoneally as a single daily dose of 0.5 ml starting with the first dose 1 hour before the operation.
- Group 3: Enoxaparin molecule (diluted in isotonic) was administered subcutaneously as a single daily dose of 100 IU/kg starting with the first dose 30 mins before the procedure.
- Group 4: Necrostatin-1 molecule was administered intraperitoneally (dissolved in DMSO+isotonic) as a single daily dose of 1.65 mg/kg starting with the first dose 1 hour before the operation.

2.2. Study Protocol

2.2.1. Necrostatin-1 molecule preparation

Necrostatin-1 molecule was purchased from Cayman Chemical company (Item no:11658) in powder form and was prepared for use in the University – Industry – Public Works Collaboration Development Practice and Research Centre (ÜSKİM). The molecules were first dissolved in DMSO solution to be 14 mg/ml, and were then diluted with isotonic solution to be at a concentration of 1mg/ml.

2.2.2. Surgical procedure

Before the surgical procedure, all the rats were injected intraperitoneally with Ketamin (50 mg/kg) (Ketalar, Eczacıbaşı, Türkiye) and Xylazine HCl (5mg/kg) (RompunR, Bayer, Türkiye), and thus general anaesthesia was obtained. The landmarks for the area where the flap was planned on the dorsal region of each rat were marked as both scapulae and the posterior iliac notches. A caudal-based McFarlane skin flap, 3 x 9 cm in size, was drawn with a skin pen, and the flap plan was formed. The surgical area was stained with povidone iodine, then following sterile draping, the flap was raised in the loose avascular plane over the dorsal muscles including the subcutaneous panniculus carnosus muscle. Hemostasis was obtained then the incision areas were closed by primary suturing returning the flap to its place. The surgical procedure was terminated (Figure 1). Postoperatively, the rats were followed up daily for 7 days, and at the end of 7 days, all the rats were sacrificed with high-dose anaesthesia.

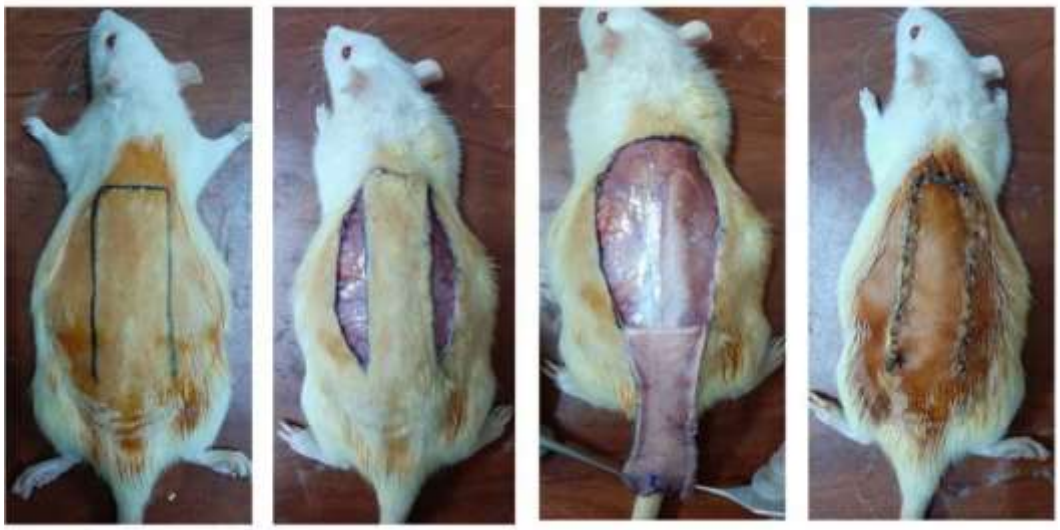

Figure 1. Surgical stages of the McFarlane skin flap.

2.3. Calculation of the Flap Necrosis Area

On postoperative day 8 after the rats were sacrificed, photographs were taken. The digital photographs obtained were uploaded to a computer, and using ImageJ 1.53 program, the total flap area and necrotic area were calculated for all the groups. The necrotic area was stated as the necrotic area percentage of the total flap area (Figure 2).

2.4. Histopathological and Immunohistochemical Evaluations

On postoperative day 8 after the rats were sacrificed, a full layer approximately 1 x 1 cm^2 tissue sample was

taken from the demarcation zone passing through the live line-necrosis line of the flap tissue. All the tissue samples were stained with hematoxylin and eosin (HandE) and Masson Trichrome (MT), then the capillary proliferation, inflammation, necrosis, fibroblast proliferation, and fibrosis parameters were examined (×200). The capillary proliferation values were stated numerically by counting. Inflammation (neutrophil infiltration) was graded as mild (1 point), moderate (2 points), or severe (3 points). In the necrosis parameter, complete epidermal and dermal organization was scored as 1, moderate epidermal and dermal organization (erosive) as 2 and little epidermal and dermal organization (necrotic) as 3. In fibroblast proliferation, fine granulation tissue was categorized as 1 point, moderate granulation tissue as 2 points, and thick granulation tissue as 3 points. In the MT histochemical staining, the presence of irregular collagen fibres (fibrosis) was graded as 1 point, moderate as 2 points, and severe as 3 points.

Figure 2. Calculation of the flap total area and necrotic area using ImageJ 1.53 program.

RIPK-1 (1/100 dilution, 24 mins, ABCAM, ab106393) was examined immunohistochemically in compliance with validated procedures using a Roche Diagnostic Ventana Benchmark XT staining device. A brown colour showing the presence of antibody-dependent antigen was examined under a light microscope (Olympus Corporation, Tokyo, Japan) (x200) fitted with a computer-controlled digital camera and imaging software and the RIPK-1 (+) stained cell percentage was determined for each sample.

Before the rats were sacrificed, a 1.5 cc cardiac blood sample was taken. The samples were centrifuged at 1000 rpm for 15 mins and plasma was obtained. The samples obtained were evaluated using the Elabscience rat TNF-α kit according to the protocol defined by the manufacturer.

2.5. Statistical Analysis

Data obtained in the study were analyzed statistically using SPSS vn. 28.0 software. Conformity of the data to normal distribution was assessed using the Shapiro-Wilk and Kolmogorov-Smirnov tests. In the comparisons of three groups of independent variables showing normal distribution, the One-Way ANOVA test was used. In the post-hoc tests of the 3-group comparisons where a significant difference was determined, the LSD test was used when variances showed homogenous distribution and the Tamhane T2 test when not homogenous. A value of $P<0.05$ was accepted as statistically significant.

3. Results

When examined according to the groups, the mean macroscopic necrotic area was determined to be 41.36% in Group 1 and 40.66% in Group 2 as the control groups, followed by the drug groups as 36.56% in Group 3 and 31.67% in Group 4. Although the percentages were lower in the necrostatin-1 group (Group 4) and the enoxaparin group (Group 3), no statistically significant difference was determined between the groups ($P>0.05$) (Table 1). The inflammation scores were calculated for all the groups, as 2.375 for Group 1 and 2.250 for Group 2 as the control groups, and 2.000 for Group 3 and 1.625 for Group 4 as the drug groups. The values of Groups 3 and 4 were lower but in the statistical analysis, the difference was not significant ($F=2.649$, $P>0.05$).

The capillary proliferation values were determined as mean 15.375 and 22.375 for Group 1 and Group 2, respectively, and 37.0 and 47.75 for Group 3 and Group 4, respectively. The difference between the groups was determined to be statistically significant ($F=16.330$, $P<0.05$). Post-hoc paired comparisons were performed to determine from which groups the difference originated (Table 2).

Table 1. Comparisons of the necrosis percentages of the groups

		N	Mean (%)	SD*	95% CI		F	P*
					Min	Max		
Necrosis percentage	Group 1	8	41.362	8.571	34.196	48.528	1.687	.192
	Group 2	8	40.662	13.511	29.367	51.958		
	Group 3	8	36.562	6.149	31.421	41.703		
	Group 4	8	31.675	9.147	24.027	39.322		
	Total	32	37.565	10.032	33.948	41.182		

*SD= standart deviations. P value <0.05 was accepted as statistically significant. Group 1= no tratment group, Group 2= dmso + isotonic group, group 3= enoxparin group and group 4= necrostatin-1 group.

Table 2. Post-hoc analyses of the capillary proliferation counts of the groups*

	(I) Groups	(J) Groups	Mean difference (I-J)	SH	P	95% CI	
						Min	Max
Capillary proliferation	Group 1	Group 2	-7.00000	5.08960	.180	-17.4256	3.4256
		Group 3	-21.62500	5.08960	<.001	-32.0506	-11.1994
		Group 4	-32.37500	5.08960	<.001	-42.8006	-21.9494
	Group 2	Group 1	7.00000	5.08960	.180	-3.4256	17.4256
		Group 3	-14.62500	5.08960	.008	-25.0506	-4.1994
		Group 4	-25.37500	5.08960	<.001	-35.8006	-14.9494
	Group 3	Group 1	21.62500	5.08960	<.001	11.1994	32.0506
		Group 2	14.62500	5.08960	.008	4.1994	25.0506
		Group 4	-10.75000	5.08960	.044	-21.1756	-.3244
	Group 4	Group 1	32.37500	5.08960	<.001	21.9494	42.8006
		Group 2	25.37500	5.08960	<.001	14.9494	35.8006
		Group 3	10.75000	5.08960	.044	.3244	21.1756

*In the post-hoc paired comparisons of the groups, the capillary proliferation counts recorded for Group 3 and Group 4 were determined to be statistically significantly higher than the values of Group 1 (P<0.001) and Group 2 (P<0.001). In the comparison of the two drug groups, the capillary proliferation value of Group 4 was determined to be significantly higher than that of Group 3 (P: 0.044). CI= confidence interval, P value <0.05 was accepted as statistically significant.

Accordingly, the capillary proliferation counts recorded for Group 3 and Group 4 were determined to be statistically significantly higher than the values of Group 1 (P<0.001) and Group 2 (P<0.001). In the comparsion of the two drug groups, the capillary proliferation value of Group 4 was determined to be significantly higher than that of Group 3 (P=0.044). The necrosis scores were determined as mean 2.50 and 2.370 for Group 1 and Group 2, respectively, and 2.125 and 1.750 for Group 3 and Group 4, respectively (Table 3). The difference between the groups was determined to be statistically significant (F=2.2970, P<0.05). In the post-hoc paired comparisons, the necrosis score of Group 4 was determined to be significantly lower than the score of Group 1 (P=0.010) and Group 2 (P=0.029). In the comparison between Group 3 and Group 4, the mean necrosis score was observed to be lower but the difference between the groups was not statistically significant (P=0.178) (Figure 3).

Table 3. Comparisons of the necrosis scores of the groups*

		N	Mean	SD	Min	Max	F	P
Necrosis	Group 1	8	2.500	.534	2.053	2.946	2.970	.049
	Group 2	8	2.370	.515	1.942	2.807		
	Group 3	8	2.125	.687	1.589	2.660		
	Group 4	8	1.750	.462	1.363	2.137		
	Total	32	2.185	.599	1.974	2.401		

*Table 3 shows the comparison of necrosis scores by groups. One-Way ANOVA test was used to compare normally distributed variables in three independent groups. Accordingly, necrosis scores showed a significant difference between groups, F=2.2970, p<0.05. P value <0.05 was accepted as statistically significant.

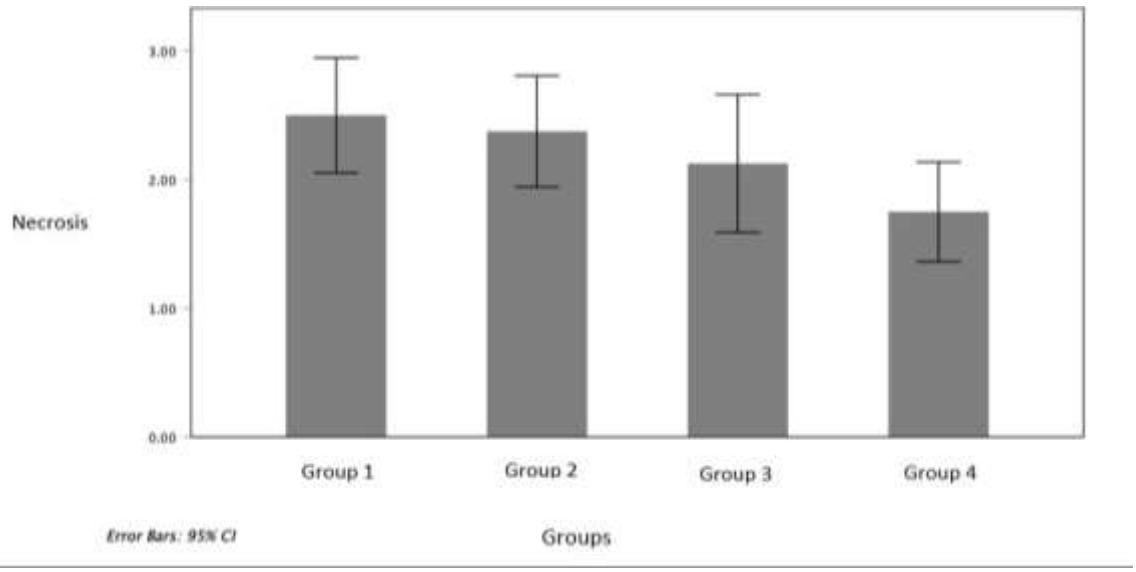

Figure 3. Comparisons of the necrosis scores of the groups. The necrosis scores were determined as mean 2.50 and 2.370 for Group 1 and Group 2, respectively, and 2.125 and 1.750 for Group 3 and Group 4, respectively. In the post-hoc paired comparisons, the necrosis score of Group 4 was determined to be significantly lower than the score of Group 1 (P=0.010) and Group 2 (P=0.029). In the comparison between Group 3 and Group 4, the mean necrosis score was observed to be lower but the difference between the groups was not statistically significant (P=0.178).

The fibroblast proliferation scores were determined as mean 1.375 and 1.625 for Group 1 and Group 2, respectively, and 2.500 and 2.375 for Group 3 and Group 4, respectively. The difference between the groups was determined to be statistically significant (F=5.896, P<0.05). In the post-hoc paired comparisons of the groups, the fibroblast proliferation scores of Group 3 and Group 4 were determined to be statistically significantly higher compared to Group 1 and Group 2 (P<0.05) (Table 4).

Table 4. Post-hoc analyses of the fibroblast proliferation scores of the groups

	(I) Groups	(J) Groups	Mean difference (I-J)	SH	P	Min	Max
Fibroblast proliferation	Group 1	Group 2	-.25000	.32217	.444	-.9099	.4099
		Group 3	-1.12500	.32217	.002	-1.7849	-.4651
		Group 4	-1.00000	.32217	.004	-1.6599	-.3401
	Group 2	Group 1	.25000	.32217	.444	-.4099	.9099
		Group 3	-.87500	.32217	.011	-1.5349	-.2151
		Group 4	-.75000	.32217	.027	-1.4099	-.0901
	Group 3	Group 1	1.12500	.32217	.002	.4651	1.7849
		Group 2	.87500	.32217	.011	.2151	1.5349
		Group 4	.12500	.32217	.701	-.5349	.7849
	Group 4	Group 1	1.00000	.32217	.004	.3401	1.6599
		Group 2	.75000	.32217	.027	.0901	1.4099
		Group 3	-.12500	.32217	.701	-.7849	.5349

*In the post-hoc paired comparisons of the groups, the fibroblast proliferation scores of Group 3 and Group 4 were determined to be statistically significantly higher compared to Group 1 and Group 2 (P<0.05). P value <0.05 was accepted as statistically significant.

The fibrosis scores were determined as mean 2.370 and 1.750 for Group 1 and Group 2, respectively, and 2.375 and 1.375 for Group 3 and Group 4, respectively. The difference between the groups was determined to be statistically significant (F=4.903, P<0.05).

The RIPK-1 immunohistochemically staining percentages were determined as mean 53.75% and 55.525% for Group 1 and Group 2, respectively, and 61.250% and 21.875% for Group 3 and Group 4, respectively. In the post-hoc paired comparisons of the groups, the RIPK-1 staining percentage of Group 4 was determined to be statistically significantly higher compared to Group 1 (P= 0.002), Group 2 (P=0.003), and Group 3 (P<0.001) (P<0.05) (Figure 4).

The descriptive statistics of the variables examined are shown as mean and standard deviation values in Table 5.

No statistically significant difference was determined between the groups in respect of the TNF-α levels (F=1.759, P>0.05).

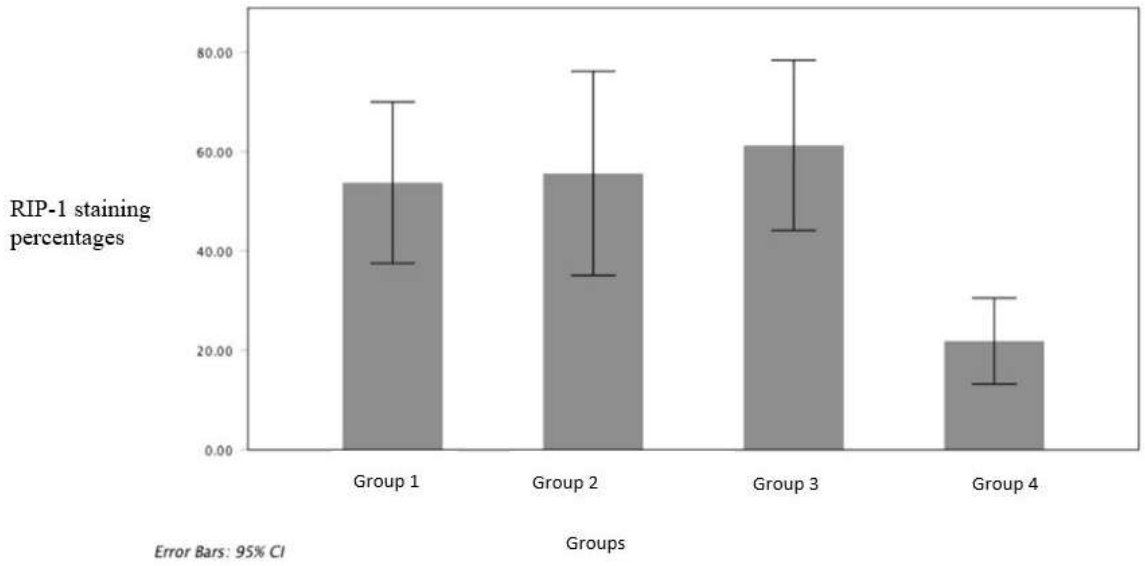

Figure 4. Comparisons of the RIP-1 staining percentages of the groups. The RIPK-1 immunohistochemically staining percentages were determined as mean 53.75% and 55.525% for Group 1 and Group 2, respectively, and 61.25% and 21.875% for Group 3 and Group 4, respectively. In the post-hoc paired comparisons of the groups, the RIPK-1 staining percentage of Group 4 was determined to be statistically significantly higher compared to Group 1 (P= 0.002), Group 2 (P=0.003), and Group 3 (P<0.001) (P<0.05).

Table 5. Descriptive findings of the variables*

	Groups							
	Group 1		Group 2		Group 3		Group 4	
	Mean	SD*	Mean	SD	Mean	SD	Mean	SD
Necrosis area percentage	41.36	8.57	40.66	13.51	36.56	6.15	31.67	9.15
Capillary proliferation	15.37	1.69	22.38	10.91	37.00	13.49	47.75	10.51
Inflammation	2.38	.52	2.25	.71	2.00	.53	1.63	.52
Necrosis	2.50	.53	2.38	.52	2.13	.64	1.75	.46
Fibroblast proliferation	1.38	.52	1.63	.52	2.50	.76	2.38	.74
Fibrosis	2.38	.74	1.75	.71	2.38	.52	1.38	.52
RIP-1 staining percentage	53.75	19.41	55.63	24.56	61.25	20.49	21.88	10.33
TNF-alpha level	17.89	7.21	20.91	12.43	16.44	7.74	11.66	1.96

*Descriptive statistics of the studied variables are shown in Table 5. Accordingly, the means and standard deviations of the variables are shown. SD= standard deviations.

4. Discussion

In this study of a flap necrosis model, two molecules were examined. The study results demonstrated that significantly better results were obtained, especially in the necrostatin-1 group, in respect of necrosis percentage, capillary proliferation, necrosis scores, fibroblast proliferation, and fibrosis scores.

In a study by Koudstaal et al. (2015) the effects of nec-1 molecule were examined on I/R damage formed in pig myocardium. Groups administered 1mg/kg and 3.3 mg/kg nec-1 was compared with a control group. The results showed that the infarct area of the group administered 1 mg/kg nec-1 was less but the difference was not statistically significant, whereas in the group administered 3.3 mg/kg nec-1, the infarct area developing associated with I/R damage was seen to be statistically significantly less compared to the control group.

In the current study, no statistically significant difference was observed in the flap necrosis percentage of the nec-1 group (Group 4) compared to the control groups of Group 1 and Group 2. However, the flap necrosis percentage of the nec-1 group (31.675%) was observed to be evidently lower than that of the other two groups (41.36%, 40.66%, respectively). The drug dose in the current study was determined as 1.65 mg/kg/day (Liu et al., 2019b). In the Koudstaal et al. (2015) study, the use of 3.3mg/kg nec-1 was shown to be more effective than the dose of 1mg/kg in inhibiting necroptotic cell death and decreasing the tissue infarctus area. In the light of these data, it can be considered that the use of a higher dose of nec-1 in a random pattern flap model will increase flap viability more than the use of a low dose.

Neonatal hypoxic-ischaemic rat model, nec-1 treatment was seen to decrease neuro-inflammation and inhibit NF-KB activation and IL-1β, IL-6, and TNF-α expression by blocking the interaction of RIPK-1 and RIPK-3 in neurons. As a result of that study, it was seen that nec-1 could have a protective effect against hypoxic-ischaemic brain damage in newborns (Chavez-Valdez, et al., 2012; Azboy et al., 2014; Cao and Mu, 2021;). Wen et al. (2017) conducted an experimental study examining the effects of nec-1 molecule on intestinal I/R damage, and as a result of that study, it was found that the intestinal mucosal structure of the animals in the group administered nec-1 molecule (1.0 mg/kg) was significantly protected and they had lower mucosal damage scores compared to the control group. In the current study, the tissue RIPK-1 staining levels were compared immunohistochemically, and consistent with similar studies in the literature, RIPK-1 (+) stained cells were determined at a high rate (53.75%) in the control group, and the percentage of RIPK-1 (+) stained cells was observed to be significantly low (21.87%) in the rats administered nec-1.

There are different results in literature related to the use of enoxaparin molecule in a random pattern flap model (Miyawaki et al., 2002; Chung et al., 2006). Fatemi et al. (2012) investigated the effects of enoxaparin and clopidogrel molecules in a random pattern skin flap model and the results of the study showed no statistically significant difference between the enoxaparin molecule and the control group in reducing the flap necrosis percentage. In contrast, Aral et al. (2015) reported that enoxaparin molecule was effective in reducing flap necrosis and increasing flap viability in a random pattern flap model. In Group 3 of the current study, administered with 100 IU/kg enoxaparin, the flap necrosis percentage was determined to be 36.56%. Although this rate was lower than that of Group 1 (41.36%) and Group 2 (40.66%), no statistically significant difference was determined between the groups. These results in the current study are not consistent with the findings of the similar study by Aral et al. This difference could be due to the fact that Aral et al. administered a dose of 320 IU/kg enoxaparin molecule subcutaneously directly into the flap tissue (Aral et al., 2015).

When the flap necrosis percentages were compared between the nec-1 and enoxaparin groups, the flap necrosis percentages were observed to be 31.675% and 36.56%, respectively. Although the necrosis percentage was lower in the nec-1 group, the difference was not found to be statistically significant.

All the groups in the current study were compared in respect of inflammation (neutrophil inflammation), capillary proliferation, fibroblast proliferation, fibrosis and necrosis parameters. No statistically significant difference was determined in respect of the inflammation scores, but the mean inflammation score of the nec-1 group was determined to be lower than that of both the enoxaparin group and the control groups, Groups 1 and 2. Previous studies in literature have shown the anti-inflammatory effect of both nec-1 molecule and enoxaparin molecule (Iba and Miyasho, 2008; Iba et al., 2012; Iba et al., 2013; Wen et al., 2017). When the necrosis scores were compared between the two drug groups, lower scores were observed in the nec-1 group but the difference was not statistically significant. These histopathological results suggest that there are positive effects of nec-1 molecule on tissue healing in a flap necrosis model, but when the data of the enoxaparin group were examined, this effect was seen to be limited.

A limitation of this study was that only a single dose of nec-1 molecule was administered. The administration of different doses could provide clearer information for the interpretation of the data in the examination of a flap necrosis model.

There is a need for further research of more extensive animal groups to investigate the effects of different doses and potential side-effects of necrostatin, which is an experimental molecule. Moreover, there are different results in literature related to the effect of the enoxaparin molecule in random pattern flaps. When the flap necrosis percentage and histochemical parameters of the enoxaparin molecule were evaluated in this study, the effects on flap viability were considered to be limited. Further studies of enoxaparin molecule with larger samples and drugs used at different doses and applications would be helpful in clarifying the different results in literature.

5. Conclusion

When the flap necrosis percentages and immunohistochemical and histopathological data obtained in this study are taken into consideration, they suggest that necrostatin molecule has significant therapeutic potential for increasing flap viability in the random pattern flap model. When the flap necrosis percentage and histochemical parameters of the enoxaparin molecule were evaluated in this study, the effects on flap viability were considered to be limited.

Author Contributions

The percentage of the author(s) contributions is presented below. All authors reviewed and approved the final version of the manuscript.

	Ö.F.Ç.	N.B.	E.K.G.	M.S.	İ.O.	M.G.Y.
C	40	20	20	20		
D	50	25			25	
S	100					
DCP	40	20			20	20
DAI			50	50		
L	50	20				30
W	50	25				25
CR	20	20	20	20	20	
SR	50					50
PM	20	40			20	20
FA	50	50				

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Acknowledgments

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Ethical Approval/Informed Consent

All the study protocols were approved by the Animal Experiments Ethics Committee of Kahramanmaras Sutcu Imam University Medical Faculty (approval date: October 13, 2021, protocol code: 18-2021/07-01). All procedures were in compliance with the National Health Institutes regulations for the use and care of laboratory animals.

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