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## Original Research

# Neutrophil Elastase Inhibitor Increases Flap Survival in Experimental Degloving Injuries

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### Abstract

**Objectives:** Degloving hand injuries have generally been viewed as among the most difficult of injuries to manage due to the extensive nature of associated damage. The traditional approach to the circumferentially degloved segment of problematic flap viability has been to resuture the flap and to wait and see. However, the waiting period or the specific hemorheological protocol remains uncertain. The purpose of this study is to acknowledge if Sivelestat, known to ameliorate ischemia reperfusion injury, enhances the survival of avulsed flaps in a hind limb degloving model of rats and to compare Sivelestat's effects to Pentoxifylline.

**Methods:** Total flap area (cm<sup>2</sup>), area of necrosis in the flap (cm<sup>2</sup>), and the ratio between the necrotic and total areas (percentage) were determined. Angiogenesis among the groups were documented with CD31, anti-PECAM stainings. TUNEL assay was performed to allow the visualization of cell nuclei containing fragmented DNA, a typical feature of apoptosis.

**Results:** This study demonstrated that, Sivelestat administered at 10 mg/kg/hour dosage will inhibit the ischemia reperfusion injury more pertinently than Pentoxifylline which exerts only hemorheological effects.

**Conclusion:** The anti-inflammatory effects of Sivelestat, will be beneficial for decreasing the early complications of degloving injury, such as inflammation, sepsis, and edema, better than Pentoxifylline which exerts only hemorheological effects.

**Keywords:** Degloving injury; neutrophil elastase inhibitor; ischemia reperfusion injury.

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A vulsion and degloving injuries are caused by a vast array of mechanisms, machines and situations, in which the hand is injured with low energy shearing forces in a rigid position.<sup>[1]</sup> These types of injuries have generally been viewed as among the most problematic of injuries to manage due to their extensive nature of associated damage. During degloving injuries, blood vessels having great

elasticity, are disembodied and separated with irreversible injury along the entire length of involvement and so are the tendons and nerves. Furthermore in circumferential degloving injuries, the distal flap goes through a state of abrupt ischemia followed by reverse perfusion forming after the reattachment of the flaps back to its' origin. Arnez et al, recently reclassified degloving injuries according to the

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extension of the injury mechanism into 4 groups: a) circum-scribed degloving injury caused by tangential forces, b) non-circumferential degloving, c) single and d) multiplane circumferential degloving injury.<sup>[2]</sup> Among many options of treating a degloving injury are: salvaging the degloved segment through conservative therapy with dressing, debriding and skin grafting, maintaining vascularity by arterial anastomosis or arteriovenous shunting; and coverage by free flaps if the segments are not salvageable.<sup>[3-5]</sup> The traditional approach to the circumferentially degloved segment of problematic flap viability has been to resuture the flap and to wait and see. However, the waiting period or the specific treatment protocol remains uncertain. Sivelestat<sup>®</sup>, N-{2-[[{4-[(2, dimethylpropanoyl)oxy]phenyl}sulfonyl]amino]benzoyl glycine (Ono Pharmaceutical, Osaka, Japan) is a specific neutrophil elastase inhibitor which found use in ameliorating acute systemic inflammatory response syndrome, acute respiratory distress syndrome and in ischemia reperfusion injury forming after crush syndrome.<sup>[6-8]</sup>

Pentoxifylline is a competitive nonselective phosphodiesterase inhibitor, which raises the intracellular cAMP, activates pKA, inhibits TNF and leukotrien synthesis. In addition, pentoxifylline improves red blood cells deformability, and decreases the potential for platelet aggregation.<sup>[9-11]</sup> Experimental studies show that pentoxifylline increases perfusion in myocutaneous and skin flaps.

Allopurinol is a purine analog and an inhibitor of xanthine oxidase and through its antioxidant effects, it is found to be beneficial in ischemia reperfusion injuries.<sup>[9-11]</sup>

The purpose of this study is to acknowledge if Sivelestat, known to ameliorate IRI, enhances the survival of avulsed flaps in a hind limb degloving model of rats which was described by Milcheski et al and to compare Sivelestat's effects to a well known hemorheological agent, Pentoxifylline.

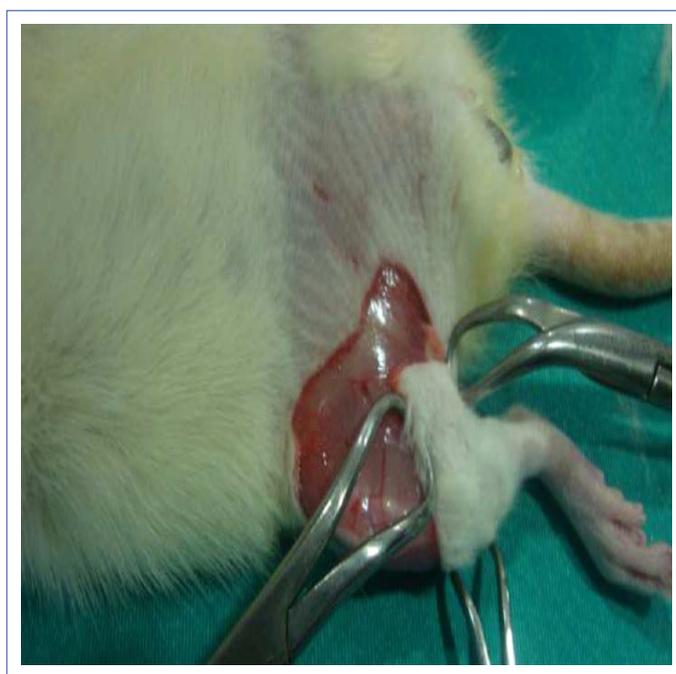
## Methods

### Experiment

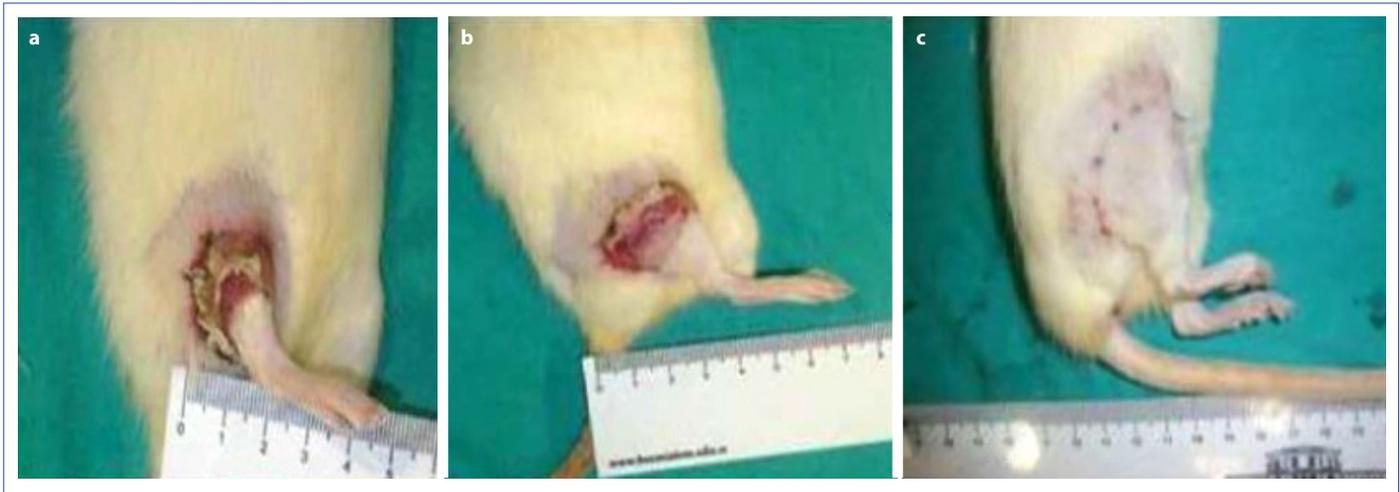
Upon obtaining the necessary ethics committee approval for animal experimentation the study was initiated. A total of 24 male Wistar rats, weighing between 250 and 350 g, were used in this experiment and divided into 3 groups, 8 rats in each group. Animals were anesthetized with 35 mg/kg Ketamin (Ketalar<sup>®</sup>, Pfizer, Istanbul) and 5 mg/kg Xylazine (Rompun<sup>®</sup>, Bayer, Istanbul) intramuscularly. An incision was made on the right groin over the inguinal ligament including the skin and subcutaneous tissue. Degloving injury model has been adapted from Laboratory of Microsurgery, Division of Plastic Surgery of Sao Paulo.<sup>[10]</sup> Briefly the right

hindlimb was incised circumferentially at the inguinal ligament level. Four Backhaus clamps were positioned in the 4 quadrants (3, 6, 9, and 12 o'clock positions). A distal traction force was applied to the flap, enough to pull the flap to the ankle region (Fig. 1). This maneuver created a reverse-flow flap going through the ischemia-reperfusion cycle. After a downtime of 5 minutes, the flap was sutured back to its original place and the incision was closed with 5/0 nylon sutures. The rat's tail vein was cannulated with a 16 G catheter for pharmacological interventions.

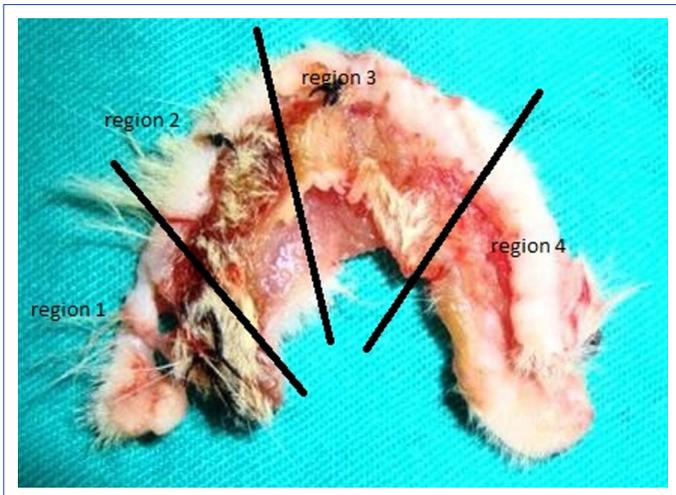
In group 1, also described as the control group, 1 cc of saline through the tail vein was administered. In group 2, which is described as the Pentoxifylline group, 1 cc of pentoxifylline (25 mg/kg), (Trental<sup>®</sup>, Hoechst pharmaceutical industry, Istanbul) was administered through the tail vein as a single injection given gradually. In group 3, which is the Sivelestat group, 1 cc (10 mg/kg) of Sivelestat<sup>®</sup> was administered after creating the degloved flap.<sup>[12]</sup> Fluid replacement with saline was conducted 2 cc/kg/hour dosage throughout the experiment. Bacitracin was utilized for wound dressing purposes and the rats were observed daily in terms of flap survival for 7 days. At the end of this period, the rats were sacrificed by an overdose of Thiopental. No signs of autophagy was observed in the avulsed flaps. In group 1 animals, signs of infection was observed in 3 animals. Total area of flap necrosis was calculated by using Image J software (National Institutes of Health, NIH, USA). Total flap area (cm<sup>2</sup>), area of necrosis in the flap (cm<sup>2</sup>), and the ratio between the necrotic and total areas (percentage) were determined (Fig.



**Figure 1.** Degloving injury model of the lower extremity.



**Figure 2.** Macroscopic view of the avulsed flap on postoperative day 7 in groups a, b and c.



**Figure 3.** Schematic view of the avulsed flap segment divided into 4 regions.

2a, 2b, 2c, Fig. 3) (Table 1). In order to determine the amount of angiogenesis among the groups, CD31, anti-PECAM staining was performed as described below. The amount of angiogenesis was determined by using Image J analysis (Fig. 4a, 4b, 4c). Analysis of apoptotic cells were made by TUNEL assay (Terminal deoxynucleotidyl transferase dUTP Nick End Labeling) method.

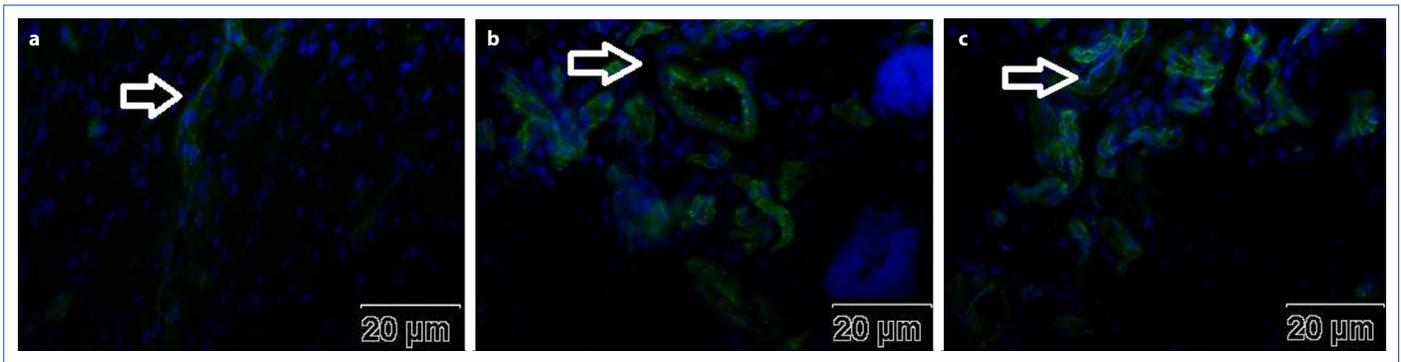
### Immunofluorescence Protocol

The degloved flap was excised en-bloc as a pathology specimen, frozen on dry ice and cut on a cryostat into 20 µm horizontal sections (Leica Instruments, Wetzlar, Germany). Skin sections were fixed in 4% paraformaldehyde in 0.1M phosphate-buffered saline, rinsed, pretreated for antigen retrieval with 0.1M phosphate-buffered saline containing 1% sodium dodecyl sulfate, rinsed and immersed for 1 h in 0.1M phosphate-buffered saline containing 0.3% Triton X-100 and 10% normal donkey serum. The sections were incubated overnight at 4°C with polyclonal goat anti-PECAM-1 (CD31) (#sc1506; SANTA CRUZ), (diluted 1:100 in 0.1M phosphate-buffered saline) that were detected with Alexa Fluor 488 donkey anti-goat (A11055; INVITROGEN) secondary antibody. Sections were counterstained with 40-6-diamidino-2-phenylindole (DAPI). Sections were evaluated under a fluorescence microscope (NIKON ECLIPSE Ni) connected to a CCD camera (NIKON DS-Fi1c). Rate of angiogenesis (CD31+ cells) was analysed in a blinded way by 5 independent examiners through counting numbers of cells or profiles in four randomly defined regions of interests per tissue measuring 62.500 µm<sup>2</sup>. Two sections were processed for each animal. Mean values were calculated for both sections for all specimen (Fig. 3).<sup>[13]</sup>

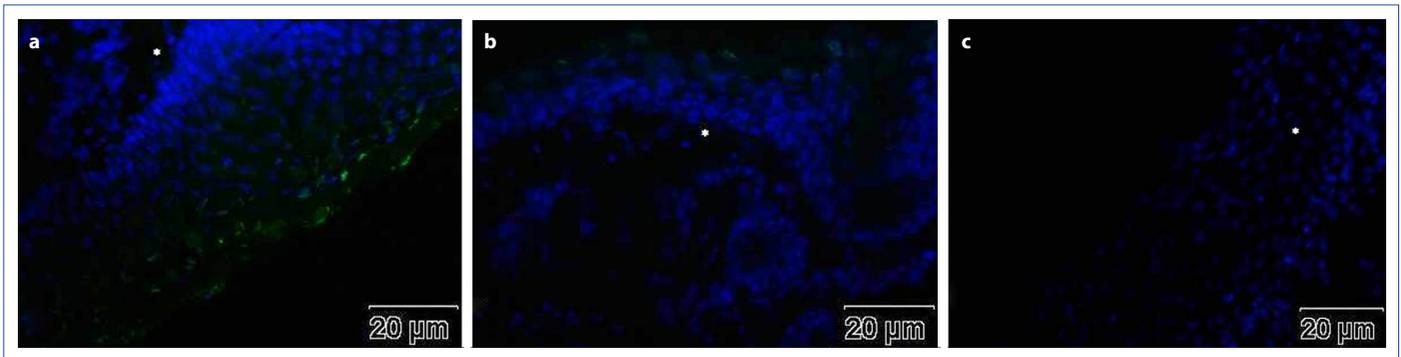
**Table 1.** Total Flap area (cm<sup>2</sup>), Necrotic Area in the flap (cm<sup>2</sup>), and Ratio between necrotic and Total Areas (%)

Groups	Total Flap Area (cm <sup>2</sup> )			Necrotic Area (cm <sup>2</sup> )			Necrosis Ratio (%)		
	Gr 1	Gr 2	Gr 3	Gr 1	Gr 2	Gr 3	Gr 1	Gr 2	Gr 3
Lower Value	5.245	5.17	5.23	3.127	2.98	1.58	60.48	45.81	30.21
Median Value	5.431	5.23	5.45	3.87	3.28	1.72	66.72	61.34	31.55
Higher Value	6.19	5.78	6.71	4.46	3.75	2.56	73.99	70.58	38.5
Significance Level	p=0.8165			p<0.05			p<0.05		

Gr 1: Group 1, Control Group; Gr 2: Group 2, Pentoxifylline Group; Gr 3: Group 3, Sivelestat Group.



**Figure 4.** (a) Immunohistochemical stainings for the endothelial marker of angiogenesis CD31 were assessed in the control group. Arrow shows capillary formation stained with CD31. (b) Immunohistochemical stainings for the endothelial marker of angiogenesis CD31 were assessed in the pentoxifylline group. Arrow shows capillary formation stained with CD31. (c) Immunohistochemical stainings for the endothelial marker of angiogenesis CD31 were assessed in the sivelestat group. Sivelestat treated group showed an increase in the capillaries to approximately 200% of baseline from saline treated rats. Arrow shows capillary formation stained with CD31.



**Figure 5.** TUNEL and Roche staining of nonviable flaps. Apoptotic cell nuclei stained by TUNEL in the dermis of the flap. (a) Saline (b) Pentoxifylline (c) Sivelestat. TUNEL+ cell nuclei revealed brighter fluorescence shown by arrow. Asterisk shows chromatin condensation. There was a significant difference between saline and sivelestat groups, also between pentoxifylline and sivelestat groups ( $p < 0.05$ )

**TUNEL Assay**

Analysis of DNA-fragmented cells from the sections harvested from animals were stained by terminal transferase fluorescein-dUTP nick end labeling (TUNEL), using a commercially available kit (11684795910, ROCHE, Switzerland). In these sections, DNA-fragmented cells were counted in blinded manner in four randomly defined regions in epidermis (each 62.500 µm<sup>2</sup>) as above.<sup>[14]</sup> Apoptotic cells were

identified by the characteristics of nuclear condensation, fragmentation and margination (Fig. 5a-c) (Table 2, 3).

**Statistical Analysis**

The results are expressed in the form of mean±SD. Serially measured parameters were compared with two-way analysis of variance (ANOVA-Tukey test). Kruskal-Wallis non-parametric test for independent samples among the 3 groups were performed. Inter-observer results correlated well and significance level was 95% ( $p < 0.05$ ).

**Table 2.** Summary of Manual Microvessel Counts

Examiner	Group Saline*	Group Pentoxifylline*	Group Sivelestat *	p
1	7.5±1.18	16.84±3.51	30.6±4.28	0.006
2	9.25±1.01	14.0±3.28	26.0±3.61	0.012
3	7.45±1.34	15.21±2.44	27.1±1.51	0.032
4	9.01±1.05	17.11±3.21	37.03±1.24	0.054
5	6.81±1.68	16.0±2.64	29.02±1.06	0.008
Summary**				$p < 0.05$

\*Mean microvessel count per high power field (200x)± Standard Error; \*\*p value determined by ANOVA, Overall statistical significance as determined by repeated measures analysis of variance.

**Table 3.** TUNEL + cells counted by region

	Group Saline (Med±S.D)	Group Pentoxifylline (Med±S.D)	Group Sivelestat (Med±S.D)	p
Region 1	31.7±22.5	25.7±16.1	3.0±4.1	0.019
Region 2	36.5±22.8	20.2±13.1	14.3±8.1	0.07
Region 3	41.3±23.1	29.0±19.7	7.3±9.9	0.019
Region 4	48.5±28.6	34.7±17.2	9.3±8.8	0.012

ANOVA(Tukey Test);  $p < 0.05$ , statistically significant.

## Results

**Animals:** One animal from Group 2 (Pentoxifylline) was excluded due to unsuspected death in the first postoperative night, according to the exclusion criteria. Other exclusion criteria consisted of diarrhea, and significant weight loss throughout the experiment. Sivelestat group rats displayed minor inflammation on injury sites macroscopically during the wound healing process of the avulsed flap.

**Total flap area planimetry:** The total flap area (cm<sup>2</sup>), necrotic area in the flap (cm<sup>2</sup>), and ratio between necrotic and total areas (%) were determined with Image J analysis in each group after 7 days. Total flap area (cm<sup>2</sup>), was 6.19 for the control group, 5.78 for the Pentoxifylline group, and 6.71 for the Sivelestat group. Total flap areas were statistically similar ( $p=0.8165$ ). The ratio between the avulsed flap necrotic area and total area in the control group was 66.72%, 61.3% in the Pentoxifylline group and 31.5% in the Sivelestat applied group. ANOVA-Tukey analysis showed significant differences between pairs, control and pentoxifylline group ( $p<0.01$ ) and control and Sivelestat ( $p<0.01$ ). There was also a statistically significant difference between the pentoxifylline and sivelestat groups ( $p<0.05$ ). These data are listed in Table 1.

## CD31-antiPECAM Immunostaining Results

Sivelestat increased CD31-antiPECAM (Platelet endothelial cell adhesion molecule) staining, showing marked angiogenesis and attenuated apoptosis in degloved flaps.

Ischemia reperfusion injury treated by Sivelestat and Pentoxifylline, were followed by an increase in the density of CD31 + capillaries. In Sivelestat treated animal, capillaries increased to almost 150% of baseline in Sivelestat treated animals and in Pentoxifylline treated animals, to 100% of baseline. The rate of angiogenesis (CD31+ cells) was analyzed in a blinded way by counting numbers of cells or profiles in four defined regions of interests per tissue measuring 62.500  $\mu\text{m}^2$  and the results are shown in Table 2. These results suggest that Sivelestat could effectively promote vascular endothelial cells leading to angiogenesis by neutrophil-elastase inhibition compared with Pentoxifylline treated groups ( $p<0.05$ ).

**TUNEL assay results:** Positive TUNEL-stained cells were detected and quantified in the dermis and hypodermis of avulsed flaps during the wound healing in all groups. Nucleus of TUNEL (+) cells were marked by an increase in intensity of nuclear fluorescence by changing their fluorescence from dark blue (normal cells) to a bright blue, white color, indicating chromatin condensation. As shown in figure 5a-c, the TUNEL positive cell number was significantly decreased by Sivelestat treatment on day 7, compared

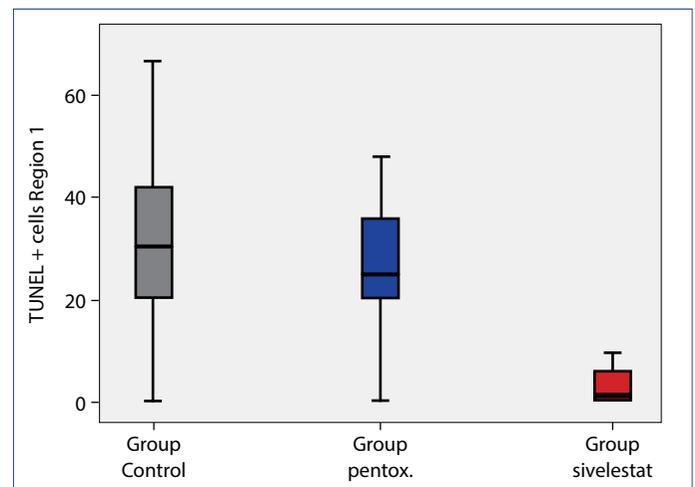
to the other 2 groups ( $p<0.05$ ,  $p<0.01$ , respectively). The apoptotic cells were counted in 4 randomly chosen areas of the dermis and the comparison between the groups are presented in figure 6.

## Discussion

Treatment of circumferentially avulsed flaps with selective neutrophil-elastase inhibitor, Sivelestat<sup>®</sup> reduced apoptotic cell death and enhanced endothelial wound healing, as well as angiogenesis, which was shown through TUNEL assay and activity of CD31 anti-PECAM antibodies. To the best of our knowledge, this is the first study to examine the consequences of degloving injury on the basis of an ischemia reperfusion injury, since, the circumferentially degloved segment becomes a reversely perfused flap going through the phases of the IRI sequela.

We have previously shown that Sivelestat, ameliorates ischemia reperfusion injury in a muscle flap model when administered at a dosage of 10 mg/kg/hour. In this study, Sivelestat was utilized on an animal degloving injury model described by Milcheski et al and the ischemia reperfusion ameliorating effects of Sivelestat was compared with Pentoxifylline. However these prementioned studies were designs of experimental degloving injury models. They only compared the flap results in terms of planimetry and percentages of necrotic areas which were determined only macroscopically.

It is a well-known fact that many factors play a role before a decision is made about the treatment protocol in degloving injuries. The age of the patient, hand dominance, and occupation of the patient are important considerations in choosing the reconstruction method. The principles of management include preserving as much of the struc-



**Figure 6.** Representation of the statistical analysis between groups with regards to TUNEL + cells in region 3 of the avulsed flap.

tures as possible, providing early primary skin coverage and allowing early return of function. Choices of treatment methods are thoroughly discussed throughout the literature.<sup>[15]</sup> In instances of circumferential single plane degloving of the skin, the degloving is usually between the deep fascia and the subcutaneous fascia and skin. Mac Collum recommended conservative management in 26 “wringer” injuries that caused circumferential single plane degloving.<sup>[16]</sup> In his treatment algorithm, conservative management composed of removing the subcutaneous hemorrhage and nonviable fat tissue followed by drainage and pressure dressings. The simplest and most frequently used treatment in degloving injuries over the deep fascia, is to re-suture the avulsed flap. Numerous authors recommend wound debridement and coverage of the defect with skin grafts, perhaps harvested from the avulsed flaps.<sup>[17]</sup> Microsurgical techniques may be used as described by Waikakul, where avulsed flaps are used as perforator-based flaps.<sup>[18]</sup> The VAC™ therapy (KCI, Inc., San Antonio, Texas) has been proven to be useful in the management of the degloved foot, since it provides a secure contact of the graft with the bed. Dermal substitutes have been tried in degloving injuries with relative success.<sup>[19, 20]</sup> However the cost-effectivity of the latter 2 techniques are arguable.

Endothelial dysfunction is one of the characteristics of ischemia reperfusion injury. De With et al. reported that reperfusion injury impairs ascending vasodilation in the feeding arteries of hamster skeletal muscle.<sup>[21]</sup> In our study, Sivelestat induced angiogenesis through providing endothelial recovery in the avulsed flap which have been shown by CD31 immunofluorescence stainings and the results are statistically significant compared with the control group and the pentoxifylline group. Upholding endothelial recovery enhanced the surviving length of the degloved segment of the flap.

Degloving injury may exert both early and late complications. Early complications such as serious infection, distal ischemia, may currently be dealt with the use of neutrophil-elastase inhibitor. Therefore, another important part that needs to be put forward in this study is the dominant anti-inflammatory properties of neutrophil elastase inhibitors. Neutrophil elastase inhibitors reduce the level of inflammatory mediators by inhibiting NF- $\kappa$ B. Elastase is stored in cytoplasmic granules, its' primary role being intracellular degradation of foreign proteins during phagocytosis. It is for this reason, that Sivelestat has found use in acute systemic respiratory distress syndrome and in the treatment of shock.<sup>[22, 23]</sup> The release of elastase during postischemic reperfusion may be an important mechanism by which neutrophils cause tissue injury. We have shown in this study that Sivelestat treated groups, present with less

inflammation. Pentoxifylline, on the other hand, doesn't present a marked anti-inflammatory effect. The vasodilatory effects of Pentoxifylline, and anti-oxidant properties of Allopurinol stand out more significantly in treating degloving injuries.<sup>[10]</sup>

Our study showed that, the amount of DNA fragmentation and chromatin condensation is significantly decreased by the administration of Sivelestat and the results when compared to Pentoxifylline, are more effective in the salvage of the degloved segment( $p < 0.05$ ).

It is evident that a reversely-perfused flap can survive via formation of direct vascular anastomosis between the recipient and donor area, which usually takes place during the first 72 hours.<sup>[24]</sup> Sivelestat increases angiogenesis, therefore, the amount of direct vascular anastomosis significantly increases when Sivelestat is administered at 10 mg/kg/hour dosage.<sup>[25-27]</sup>

In conclusion, we have demonstrated that Sivelestat treatment improves flap survival in degloved segments by simultaneously attenuating apoptosis and enhancing angiogenesis, which is a unique result of this study. It is our belief that, the modulation of anti-PECAM antibodies play an important role in this response. These findings warrant further investigation of intravenous Sivelestat effects in improving the degree of tissue preservation in degloving injuries of the extremities. Increasing the survived length of avulsed flap, raises the possibility of being able to restore a more normal gait than would be possible using a free flap or other techniques. This conservative treatment modality should be kept in mind for the treatment of extreme soft tissue degloving injuries. It is our premise that an improved understanding of degloving injury mechanisms will result in improved clinical approaches to heal these complex injuries.

#### Disclosures

**Ethics Committee Approval:** The study was approved by the Local Ethics Committee.

**Peer-review:** Externally peer-reviewed.

**Conflict of Interest:** None declared.

**Authorship contributions:** Concept – E.Y., K.Z.S.; Design – K.Z.S.; Supervision – S.K.; Materials – F.I., D.D.; Data collection &/or processing – F.I., D.D.; Analysis and/or interpretation – K.Z.S., M.V.K.; Literature search – E.Y.; Writing – E.Y.; Critical review – S.K.

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