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. This study aims 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: In this study, total flap area (cm²), area of necrosis in the flap (cm²), and the ratio between the necrotic and total areas (percentage) were determined. Angiogenesis among the groups was documented with CD31, anti-PECAM staining. TUNEL assay was performed to allow the visualization of cell nuclei containing fragmented DNA, a typical feature of apoptosis.

Results: The findings obtained in this study showed 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.


Avulsion and degloving injuries arises from a vast array of mechanisms, machines and situations, in which the hand is injured with low energy shearing forces in a rigid position. 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 high 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 extension of the injury mechanism into four groups: a) circumscribed degloving injury that arises from tangential forces, b) non-circumferential degloving, c) single and d) multiplane circumferential degloving injury. 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. 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®, N-[2-[[4-[[2,4-dimethylpropanoyloxy]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. Sivelestat® 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. Sivelestat® 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. Sivelestat® 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. Sivelestat® 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. Sivelestat® 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. Pentoxifylline is a competitive nonselective phosphodies- terase inhibitor, which raises the intracellular cAMP, activates pKA, inhibits TNF and leukotriene synthesis. In addition, pentoxifylline improves red blood cell deformability and decreases the potential for platelet aggregation. 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. This study aims 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, this study was initiated. A total of 24 male Wistar rats, weighing between 250 and 350 g, were used in this experiment and divided into three groups, eight rats in each group. Animals were anesthe- tized with 35mg/kg Ketamine (Ketalar®, Pfizer, Istanbul) and 5 mg/kg Xylazine(Rompun®, 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 the Laboratory of Microsurgery, Division of Plastic Surgery of Sao Paulo.

Briefly, the right hind limb was incised circumferentially at the inguinal ligament level. Four Backhaus clamps were positioned in the four 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 five 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, which was also described as the control group, 1 cc of saline through the tail vein was administered. In group 2, which was described as the Pentoxifylline group, 1 cc of pentoxifylline (25 mg/kg), (Trental®, Hoechst pharmaceutical industry, Istanbul) was administered through the tail vein as a single injection given gradually. In group 3, which was the Sivelestat group, 1cc (10 mg/kg) of Sivelestat ® was administered after creating the degloved flap. 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 seven days. At the end of this period, the rats were sacrificed by an overdose of Thiopental. No signs of autophagy were observed in the avulsed flaps. In group 1 animals, signs of infection were observed in three animals. The total area of flap necrosis was calculated using Image J software (National Institutes of Health, NIH, USA). Total flap area (cm²), area of necrosis in the flap (cm²), and the ratio between the necrotic and total areas (percentage) were determined (Fig. 2a-c, Fig. 3)
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 using Image J analysis (Fig. 4a-c). Analysis of apoptotic cells was made by the 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 ºC 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 five independent examiners through counting numbers of cells or profiles in four randomly defined regions of interests per tissue measuring 62.500 µm². Two sections were processed for each animal. Mean values were calculated for both sections for all specimens (Fig. 3).[13]

![Figure 2. Macroscopic view of the avulsed flap on postoperative day seven in groups 1, 2 and 3.](image)

![Figure 3. Schematic view of the avulsed flap segment divided into four regions.](image)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total Flap Area (cm²)</th>
<th>Necrotic Area (cm²)</th>
<th>Necrosis Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gr 1</td>
<td>Gr 2</td>
<td>Gr 3</td>
</tr>
<tr>
<td>Lower Value</td>
<td>5.245</td>
<td>5.17</td>
<td>5.23</td>
</tr>
<tr>
<td>Median Value</td>
<td>5.431</td>
<td>5.23</td>
<td>5.45</td>
</tr>
<tr>
<td>Higher Value</td>
<td>6.19</td>
<td>5.78</td>
<td>6.71</td>
</tr>
<tr>
<td>Significance Level</td>
<td>p=0.8165</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
</tr>
</tbody>
</table>

Significant difference exists between groups 1 and 2 (p<0.01), groups 1 and 3 (p<0.01) and groups 2 and 3 (p<0.05).
TUNEL Assay

Analysis of DNA-fragmented cells from the sections harvested from animals was 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 a blinded manner in four randomly defined regions in the epidermis (each 62,500 µm²) as above. Apoptotic cells were identified by the characteristics of nuclear condensation, fragmentation and margination (Fig. 5a-c) (Table 2, 3).

Statistical Analysis

The results were 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 three groups was performed. Inter-observer results correlated well and the significance level was 95% (p<0.05).

Results

Animals: One animal from Group 2 (Pentoxifylline) was excluded from this study 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 (cm²), the necrotic area in the flap (cm²), and the ratio between necrotic and total areas (%) were determined using Image J analysis in each group after seven days. The total flap area (cm²) 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. The ANOVA-Tukey analysis showed significant differences between pairs, con-
trol 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 was followed by an increase in the density of CD31+ capillaries. In Sivelestat treated animal, capillaries increased to almost 150% of the 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 µm² 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 seven, compared to the other two groups (p<0.05, p<0.01, respectively). The apoptotic cells were counted in four 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® 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 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 concerning planimetry and percentages of necrotic areas, which were determined only macroscopically.

Many factors play a role before a decision is made about

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

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

*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**

<table>
<thead>
<tr>
<th></th>
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<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Region 1</td>
<td>31.7±22.5</td>
<td>25.7±16.1</td>
<td>3.0±4.1</td>
<td>0.019</td>
</tr>
<tr>
<td>Region 2</td>
<td>36.5±22.8</td>
<td>20.2±13.1</td>
<td>14.3±8.1</td>
<td>0.07</td>
</tr>
<tr>
<td>Region 3</td>
<td>41.3±23.1</td>
<td>29.0±19.7</td>
<td>7.3±9.9</td>
<td>0.019</td>
</tr>
<tr>
<td>Region 4</td>
<td>48.5±28.6</td>
<td>34.7±17.2</td>
<td>9.3±8.8</td>
<td>0.012</td>
</tr>
</tbody>
</table>

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

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Figure 6. Representation of the statistical analysis between groups with regards to TUNEL (+ cells in region 3 of the avulsed flap.
the treatment protocol in degloving injuries. The age of
the patient, hand dominance, and occupation of the pa-
ient are important considerations in choosing the recon-
struction method. The principles of management include
preserving as much of the structures as possible, provid-
ing early primary skin coverage, and allowing the early re-
turn of function. Choices of treatment methods are thor-
oughly discussed throughout the literature. In instances
of circumferential single plane degloving of the skin, the
degloving is usually between the deep fascia and the sub-
cutaneous fascia and skin. Mac Collum recommended con-
servative management in 26 “wringer” injuries that caused
circumferential single plane degloving. In his treatment
algorithm, conservative management composed of re-
moving 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.
Many authors recommend wound debridement and cover-
age of the defect with skin grafts, perhaps harvested from
the avulsed flaps. Microsurgical techniques may be used
as described by Waikakul, where avulsed flaps are used as
perforator-based flaps. The VAC™ therapy (KCI, Inc., San
Antonio, Texas) has been proven to be useful in the man-
agement 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.
However, the cost-effectiveness of the latter two techniques
is 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. In our study,
Sivelestat induced angiogenesis through providing endo-
thelial recovery in the avulsed flap, which have been shown
by CD31 immunofluorescence staining and the results are
statistically significant compared with the control group
and the pentoxifylline group. Upholding endothelial re-
covery enhanced the surviving length of the degloved seg-
ment of the flap.

Degloving injury may exert both early and late complica-
tions. Early complications, such as serious infection, distal
ischemia, may currently be dealt with the use of neutro-
phil-elastase inhibitor. Therefore, another important part
that needs to be put forward in this study is the dominant
anti-inflammatory properties of neutrophil elastase in-
hibitors. Neutrophil elastase inhibitors reduce the level of
inflammatory mediators by inhibiting NF-κβ. Elastase is
stored in cytoplasmic granules, its’ primary role being in-
tracellular degradation of foreign proteins during phago-
cytosis. It is for this reason that Sivelestat has found use
in acute systemic respiratory distress syndrome and in
the treatment of shock. 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, does not
present a marked anti-inflammatory effect. The vasodila-
tory effects of Pentoxifylline and anti-oxidant properties
of Allopurinol stand out more significantly in treating deglo-
ing injuries.

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

A reversely-perfused flap can survive via the formation of
a direct vascular anastomosis between the recipient and
donor area, which usually takes place during the first 72
hours. Sivelestat increases angiogenesis. Therefore, the
amount of a direct vascular anastomosis significantly in-
creases when Sivelestat is administered at 10 mg/kg/hour
dosage.

**Conclusion**

In conclusion, we have demonstrated that Sivelestat treat-
ment improves flap survival in degloved segments by simul-
taneously attenuating apoptosis and enhancing angiogen-
esis, which is a unique result of this study. It is our belief that
the modulation of anti-PECAM antibodies plays an impor-
tant role in this response. These findings warrant further in-
vestigation of intravenous Sivelestat effects in improving the
degree of tissue preservation in degloving injuries of the ex-
tremities. Increasing the survived length of the avulsed flap
raises the possibility of being able to restore a more normal
gait than would be possible using a free flap or other tech-
iques. This conservative treatment modality should be kept
in mind for the treatment of extremely 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 Lo-
cal 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 pro-
Literature search – E.Y.; Writing – E.Y.; Critical review – S.K.