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Necrostatin-1 protects against ischemia/reperfusion injury by inhibiting receptor-interacting protein 1 in a rat flap model

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KEYWORDS
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Summary *Introduction:* The failure of reconstructive surgeries remains a challenge for plastic surgeons. Ischemia reperfusion (I/R) injury is considered to be one of the major problems in flap surgery. Necroptosis is a recently discovered and caspase-3-independent programed necrosis. Necrostatin-1 (Nec-1) is a specific inhibitor of necroptosis. Reports indicate that Nec-1 provides protection in ischemic models, such as brain, kidney, and heart. The aim of this study is to investigate the influence of Nec-1 on the I/R process in rat abdominal skin flaps.
Methods: Twenty male Sprague-Dawley rats, weighing 280–320 g, were randomly divided into three groups. The extended epigastric skin flap (6 cm × 9 cm) of rats was used. Three hours of complete ischemia was performed using a clamp, and the clamp was then removed to reperfusion the flap. Twenty-four hours after the onset of the reperfusion, the rats were assessed for flap survival and perfusion analysis. One sample (1 cm × 1 cm) was taken for H&E, TUNEL, electron microscopy, IHC staining for RIP-1, and ELISA analysis for caspase-3 activity.
Results: Compared to the CTL group, the flap in the Nec-1 group showed a higher survival rate and better blood perfusion. In histological observation, skin flap in the Nec-1 group showed less inflammatory infiltration than the CTL group. The AI in the CTL group was higher than that in the Nec-1 group and showed typical morphological changes of apoptotic cells. In IHC study, RIP-1 expression was higher in the CTL group. But there was no significant difference between the two groups in caspase-3 activity detection.

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Conclusion: Nec-1 has a protective effect against I/R injury through the inhibition of RIP-1 on the skin flap model; this makes it a promising novel strategy in clinical setting.

Introduction

Skin flap transfers are widely used in wound coverage and reconstruction, but soft tissue necrosis remains a challenging problem. In Chen's study,[1] out of 1142 free flap operations, 113 operations had complications, and 82% of these 113 cases presented with some form of circulatory compromise within 24 h after surgery. The main cause of flap loss is ischemia/reperfusion (I/R) injury. During I/R injury, cytological and morphological changes occur, resulting in cell death. The classical pathways of cell death are necrosis and apoptosis, but recently, our study[2] demonstrated a new cell death pathway called necroptosis that exists in flap I/R injury models. In general, necrosis is related to unprogramed cell death, while programed cell death occurs through apoptosis. Necroptosis is a well-defined viral defense mechanism, allowing cells to undergo "cellular suicide" in a caspase-independent manner in the presence of viral caspase inhibitors.[3]

In Degterev's study,[4] necrostatin-1 (Nec-1) was identified as a specific and potent small molecule inhibitor that blocks a critical step in necroptosis. Nec-1 has protective effects against necroptosis during I/R injury in many tissues and organs, such as hippocampal neurons,[5] myocardia,[6] kidneys,[7] and brain.[8] In cell research, Nec-1 also attenuates mitochondrial dysfunction in neurons and astrocytes following neonatal hypoxia-ischemia.[9] The possible mechanism underlying this effect might be inhibition of receptor-interacting protein 1 (RIP-1) by Nec-1. Degterev also demonstrated that RIP-1 is the primary cellular target responsible for the anti-necroptotic activity of Nec-1.[10] Based on all these theories, this study aimed to test whether Nec-1 has any protective effects that attenuate I/R injury to improve skin flap survival in rats.

Materials and methods

Animals

All protocols were approved by the Committee on Animal Rights Protection at Peking Union Medical College Hospital and were in accordance with the National Institutes of Health guidelines for the care and the use of laboratory animals. Twenty adult male Sprague-Dawley rats, weighing 280–320 g, were housed in individual cages under standard conditions at a temperature of 22–25 °C, with a 12-h light-dark cycle. Pre- and postoperatively, the rats were fed a normal diet with sufficient food and drink.

Experimental protocol and study groups

Twenty qualified laboratory Sprague-Dawley rats were randomly divided into 3 groups: a sham surgery (SH) group, a control (CTL) group, and a Nec-1 treatment (Nec-1) group. The surgical procedure was similar to the method reported by Liu.[11] The rats were anesthetized with intraperitoneal pentobarbital (40 mg/kg) injections. A rectangular 6 cm × 9 cm skin flap was created and marked on the abdomen. The flap was then elevated along the marked line. The left superficial epigastric artery was ligated in the CTL and Nec-1 groups. A 3-h period of flap ischemia was induced in both the CTL and Nec-1 groups by clamping the right superficial epigastric artery. Ischemia was not induced in the SH group, but the left superficial epigastric artery was still ligated. Then, the flap was resutured with a 0.1 mm silicone sheet to prevent neovascularization from the recipient bed. Fifteen minutes before and after removing the clamp, phosphate-buffered saline (PBS) (200 μl) was administered intraperitoneally in the CTL and SH groups. In the Nec-1 group, Nec-1 (1.65 mg/kg[12] in 200 μl total volume) was applied the same way as the PBS in the CTL group.

Evaluation of skin flap survival rate and blood perfusion

Twenty-four hours after the reperfusion initiation, the flap survival rate and average blood perfusion were measured by a laser Doppler flowmeter (Perimed AB, Stockholm, Sweden) and laser speckle contrast analysis cameras (Perimed AB, Stockholm, Sweden). The rats were anesthetized and secured on the operative bed to ensure that the whole flap was exposed, including the part of the normal abdominal skin. The laser Doppler flowmeter was used to measure the average blood flow in the skin flap (distance between skin flap and sensor = 20 cm). Perfusion in the flap area was evaluated automatically by delineating the specific area in the image with laser speckle contrast analysis cameras. Vascular flow was measured using perfusion units (PU, ml•100 g^{-1}•min^{-1}). Surviving flaps were harvested for sampling before the rats were killed by anesthesia overdose.

Histological analysis

A piece of skin tissue (1 cm × 1 cm) was taken from the middle part of the whole flap area for further analysis: hematoxylin and eosin (H&E) staining, TdT-mediated dUTP-X nick-end labeling (TUNEL) staining, electron microscopic examination, immunohistochemical (IHC) staining, and enzyme-linked immunosorbent assays (ELISAs) for caspase-3 activity detection were conducted.

The specimen was paraffin-embedded, sectioned, and mounted on a slide. Then, the paraffin slides were deparaf-

finized in xylene and rehydrated with various concentrations of ethanol. Finally, the slides were stained with H&E for histological analysis.

Apoptosis assay

TUNEL staining was used to measure the apoptosis index (AI) and was performed by using an In Situ Cell Death Detection Kit (Roche, Basel, Switzerland). The protocols were conducted according to the manufacturer's instructions. After paraffin slides were heated to 70 °C and dewaxed, the sections were rehydrated and incubated in a working solution with 20 μg/ml proteinase K for 15 minutes at room temperature. The slides were rinsed with PBS (5 minutes, 3 times) and incubated in TUNEL reaction mixture for 1 h at 37 °C. Then, converter-POD was added to the samples for 1 h at 37 °C. The slides were rinsed with PBS and stained with 3, 3′-N-diaminobenzidine (DAB) tetrahydrochloride. Five slide fields were randomly examined using a defined rectangular field area under 40 × magnifications. Apoptotic cells were counted under 400 × magnifications. The AI is presented as the percentage of TUNEL-positive cells versus the total number of cell nuclei per field.

Morphological observation

Transmission electron microscopy was used to observe cell morphology. Observation was performed by a JEM-1400 microscope (JEOL, Japan) at the Institute of Basic Medical Sciences Chinese Academy of Medical Sciences & School of Basic Medicine Peking Union Medical College (12,000 × image magnifications, 80 kV acceleration voltages).

Immunohistochemical studies

Paraffin slides were deparaffinized in xylene and rehydrated with various concentrations of ethanol. To deactivate endogenous oxidase, 3% H_2O_2 was used. Slides underwent antigen retrieval by heating, were blocked with goat serum and incubated overnight at 4 °C with RIP-1 antibody (1:50 dilution, ZSGB-BIO, Beijing, China). The slides were then rinsed in PBS (3 minutes, 3 times) and incubated with horseradish peroxidase-conjugated secondary antibody (ZSGB-BIO, Beijing, China). After rinsing with PBS (3 minutes, 3 times), the slides were stained with DAB and then counterstained with hematoxylin. The brown color signifying the presence of antigen bound to antibody was detected by a light microscope (Olympus Corporation, Tokyo, Japan) equipped with a computer-controlled digital camera and imaging software.

Caspase-3 activity assay

A fluorometric assay kit (Biovision Research Products, Mountain View, CA, USA) was used for caspase-3 activity assays. Briefly, 40 mg of tissue was homogenized in 2 × reaction buffer and then was incubated at 37 °C for 1 h with 1 mM caspase-3 substrate (DEVD-APC). Substrate cleavage was measured with a spectrofluorometer at 400 nm.

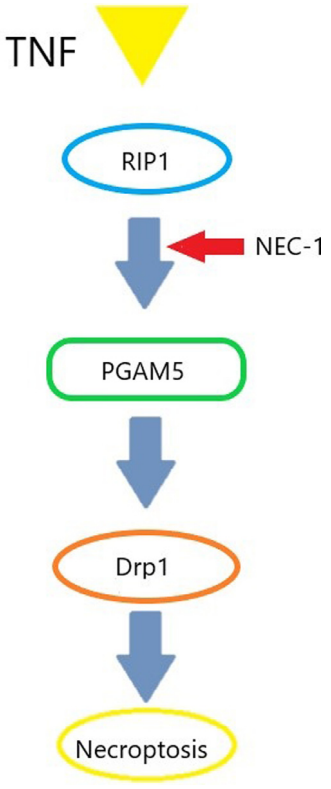

Figure 1 Necroptosis pathway. Upon TNF induction, TNF receptor 1(TNFR1) induce RIP1 to form TNFR1 complex I. This complex regulates necroptosis. It phosphorylates MLKL and PGAM5, then activate the mitochondrial fission regulator Drp1, thus leading to mitochondrial fission. Necrostatin-1 blocks necroptosis by targeting RIP1 kinases.

Statistical analysis

All data in this study are presented as means ± standard deviation (SD). Significant differences were determined by *t*-tests. Statistical significance was set at $p < 0.05$. All analyses were performed using SPSS 22.0 software.

Results

Skin flap survival and perfusion evaluation

Flap survival rate was measured by the blood distribution area, and the average blood perfusion of the flap was evaluated by blood flow (Figure 2A). In the SH group, the flap survival rate was 93.7950 ± 4.2050%, and the average blood perfusion was 82.5250 ± 12.5950 PU. In the CTL group, the flap survival rate was 73.3780 ± 9.0429%, and the average blood perfusion was 43.5250 ± 8.1661 PU. In the Nec-1 group, the flap survival rate was 81.5750 ± 9.0422%, and the average blood perfusion was 53.8950 ± 12.2251 PU. The skin flap survival rate ($p < 0.05$, Figure 2B) and average blood

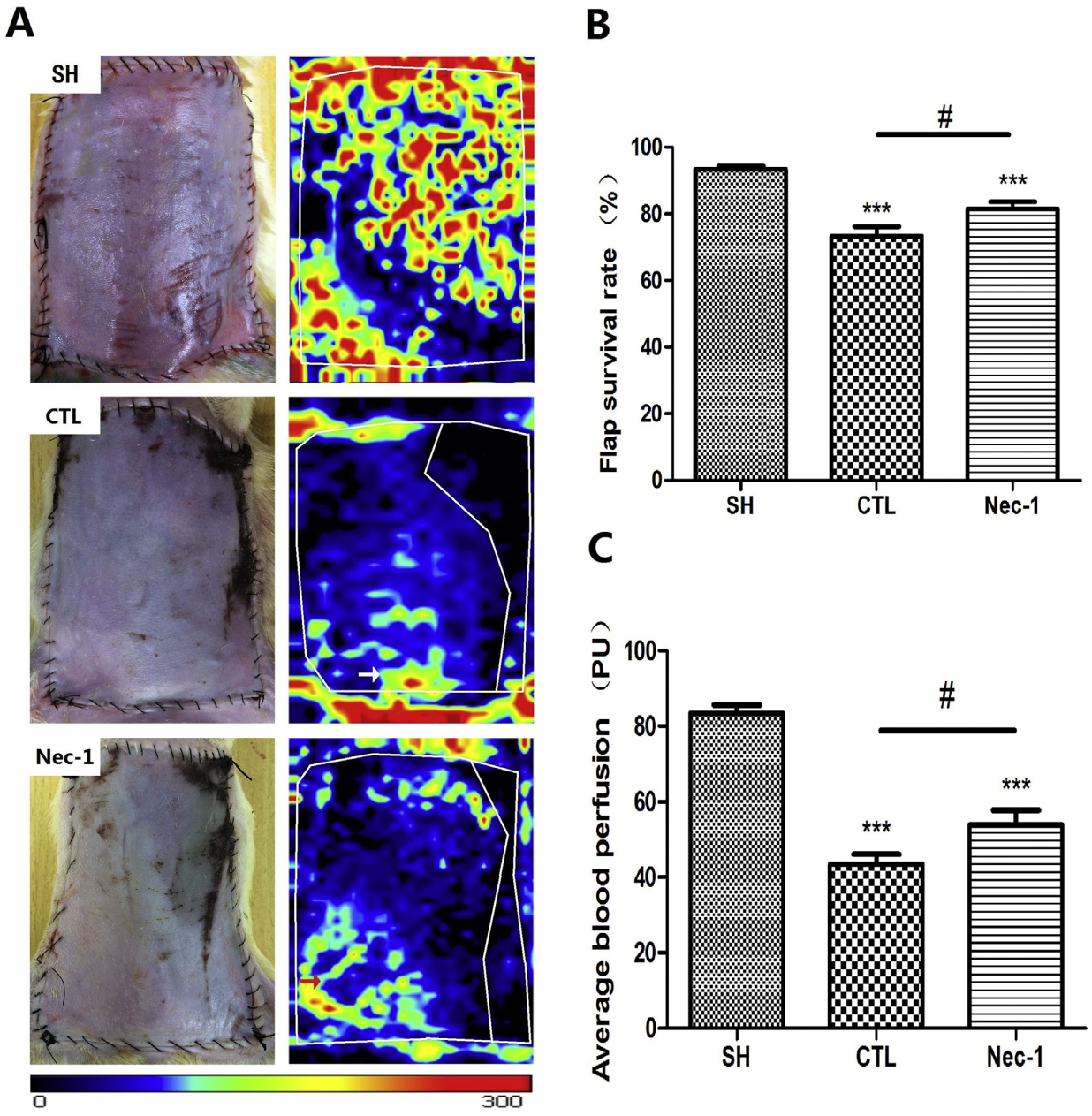

Figure 2 The survival condition of the abdominal skin flaps. White arrows pointing to black zones represent the lowest blood perfusion; in contrast, a red arrow pointing to red, yellow and adjacent blue areas represents rich blood flow. (A) Abdominal skin flap microcirculation in all three groups. (B) The flap survival rate in all the three groups. Compared with the CTL group, the SH and Nec-1 groups have higher flap survival rates. (C) The average blood perfusion in all three groups. Blood perfusion was greater in the SH and Nec-1 groups than in the CTL group. The values are means ± SD; ($n = 10$ for each group; #$p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

perfusion ($p < 0.05$, Figure 2C) were significantly different among the SH, CTL, and Nec-1 groups.

H&E staining

H&E staining was used to assess ischemic injury (Figure 3) by evaluating inflammatory infiltration. Inflammatory infiltration was observed in all the three groups, but the number of inflammatory cells was greatest in the dermal and subcutaneous layer of the CTL group skin tissue, which indicated that Nec-1 may attenuate the inflammatory response.

Detection of apoptotic cells

TUNEL-positive cells were stained brown, as shown in Figure 4A. In the same position of the flap, the number of TUNEL-positive cells was highest in the CTL group

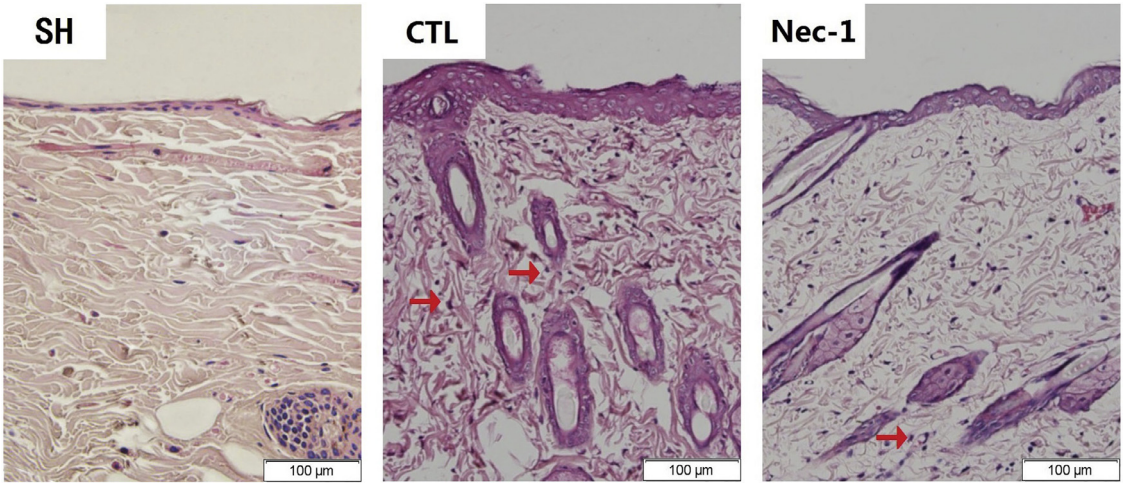

Figure 3 H&E staining in both groups. Inflammatory infiltration was observed in both groups, but the most inflammatory cells (red arrow) were found in the dermal and subcutaneous layer of the CTL group skin tissue (images, 400 ×). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

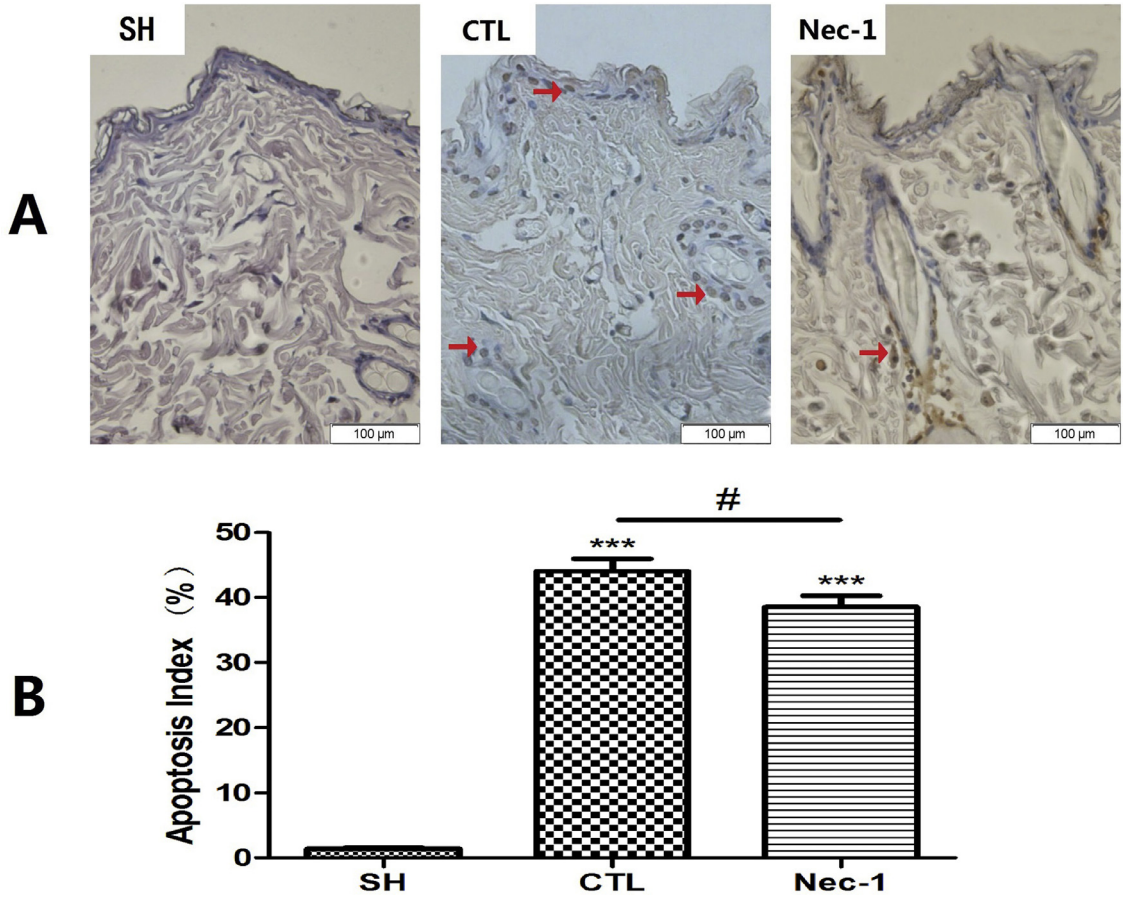

Figure 4 Evaluation of apoptotic cells by TUNEL staining in each group. (A) TUNEL staining images. Brown dots indicate apoptotic cells (red arrow) (images, 400 ×). (B) Calculation of the proportion of TUNEL-positive cells. The most TUNEL-positive cells were observed in the CTL group. Values are means ± SD; ($n = 10$ for each group; #$p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

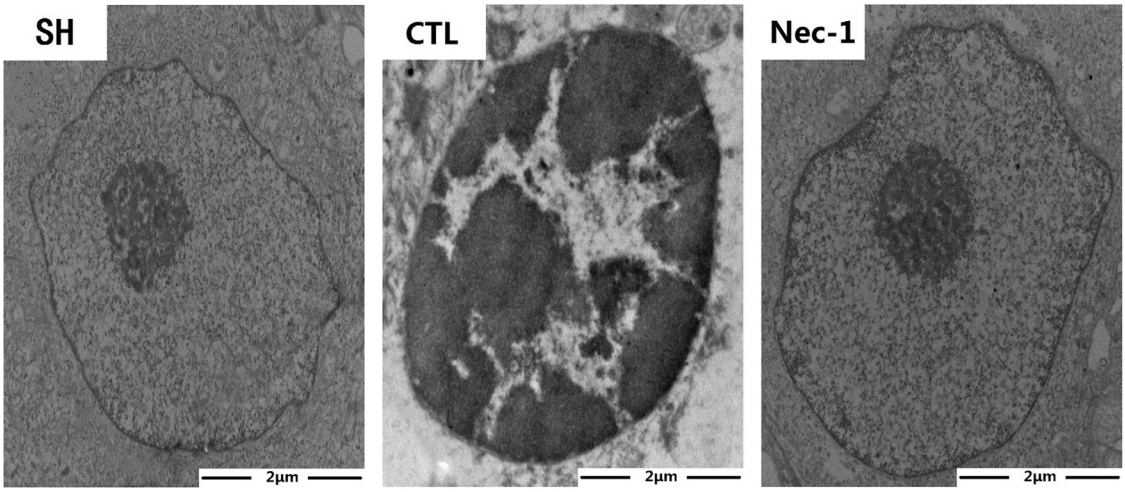

Figure 5 Morphological images of cells in each group by a transmission electron microscope. The morphological characteristics of apoptotic cells are as follows: nuclei present with chromatin pycnosis and clustering on the inner karyotheca border, condensed cytoplasm, and many cytoplasmic vacuoles. In the CTL group, typical apoptotic cells were found. In contrast, apoptotic cells were barely visible in the SH and Nec-1 groups (images, 12,000 ×).

(44.0311 ± 6.2113%). The SH (1.8903 ± 1.0603%), CTL, and Nec-1 groups (38.5397 ± 5.3106%) were significantly different ($p < 0.05$) (Figure 4B).

Electron microscopic observation

Morphologic changes in apoptotic cells were identified using a transmission electron microscope. Typical morphological characteristics of apoptotic cells are as follows: cell body shrinkage, increased cytoplasm density, and chromatin condensation are present, but the nuclear membrane, plasma membrane, and organelles are intact. In the CTL group, typical apoptotic cells were identified (Figure 5).

RIP-1 immunohistochemical studies

IHC studies reflect the expression of target proteins, such as RIP-1, which has been shown to be key necroptotic proteins. The results (Figure 6) revealed that RIP-1 expression was much higher in the CTL group. Compared with the Nec-1 (47.5397 ± 8.6263%) and SH groups (4.7832 ± 3.5012%), 61.0029 ± 9.4371% of the CTL group cells were RIP-1 positive; this difference was significant difference ($p < 0.01$). These data reveal that Nec-1 effectively decreases the key factor of necroptosis, RIP-1.

Caspase-3 activity

Caspase-3 activity was clearly upregulated 24 h after reperfusion in all the three groups (Figure 7). Compared with the CTL (0.7543 ± 0.1970) and SH rats (0.1073 ± 0.0139), the rats subjected to skin flap I/R with Nec-1 treatment (0.7757 ± 0.2109) did not present with any significant change ($p > 0.05$) in caspase-3 activity.

Discussion

Flap transfer is a challenging technique for all plastic surgeons, as evidenced by the high risk of flap complications. Even in cases of microsurgical transfer by experienced hands in the presence of a stable blood supply, skin flap loss still ranges from 1% to 5%.[13,14] There are many reasons for partial or total flap loss, including I/R injury. I/R injury is tissue damage caused by the blood supply returning to the tissue after a period of ischemia; cell death is the worst consequence of ischemia. Conventionally, two main classical forms of cell death exist: necrosis and apoptosis.[15] Necrosis is the form of cell injury that results in the premature death of cells in living tissue by autolysis.[16] Apoptosis is the process of programmed cell death that occurs in multicellular organisms.[17]

With continued studies, a novel type of cell death has been discovered and termed necroptosis. In our former study, the results revealed that necroptosis exists in I/R injury skin flaps, and necroptotic cells are commonly distributed throughout the distant flap area from the pedicle.[2] The mechanisms of necroptosis are unclear. Vanden[3] demonstrated that extracellular stimuli act on receptors of TNF-α signaling, which leads to the recruitment of TNF receptor-associated death domain (TRADD). RIP-1 activation by TRADD leads to necrosome formation. The pro-necroptotic protein, MLKL, is activated by necrosomes and initiates the necrosis phenotype by inserting into the plasma membranes of organelles, resulting in cellular content release into extracellular interspace.[18,19]

Nec-1, a specific, potent small molecule inhibitor that blocks a critical step in necroptosis, was first reported by Degterev.[4] Nec-1 inhibits the autophosphorylation of overexpressed, endogenous RIP-1, which is also considered a selective allosteric inhibitor of death domain receptor-associated adaptor kinase RIP-1 in vitro. In I/R injury studies, Nec-1 is also regarded as a new treatment approach against I/R injury. Yin's study[5] demonstrated that

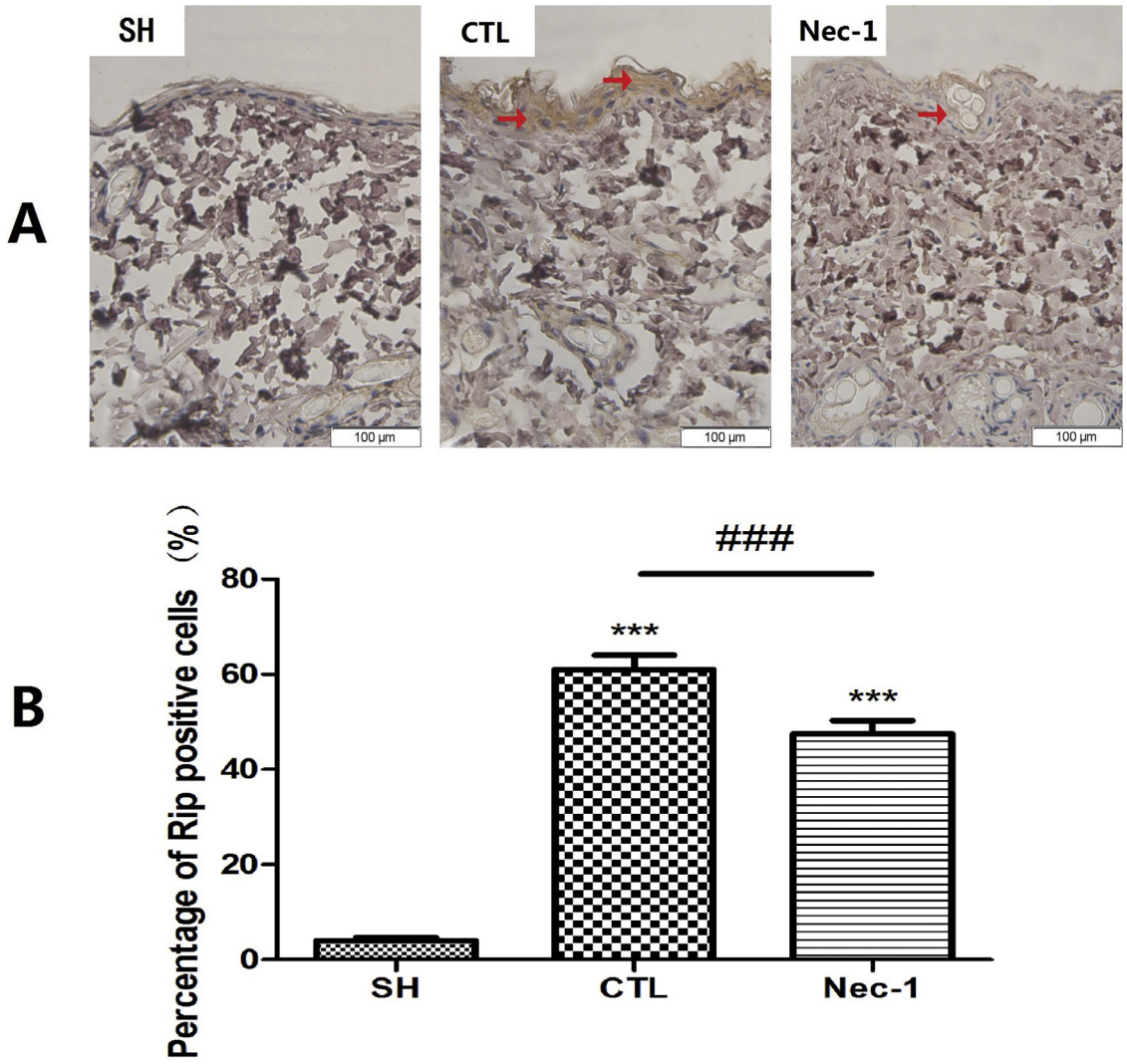

Figure 6 RIP-1 expression in both groups. (A) Representative micrographs (400 ×) of RIP-1 IHC. Brown staining indicates positive expression area, and the color shade is directly proportional to the protein expression level. In the CTL group, RIP-1 expression presented as (++~+++) and as (+~++) in the Nec-1 group and (−~+) in the SH group. (B) Percentage of RIP-1-positive cells. Flap tissues in the SH and Nec-1 groups presented with a low percentage of RIP-1-positive cells. Values are means ± SD; (n = 10 for each group; ##$p < 0.01$).

Nec-1 inhibits neuronal death induced by I/R injury through preventing RIP-3 upregulation and nuclear translocation, as well as NAD + depletion and cathepsin-B release. In 2015, Koudastaal[6] reported that intravenous Nec-1 administration prior to reperfusion was an effective strategy for significantly reducing infarct size and preserving left ventricular function. Studies on renal and cerebral I/R injury are always reaching highlights. Linkermann[7] mentioned that Nec-1 may have therapeutic potential for preventing and treating renal I/R injury. Xu demonstrated that Nec-1 conferred neuroprotection during hypoxia/ischemia/reperfusion injury in vitro and in vivo via anti-necroptotic effects. In plastic surgery, flap loss caused by I/R injury is one challenging problem. However, no evidence exists suggesting that Nec-1 has a similar protective effect against I/R injury in skin flap models. In this study, a rat abdominal skin flap I/R model was used to assess the effect of Nec-1 towards I/R injury.

In the Nec-1 group, the flap survival rate was better, the average blood perfusion was greater, and less inflammatory cell infiltration was observed than CTL group. Researchers report that the strong inflammatory response provoked by ischemia reperfusion injury might be triggered by necroptotic cells, and I/R injury-induced necroptosis is closely related to inflammation.[18–20] Neutrophil infiltration is a classic characteristic of acute inflammation during I/R injury and has been shown to worsen reperfusion injury by leukocyte activation.[21] In 2015, Liu[22] concluded that Nec-1 treatment reduced pro-inflammatory cytokine production and extracellular HMGB1 release during active necroptosis.

In the TUNEL staining results, the CTL group had the most apoptotic cells, and this finding was reflected in the AI results. The AI was significantly different among the SH, CTL, and Nec-1 groups. In Zhao's study.[23] the increased lung cell death determined by TUNEL staining was significantly

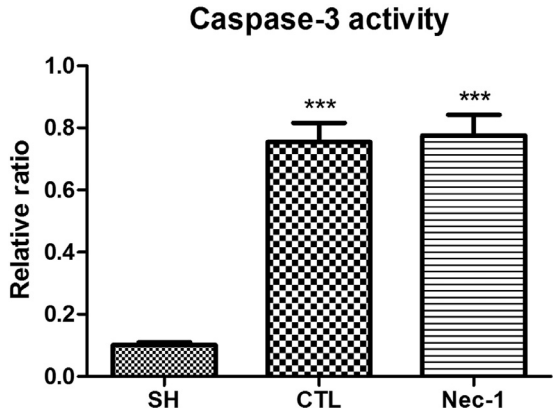

Figure 7 Caspase-3 activity in all the three groups. Compared with the SH group, the CTL and Nec-1 groups presented with a significant reduction in caspase-3 activity. However, caspase-3 activity was not significantly diminished in the Nec-1 group. Values are means ± SD; (n = 10 for each group).

reduced after Nec-1 treatment in a rat allogeneic transplantation model. Lee also arrived at a similar conclusion on ovarian cryopreservation and transplantation.[24] The number of apoptotic cells was effectively decreased in the Nec-1 group compared with the CTL group. Moreover, transmission electron microscopy was used to observe cell morphology. Typically, apoptotic cells present with cell body shrinkage, increased cytoplasm density, and chromatin condensation, which were easily observed in the CTL group. In I/R injury-induced necroptosis, RIP-1 plays a paramount role in mediating necroptotic pathways[25].

In IHC studies, RIP-1 expression presented as (++∼+++) in the CTL group and as (+∼++) in the Nec-1 group. The number of RIP-1-positive cells was significantly decreased in the Nec-1 group compared with the CTL group. Classical necroptosis requires RIP-1 for the activation of RIP-3 through the induction of RIP-1/RIP-3 necrosomes, which could be inhibited by Nec-1.[26]

Caspase-3 plays a unique role in facilitating the classic apoptotic process and is not involved in necroptosis. The in vivo data presented by Rosentreter et al. revealed that RIP-1-mediated necroptosis is not present in post-ischemic tissue, which means I/R-triggered caspase activation negatively regulates necroptosis.[25] In our results, caspase-3 activity was not significantly different among the SH, CTL, and Nec-1 groups; this finding is similar to Rosentreter's conclusion.

In this study, we found that Nec-1 can improve the skin flap survival and decrease a necroptotic key factor, RIP1 in rat abdomen flap model with I/R injury. This study reveals Nec-1 may have therapeutic potential to prevent and treat I/R injury in flap transplantation.[13] By using Nec-1, we might partly protect flaps from I/R injury after surgery. Especially in patients with a high risk of flap complications (diabetes, malnutrition, and infection). However, Takahaashi points out that Nec-1 has a paradoxical dose-response effect. Low concentrations have been suggested as toxic with improved survival at higher concentrations, while Nec-1 s (Necristamin-1 stable) did not exhibit this low dose toxicity,[29] This reminds us that clinical application of Nec-1 still needs further evaluation.

In addition to Nec-1, many other drugs have been discovered that have therapeutic effects against I/R injury, such as hydrogen-rich saline,[11,13] methane-rich saline,[27] hydroxyzine, and cimetidine.[28] However, all these drugs focus on classic cell death pathways, such as apoptosis and necrosis. Our study concentrated on a novel type of cell death, necroptosis, in I/R injury and demonstrated the protective effect of Nec-1 against I/R injury by inhibiting RIP-1 in a rat flap model. However, to achieve the desired effect of attenuating I/R injury, combining Nec-1 therapy with other drugs may be more effective than using drugs individually.

Conclusion

According to the results of our study, the skin flap survival rate was improved and the necroptotic key factor, RIP-1, was notably decreased in the Nec-1 group compared with the CTL group. Therefore, we postulate that Nec-1 may be a novel therapy for decreasing necroptotic I/R injury in rat abdominal skin flaps.

Conflict of interest statement

Funding: This study was supported by National Natural Science Foundation of China (81471885).

Conflicts of interest

None declared.

Ethical approval

All protocols were approved by the Committee on Animal Rights Protection at Peking Union Medical College Hospital and were in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

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