Journal of Inflammation Research

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

DNase I and Sivelestat Ameliorate Experimental Hindlimb Ischemia-Reperfusion Injury by Eliminating Neutrophil Extracellular Traps

Chun-Lian Wang[1], Yan Wang[2], Qi-Lan Jiang[3], Yang Zeng[4], Qing-Ping Yao[5], Xing Liu[6], Tao Li[7], Jun Jiang[1]

[1]Department of General Surgery (Thyroid Surgery), The Affiliated Hospital of Southwest Medical University, Luzhou, People's Republic of China; [2]Department of Cardiology, The Fourth Hospital of Harbin Medical University, Harbin, People's Republic of China; [3]Department of Clinical Nutrition, The Affiliated Hospital of Southwest Medical University, Luzhou, People's Republic of China; [4]Department of Orthodontics, The Affiliated Stomatological Hospital of Southwest Medical University, Luzhou, People's Republic of China; [5]Institute of Mechanobiology & Medical Engineering, School of Life Science & Biotechnology, Shanghai Jiao Tong University, Shanghai, People's Republic of China; [6]Department of Cardiology, The Affiliated Hospital of Southwest Medical University, Luzhou, People's Republic of China; [7]Key Laboratory of Medical Electrophysiology of Ministry of Education, Collaborative Innovation Center for Prevention and Treatment of Cardiovascular Disease, Institute of Cardiovascular Research, Southwest Medical University, Luzhou, People's Republic of China

Correspondence: Jun Jiang; Tao Li, Email jiangjun@swmu.edu.cn; leta49@swmu.edu.cn

Purpose: Neutrophil extracellular traps (NETs) play an important role in ischemia-reperfusion injury (IRI) of the hindlimb. The aim of this study was to investigate the effect of recombinant DNase I and sivelestat in eliminating NETs and their effects on IRI limbs.

Patients and Methods: An air pump was used to apply a pressure of 300 mmHg to the root of the right hindlimb of the rat for 2 h and then deflated to replicate the IRI model. The formation of NETs was determined by the detection of myeloperoxidase (MPO), neutrophil elastase (NE), and histone H3 in the skeletal muscles of the hindlimbs. Animals were administered 2.5 mg/kg bw/d DNase I, 15 or 60 mg/kg bw/d sivelestat by injection into the tail vein or intramuscularly into the ischemic area for 7d. Elimination of NETs, hindlimb perfusion, muscle fibrosis, angiogenesis and motor function were assessed.

Results: DNase I reduced NETs, attenuated muscle fibrosis, promoted angiogenesis in IRI area and improved limb motor function. Local administration of DNase I improved hindlimb perfusion more than intravenous administration. Sivelestat at a dose of 15 mg/kg bw/d increased perfusion, counteracted skeletal muscle fibrosis, promoted angiogenesis and enhanced motor function. However, sivelestat at a dosage of 60 mg/kg bw/d had an adverse effect on tissue repair, especially when injected locally.

Conclusion: Both DNase I and moderate doses of sivelestat can eliminate IRI-derived NETs. They improve hindlimb function by improving perfusion and angiogenesis, preventing muscle fibrosis. Appropriate administration mode and dosage is the key to prevent IRI by elimination of NETs. DNase I is more valid when administered topically and sivelestat is more effective when administered intravenously. These results will provide a better strategy for the treatment of IRI in clinical.

Keywords: ischemia-reperfusion injury, IRI, neutrophil extracellular traps, NETs, DNase I, sivelestat, hindlimb

Introduction

Hindlimb ischemia-reperfusion injury (IRI), a complex inflammatory response, may occur after recanalization of compartment syndrome, crush syndrome,[1] vascular injury, and chronic arterial occlusive diseases.[2] In addition, surgical procedures, such as the use of a tourniquet to obtain a clearer surgical field during knee replacement surgery, can also cause iatrogenic IRI.[3] After the restoration of blood flow, metabolites and oxygen free radicals generated during ischemia and hypoxia may lead to further tissue damage due to microvascular dysfunction and increased permeability of capillaries and arterioles.[4,5]

Ischemia-reperfusion (IR) promotes the release of oxygen-free radicals, cytokines, and other proinflammatory mediators, which in turn activate neutrophils.[6,7] Neutrophils aggregate and degranulate in the area of IR, destroying tissue by free radicals and other mechanisms. NETosis is one of the important causes of tissue damage caused by neutrophils. Under the induction of reactive oxygen species (ROS), protein arginine deiminase 4 (PAD4), autophagy and other factors, neutrophils release DNA as

Received: 3 November 2022
Accepted: 14 February 2023
Published: 21 February 2023

a backbone and bind granule proteins (myeloperoxidase MPO, neutrophil elastase NE, etc.) and histones into highly concentrated filamentous structures called neutrophil extracellular traps (NETs).[8–10] In acute myocardial infarction,[11,12] acute renal ischemia,[13,14] ischemic stroke[15,16] and limb ischemia-reperfusion injury,[17] NETs exacerbate disease progression and are significantly associated with worse disease outcomes.

Treatment to eliminate NETs may reduce tissue damage in the IRI region and improve vascular remodeling, improving disease prognosis.[18] DNase I and sivelestat are two NETs elimination agents that have been used in studies. The scavenger DNase I of cell-free DNA (cfDNA) exerts protective effects in models of IRI such as the heart,[19] kidney,[20] and intestine.[21] DNase I improved perfusion in ischemic limbs of mice and reduced infiltrating inflammatory cells but did not alter skeletal muscle damage.[22] Infusion of the neutrophil elastase inhibitor sivelestat reduces IRI in the myocardium,[23,24] bladder,[25] testis,[26] liver,[27] and intestine,[28] but there is no report on the treatment of hindlimb IRI. In this study, we investigated the appropriate administration mode and dosage of DNase I and sivelestat on the clearance of NETs, hindlimb perfusion, motor function, skeletal muscle injury repair, and neovascularization in the rat hindlimb IRI model (Figure 1). These results will provide a better strategy for the treatment of IRI in clinical.

Materials and Methods

Animal Model and Ethical Protocol

The experimental process complied with the China Animal Management Regulations (Document No. 55 of the Ministry of Health of China in 2001) and was approved by the Animal Protection and Utilization Committee of Southwest Medical University. 8-week-old male Sprague-Dawley rats weighing approximately 250 g were purchased from the Experimental Animal Center of Southwest Medical University and were randomly divided into 8 groups with 9 rats in each group. Rats were anesthetized under isoflurane inhalation. Except for the control group, the rest of the rats had a 5-mm-wide pressure band applied to the root of the right hindlimb to block arterial blood at a pressure of 300 mmHg. The toe claws of the rats became pale, cyanotic, and cold. The pressure was released after 2 h to achieve reperfusion. Rats were executed analgesia via subcutaneous injection of 0.05 mg/kg buprenorphine 30 min prior to surgical intervention and repeated at 24 h.[29]

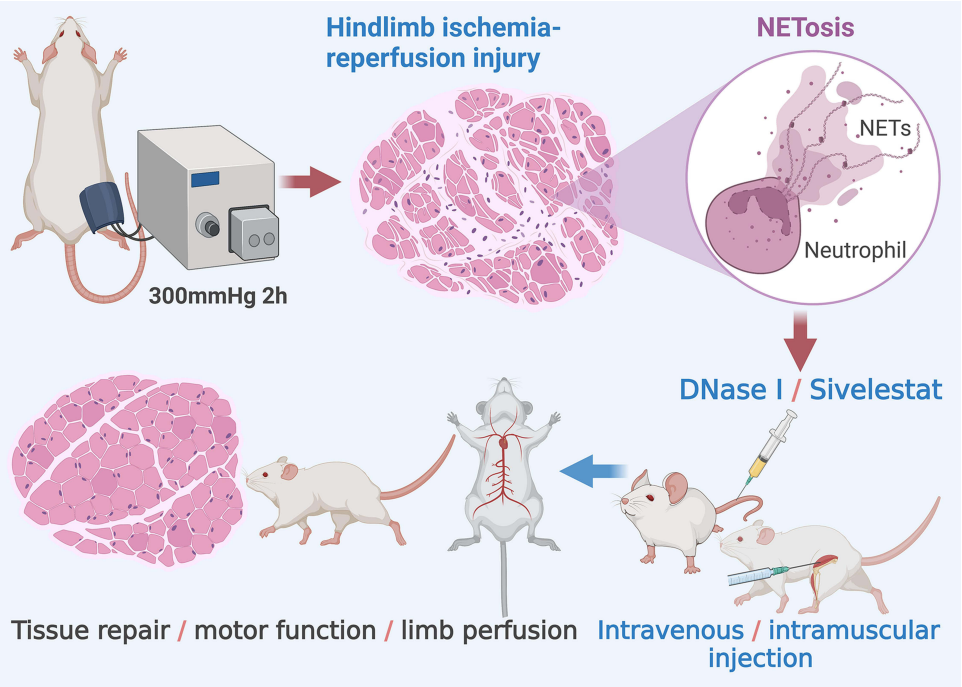

Figure 1 Experimental procedure. An air pump was used to apply a pressure of 300 mmHg to the root of the right hindlimb of rats for 2 h. Releasing the pressure resulted in hindlimb ischemic reperfusion injury (IRI). The extracellular traps (NETs) released by neutrophils lead to tissue damage in the ischemic reperfusion area. The DNA digestion enzyme recombinant DNase I and the neutrophil elastase inhibitor sivelestat are used to eliminate the components of NETs. Blood flow, motor function, angiogenesis, and muscle fibrosis in the ischemic limbs are then tested.

DNase I Treatment

Recombinant DNase I (MedChemExpress, USA) was diluted to 50 mg/mL with sterile water. DNase I solution was injected locally into the gastrocnemius muscle area of the right hindlimb of rats at a dose of 2.5 mg/kg bw/d immediately after the tourniquet was removed. Then inject once a day for the next 7 d (DNase I intramuscular injection group, im).

Or inject the same dose of DNase I solution via the tail vein immediately after the tourniquet was removed and repeated once a day for the next for 7 d (DNase I intravenous injection group, iv).

Sivelestat Treatment

Sivelestat sodium (Yuanye Bio-Technology, Shanghai, China) was diluted to 100 mg/mL in dimethyl sulfoxide. Dilute sivelestat with sterile water at the time of application. Sivelestat was intravenously injected immediately after the tourniquet was removed via the tail vein, or intramuscularly injected into the ischemic area of the mice hindlimbs, at the dose of 15 mg/kg bw/d or 60 mg/kg bw/d (15 mg iv group, 60 mg iv group, 15 mg iv group and 60 mg iv group). Then repeat once a day for the next 7 d.

Saline Treatment

Sterile 0.9% saline solution was used as a negative control of DNase I or Sivelestat treatment for local injection into the ischemic area of rats in the IRI group. As with DNase I or sivelestat treatment, one injection was administered immediately after the ischemic procedure and repeated once daily for the next 7 days.

Detection of Blood Perfusion in the Hindlimbs of Rats

Blood perfusion in the hindlimbs of rats and data analysis were performed using the RFLSI III Laser Speckle Imaging System (RWD Life Science, Shenzhen, China).

Evaluation of the Motor Function of the Rat

The function of the hindlimbs of rats was observed every day after modeling. Following a single-blind method, three researchers who were not involved in making the model were invited to measure the activities of the rats' right lower limbs, and scored them according to the motor function standards. When evaluating motor function, rats were placed in a track surrounded by cardboard, and the track wall was tapped to encourage them to run. The observation ended when the rat crawled 3 meters along the track. The motor function of the rats' ischemic hindlimbs was evaluated according to the following criteria: score 0, limping with dragging hindlimbs; score 1, limping with toes on the ground; score 2, claudication with soles on the ground; score 3, walking with soles with toe-tip activity, mild lameness; score 4, normal activities of soles, normal walking.

Histological Section and Staining of the Muscles of the Hindlimbs of Rats

Rats were euthanized at 24 h, 7 d, and 14 d after ischemia-reperfusion injury (n=3), and the right gastrocnemius muscle was harvested. The muscle tissue was fixed with 4% paraformaldehyde for 24 h, then embedded in paraffin and cut into 5 μm thick slices. The slices were stained with hematoxylin and eosin after deparaffinization.

For Masson trichrome staining, the sections were deparaffinized and stained with Weigert's ferric hematoxylin staining solution and separated color with an acidic ethanol differentiation solution. Then they were stained with Masson's blue solution (Absin, Shanghai, China) and ponceau fuchsin staining solution and separated color with acetic acid solution and phosphomolybdic acid solution. Finally, it was stained with an aniline blue staining solution and the acetic acid solution was used for color separation. After staining, muscle fibers were stained red, collagen fibers were stained blue, and nuclei were stained dark gray. Sections were viewed with an Eclipse E100 upright optical microscope (Nikon, Japan), and images were acquired with a DS -U3 imaging system (Nikon, Japan) and analyzed with Image J software (National Institutes of Health, USA).

Immunohistochemistry

Paraffin sections were deparaffinized and hydrated, and antigen retrieval was performed in a microwave oven with citrate antigen recovery buffer. Endogenous peroxidase was blocked with 3% hydrogen peroxide solution, and nonspecific antigen-antibody binding was blocked with 3% BSA. Incubate anti-myeloperoxidase (MPO) antibody (Servicebio,

Wuhan, China), anti-neutrophil elastase (NE) antibody (Affinity Biosciences, Suzhou, China) and anti-histone H3 (citrulline R2 + R8 + R17, Abcam, Cambridge, UK). Chose a secondary antibody matching the species of the primary antibody and develop the color with DAB solution. The nuclei were stained with hematoxylin. Microscopic examination, image acquisition, and analysis (as described above).

For immunofluorescence detection, the paraffin sections were deparaffinized, hydrated, and antigen recovered. Nonspecific antigen-antibody binding was blocked with 10% donkey serum. Incubate with anti-CD31 antibody (Affinity Biosciences, Suzhou, China). Bind the primary antibody with a fluorophore-conjugated secondary antibody. The cell nuclei were stained with DAPI. The slices were observed under an Eclipse C1 fluorescence microscope (Nikon, Japan) (DAPI: ultraviolet excitation wavelength 330–380 nm, emission wavelength 420 nm, blue light; CY3: excitation wavelength 510–560, emission wavelength 590 nm, red light). Images were acquired using a DS -U3 imaging system (Nikon, Japan). Analyzed with Image J software.

Statistical Analysis

All experiments were repeated at least three times. Data analysis and graphing were performed using GraphPad Prism 9 software. All data are expressed as mean ± standard deviation (SD). Comparisons between two groups were performed with Student's *t*-test. Comparisons between multiple groups were performed with one-way analysis of variance (ANOVA). Since the multi-group analysis in this study included 8 observation groups, comparing the mean of each group with the mean of every other group generated too much data. Therefore, we selected the option "Compare the mean of each column with the mean of a control column (the control group in this study)" under the "Multiple Comparisons" tab when performing the one-way analysis ANOVA in Graphpad Prism 9. The 95% confidence level was considered significant. For all tests, statistical significance of p-values is given as ns: not significant *P < 0.05, **; P < 0.01; ***P < 0.001.

Results

Replication and Evaluation of the Rat Hindlimb Ischemia-Reperfusion Injury (IRI) Model

After ischemia-reperfusion injury in the hindlimbs of rats, Laser image-speckle interferometer was used to detect blood perfusion of the bilateral hindlimb. The blood flow signal of the ischemic hindlimb was significantly lower than that of the normal contralateral hindlimb (Figure 2), demonstrating that the animal model was successfully established.

Ischemia-Reperfusion Injury of the Hindlimb Led to Neutrophil Extracellular Trap Formation in Skeletal Muscle

The effects of IRI on skeletal muscle morphology were observed by hematoxylin-eosin staining and Masson's trichrome staining. Compared with normal rat gastrocnemius muscle, skeletal muscle cells in the IRI group were swollen and

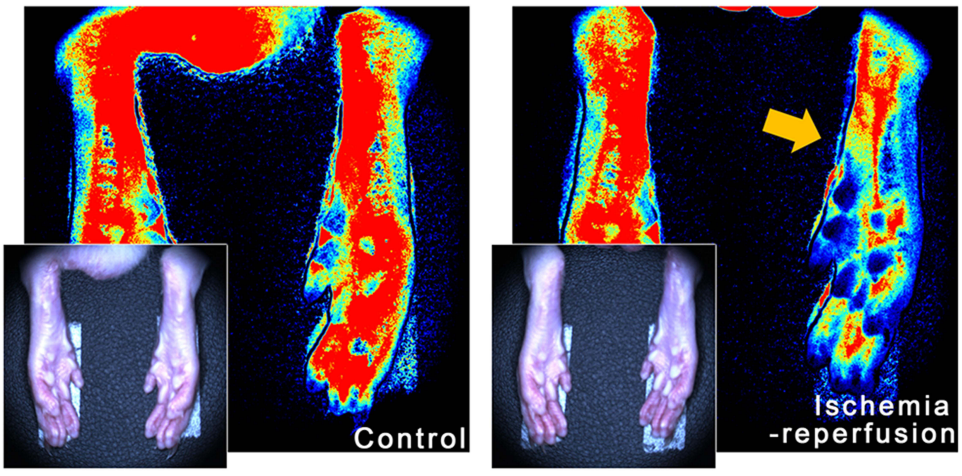

Figure 2 Compare of blood perfusion of the ischemic limb and contralateral nonischemic limb after IRI modeling. Determined and photographed with a laser image-speckle interferometer. The yellow arrow indicates the poor perfusion.

sarcolysis; muscle fibers were broken, disorganized, and irregularly shaped, and necrotic muscle fibers were vacuolated; many leukocytes were infiltrated between tissues (Figure 3A and B). Immunohistochemistry showed that neutrophil elastase (NE), myeloperoxidase (MPO), and histone H3 were presented at high levels in IRI compared with controls

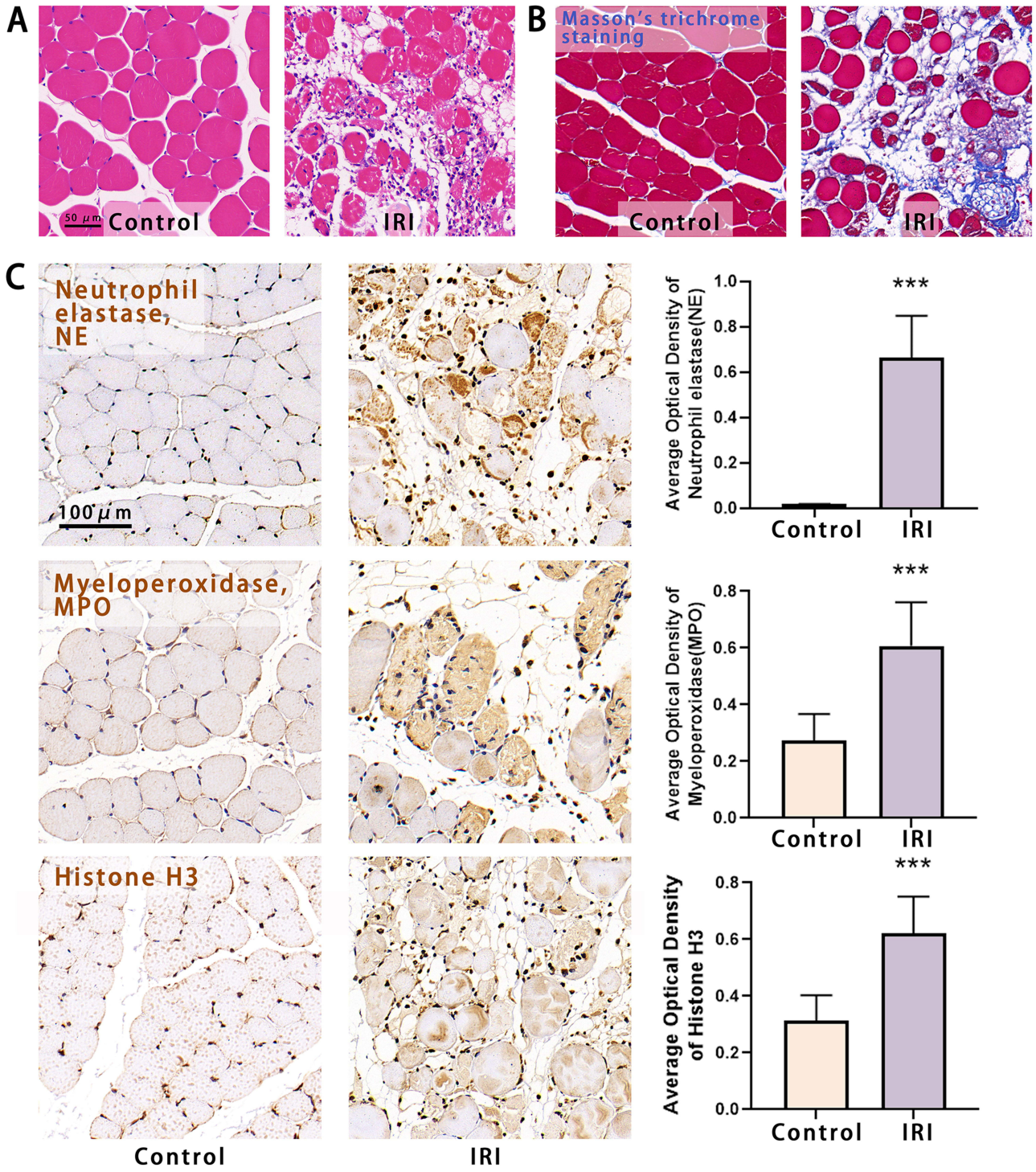

Figure 3 Effects of ischemia-reperfusion injury on muscle morphology and neutrophil Extracellular Traps (NETs) formation. (**A**) Hematoxylin-eosin staining of gastrocnemius muscle sections. IRI resulted in abnormal muscle bundle morphology and many inflammatory cells infiltration. (**B**) Masson's trichrome staining of gastrocnemius muscle sections. After IRI, necrotic skeletal muscle fibers are replaced by fibrous tissue. Scale bar = 50 μm. (**C**) Detection of NETs after IRI in the hindlimbs of rats. Between muscle fibers, neutrophil elastase (NE, n=5), myeloperoxidase (MPO, n=10), and histone H3 (n=10) stained positively, indicating the presence of NETs. Scale bar = 100 μm. Data are expressed as mean ± standard deviation, ***$p < 0.001$.

(Figure 3C). These phenomena suggest that ischemia-reperfusion injury results in large numbers of neutrophils invading local tissues and producing NETs, which is an important cause of severe skeletal muscle injury.

Both DNAse-I and Sivelestat Reduced the Content of Intermuscular NETs in the Acute Phase of Ischemia-Reperfusion Injury

DNase I or sivelestat was administered after IRI, and the levels of NE, MPO, and H3 were determined 24 h later to evaluate the elimination efficiency of NETs.

Both DNase I and sivelestat reduced MPO levels in the ischemia-reperfusion area by more than 50%. The two routes of administration of DNase I were similar in terms of efficiency of MPO reduction. The efficiency was also similar for both routes of administration of sivelestat. Among them, the effect of sivelestat injected at a dose of 60 mg/kg via the tail vein was the most significant, and the MPO was reduced by more than 60% (Figure 4). Both DNase I and sivelestat can decrease histidine H3 levels in the ischemia-reperfusion area (Figure 5). DNase I and sivelestat had similar clearance efficiencies on neutrophil elastase (NE). They decreased the level of NE in the ischemia-reperfusion area by approximately 50% (Figure 6).

Overall, both DNase I and sivelestat decreased the content of NETs in the IRI region.

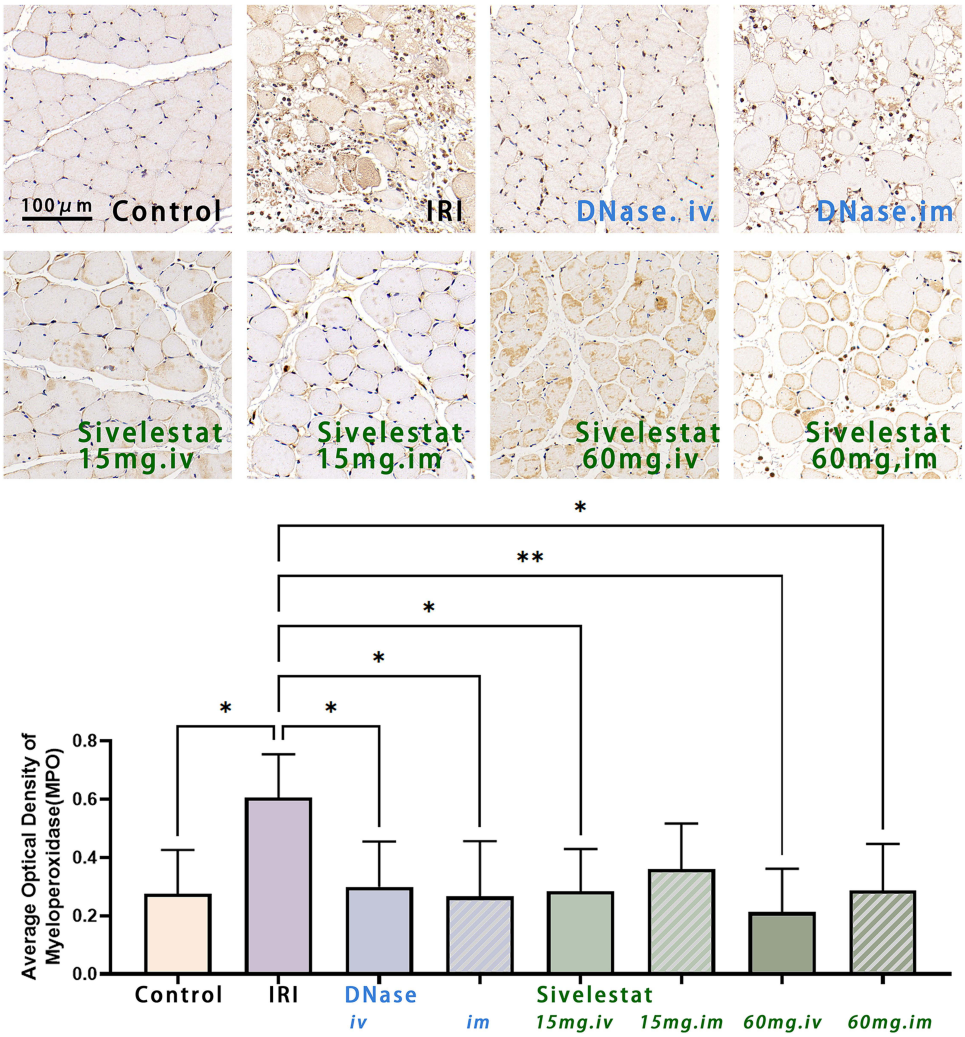

Figure 4 Myeloperoxidase (MPO) levels 24 h after ischemia-reperfusion injury in rat hindlimb muscles. MPO decreased after administration of DNAse-I or sivelestat. n=5, data are expressed as mean ± standard deviation, *$P < 0.05$, **$P < 0.01$. Scale bar = 100 μm.

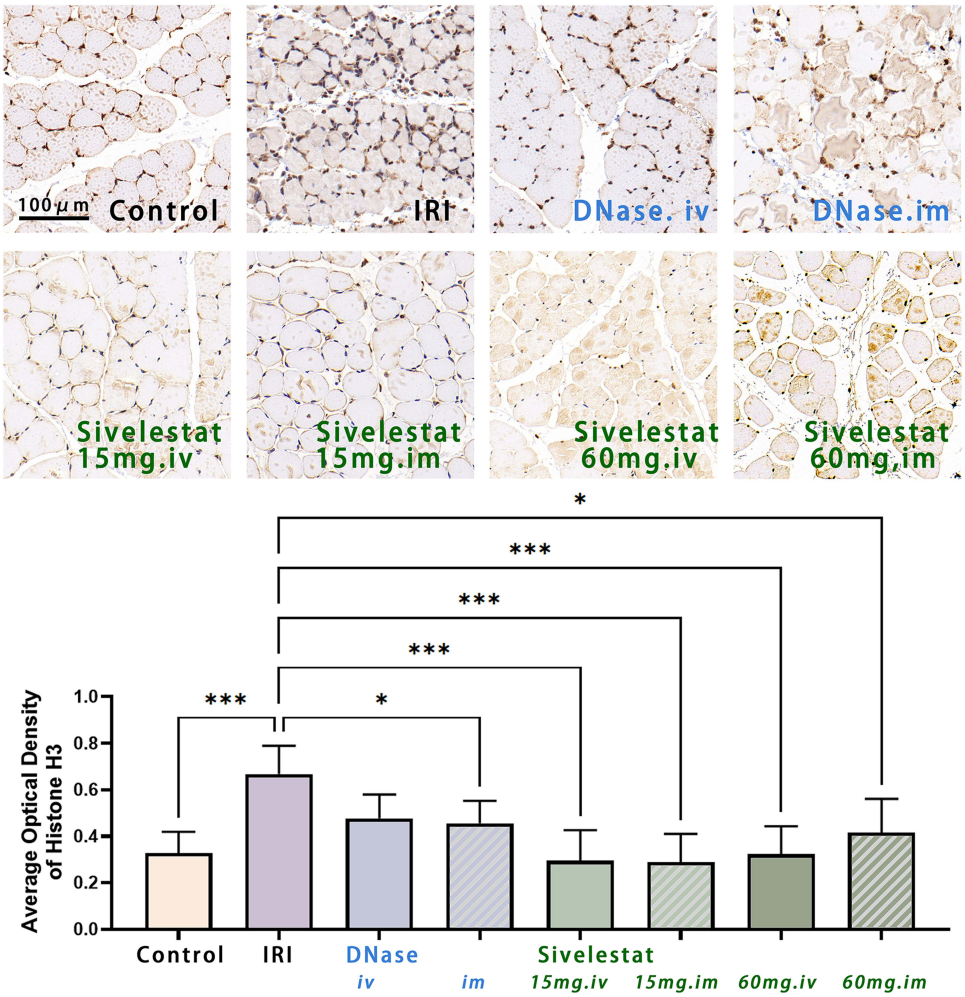

Figure 5 Histone H3 levels 24 h after ischemia-reperfusion injury in rat hindlimb muscles. H3 decreased after administration of DNAse-I or sivelestat. n=5, data are expressed as mean ± standard deviation, *$P < 0.05$, ***$P < 0.001$. Scale bar = 100 μm.

Effects of DNAse-I and Sivelestat on Blood Perfusion in Ischemic Hindlimb

After 7 d of ischemia-reperfusion injury in the hindlimbs of rats, blood perfusion of the ischemic limbs was measured by laser speckle imager, using the healthy side as a control, and the data were analyzed. In the measurement, the rat's paw was used as the region of interest (ROI), and the ratio of blood perfusion of the ischemic side to the nonischemic side of the limb was calculated. Intramuscular injection of DNase I, intramuscular injection or intravenous injection of 15 mg/kg bw/d sivelestat restored vascular perfusion of the injured hindlimb to near normal. However, intravenous injection of DNase I, intramuscular injection or intravenous injection of 60 mg/kg bw/d sivelestat did not produce any benefit in restoring blood flow to the hindlimb (Figure 7).

Effects of DNAse-I and Sivelestat on Recovery of Motor Function of Ischemic Hindlimb

Motor function was monitored daily after ischemia-reperfusion injury in the right hindlimb of rats until 14 d after injury. After injury, the hindlimbs were unable to move voluntarily and could only drag and crawl was score 0, and recovery of completely normal movement was score 4 (For specific scoring criteria, see Methods and Materials). The results showed that either DNAse-I or Sivelestat was helpful for the recovery of motor function (Figure 8), but intramuscular injection of sivelestat at a dose of 60 mg/kg bw/d into the ischemic area did not improve motor scores but decreased them. At 14

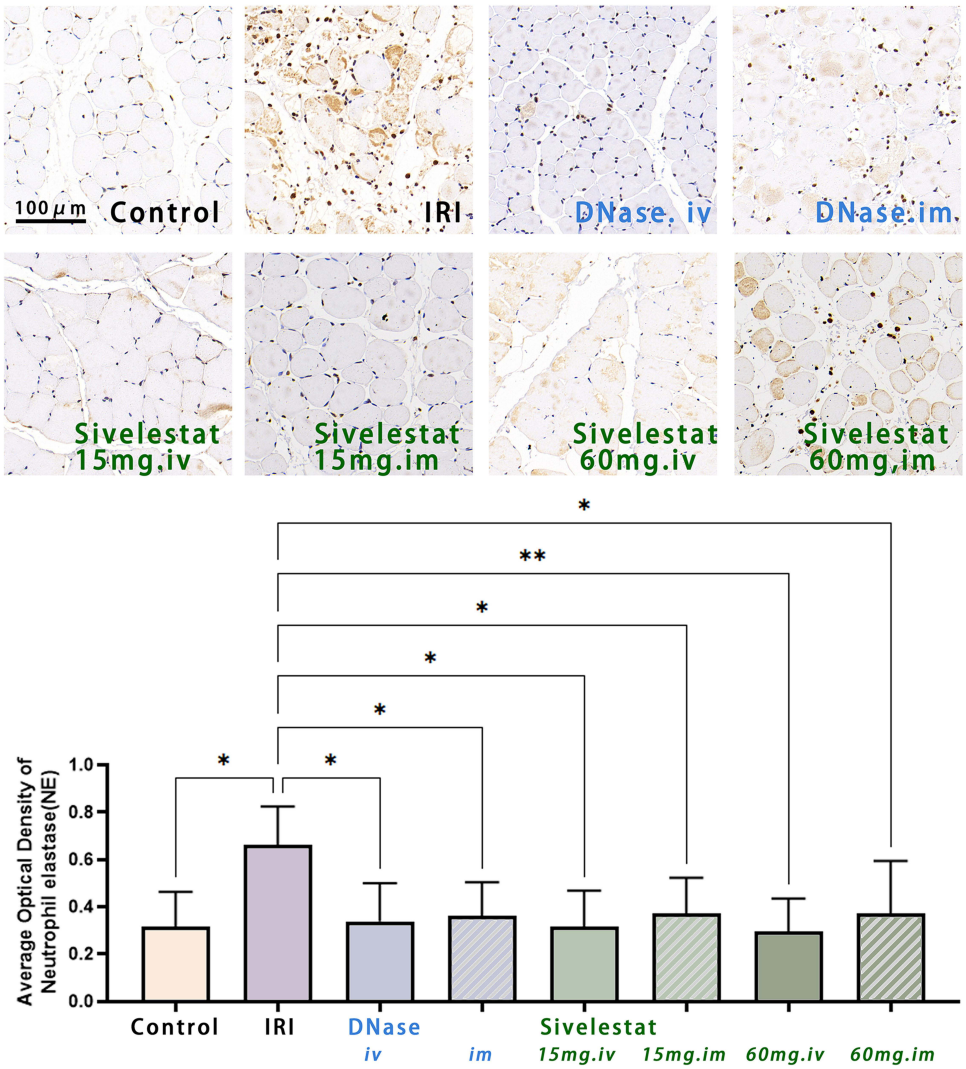

Figure 6 Neutrophil elastase (NE) levels 24 h after ischemia-reperfusion injury in rat hindlimb muscles. NE decreased after administration of DNAse-I or Sivelestat. n=5, data are expressed as mean ± standard deviation, *$P < 0.05$, **$P < 0.01$. Scale bar = 100 μm.

d after injury, except for the IRI group and the sivelestat 60 mg/kg bw/d intramuscular injection group, the motor scores of the other treatment groups were all full scores, that is, they returned to normal.

The Effect of DNAse-I and Sivelestat in Preventing Muscle Fibrosis in Ischemic Hindlimb

Ischemia-reperfusion injury caused local necrosis of the gastrocnemius muscle. The necrosis area was replaced by collagen produced by fibroblasts. At 14 d after injury, the repair of rat hindlimb skeletal muscle injury was assessed by Masson's trichrome staining. The collagen fiber regions were identified by image analysis software, and the results showed that IRI led to muscle fibrosis, and both DNase I and sivelestat treatment reduced tissue fibrosis. Among them, 15 mg/kg bw/d sivelestat injected intravenously had the most significant effect on inhibiting the proliferation of collagen fibers, while 60 mg/kg bw/d sivelestat injected intramuscularly into the ischemic area did not reduce tissue fibrosis (Figure 9).

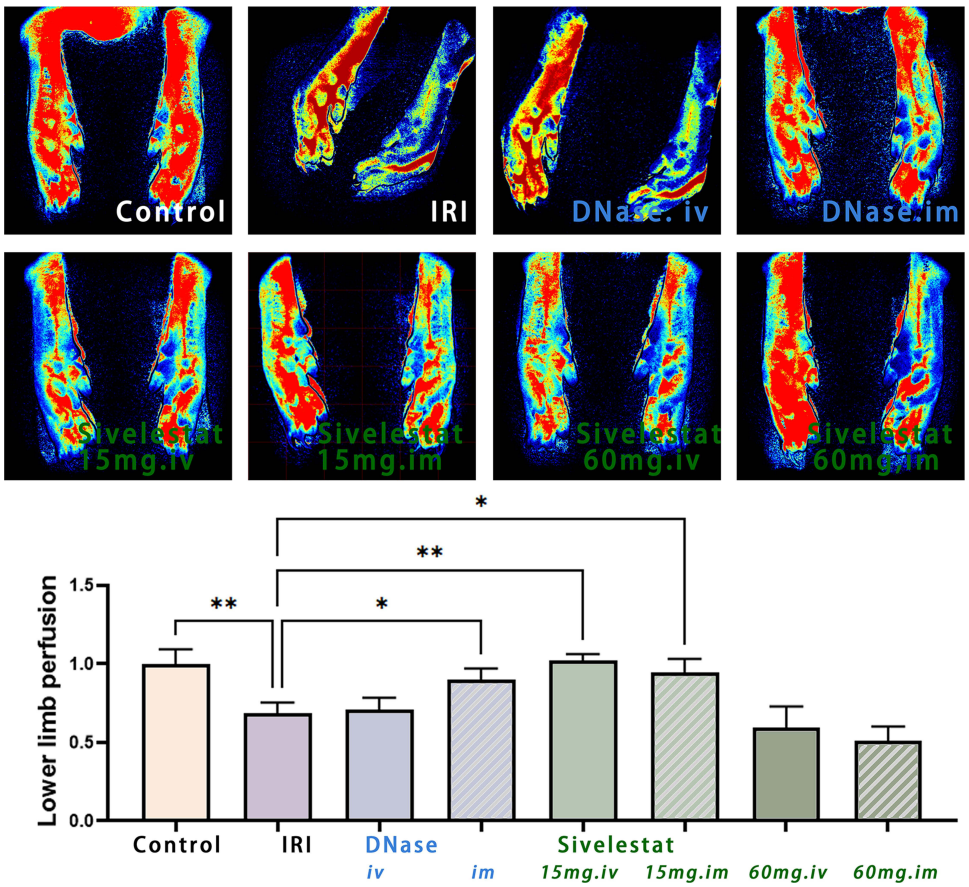

Figure 7 Blood perfusion in the hindlimbs of rats 7 d after ischemia-reperfusion injury in the hindlimbs. Blood circulation in the hindlimbs of rats was determined by laser speckle contrast imaging technique. Intramuscular DNase I, intramuscular and intravenous sivelestat at a dose of 15mg/kg bw/d were beneficial for restoring blood flow in the ischemic limbs. n=3, data are expressed as mean ± standard deviation, *$P < 0.05$, **$P < 0.01$.

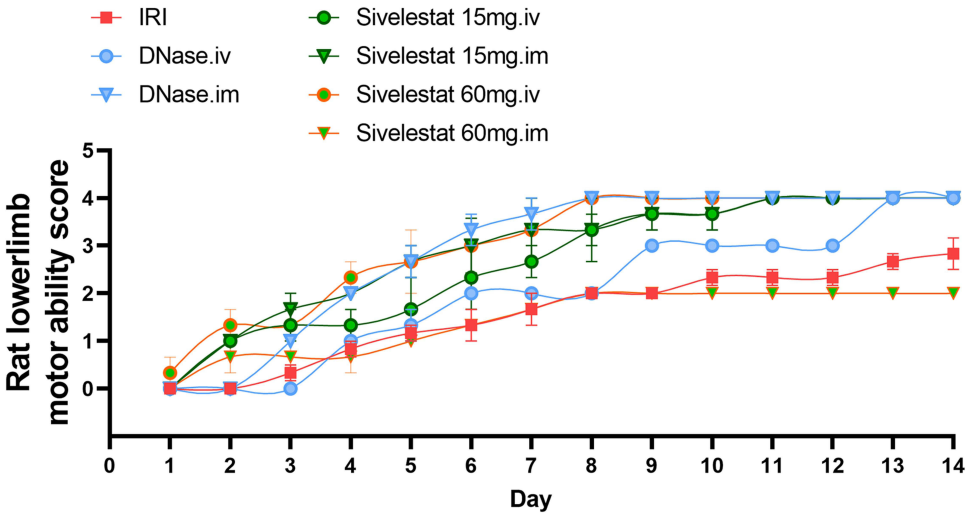

Figure 8 Hindlimb motor scores within 14 d after ischemia-reperfusion injury of hindlimb muscles in rats. Sorted by rate of recovery of motor function: DNAse (im) > sivelestat (60mg.iv) > sivelestat (15mg.iv) > sivelestat (15mg.im) > DNAse (iv) > IRI> sivelestat (60mg.im). n=3, data are expressed as mean ± standard deviation.

Effects of DNsase I and Sivelestat on Angiogenesis in Ischemic Regions

Platelet endothelial cell adhesion molecule (CD31) was used as a marker for vascular endothelial cell. At 14 d after injury, the angiogenesis in the ischemic area was evaluated by immunofluorescence labeling of CD31 in rat

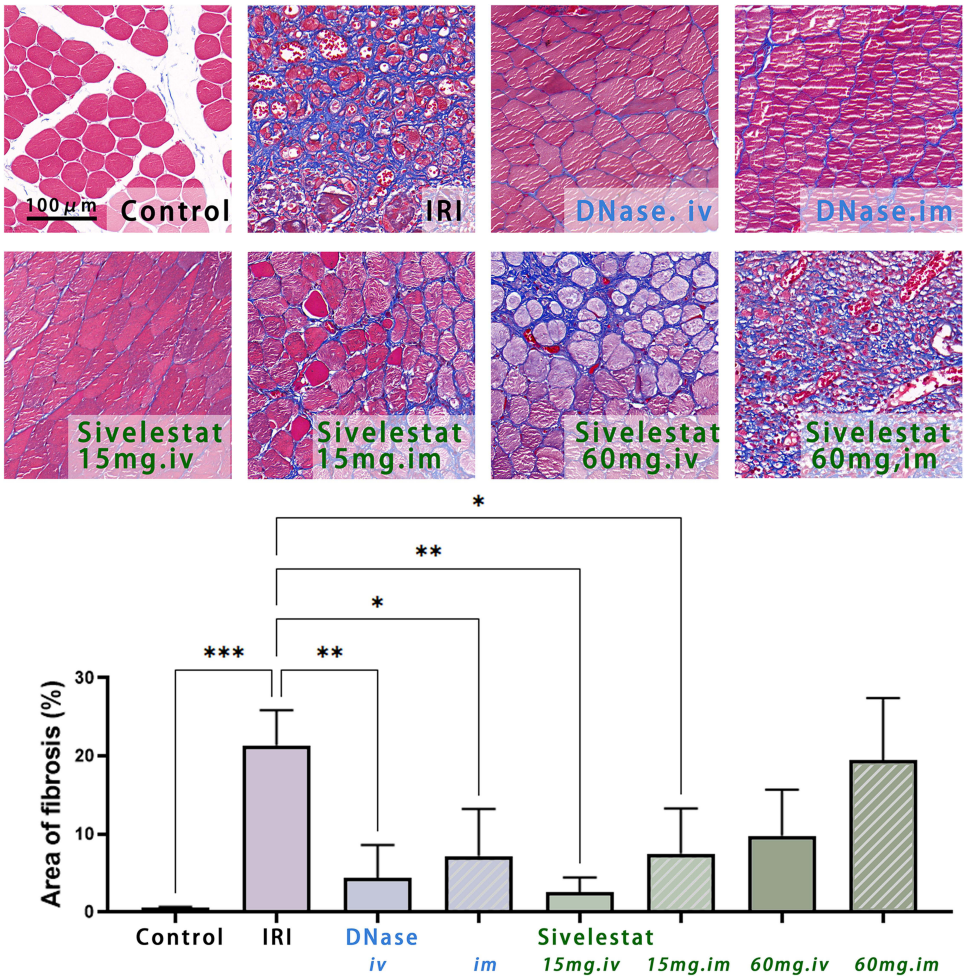

Figure 9 Masson's trichrome staining of the ischemic area 14 d after ischemia-reperfusion injury in rat hindlimb muscles. Muscle fibers are stained red, collagen is stained blue, and nuclei are stained dark brown. Necrotic myofibers are replaced by fibrin hyperplasia after ischemia-reperfusion, showing large blue areas. Intravenous injection of 15 mg sivelestat most markedly reduced collagen fibrillar hyperplasia. Scale bar = 100 μm. n=3, data are expressed as mean ± standard deviation, *P < 0.05, **P < 0.01, ***P < 0.001.

gastrocnemius muscle sections. The proportion of fluorescent area was calculated under Image J. The reason we chose day 14 was that the hindlimb function of the rats at this time had basically recovered. Immunofluorescence of CD31 showed that both DNase I and sivelestat contribute to angiogenesis in the ischemic area. Among them, intramuscular injection of DNase I in the ischemic area had the most obvious effect on promoting angiogenesis, and its fluorescence intensity was 3 times that of the IRI group (Figure 10).

Overall Evaluation of the Effect of DNase I and Sivelestat on NETs Elimination in Ischemia-Reperfusion Areas and on Tissue and Functional Repair

Taking effective removal of NETs as 1, invalid as 0; improvement of blood perfusion and motor function as 1, no improvement as 0; reduction of skeletal muscle fibrosis in ischemia-reperfusion area as 1, failure to reduce as 0; promotion of angiogenesis in the ischemia-reperfusion area as 1, failure to promote as 0, we summarized the effects of DNase I and sivelestat on NETs clearance and tissue and functional repair. The results showed that DNase I was beneficial to IRI whether it was through the tail intravenous injection or the local injection of the ischemia area, and sivelestat at a dosage of 15 mg/kg bw/d showed a similar effect. Sivelestat at a dose of 60 mg/kg bw/d had the effect of eliminating NETs but was not beneficial for tissue and functional repair (Table 1).

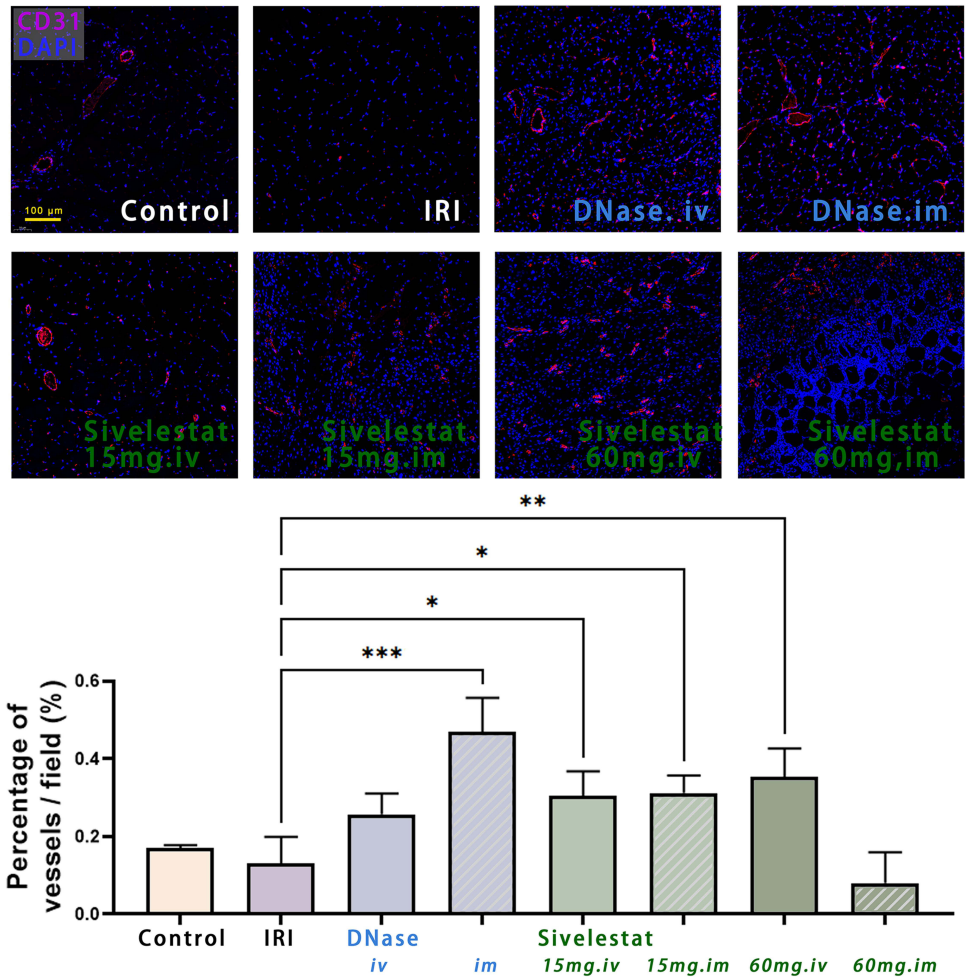

Figure 10 Immunofluorescence staining of CD31 in the ischemic area 14 d after ischemia-reperfusion injury of rat hindlimb muscles. Angiogenesis in the IRI region was assessed by labeling vascular endothelial cells with anti-CD31 antibody (red fluorescence). Single or clustered CD31-positive signals were counted as vascular vessels. Both DNase I and sivelestat treatment contributed to angiogenesis, with local injection of DNase I have the most obvious effect. Cell nuclei were stained with DAPI (blue fluorescence). Scale bar = 100 μm. n=3, data are expressed as mean ± standard deviation, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Discussion

The ischemia-reperfusion injury occurs after acute hindlimb mechanical trauma, arterial embolism, surgery of the extremities with a tourniquet, and recanalization of chronic hindlimb ischemia and is an important factor affecting limb survival and function.[30,31] Neutrophil extracellular traps play an important role in the process of hindlimb IRI. NETs are extracellular fibrillar matrices of chromatin and granular proteins formed by neutrophils upon activation by chemicals, inflammatory mediators, or foreign bodies. They are the means by which neutrophils kill extracellular pathogens, but may also have deleterious effects on the host, which can occur in infectious, inflammatory and thrombotic diseases.[32–36] The components of NETs are DNA backbones, histones, granzymes, and peptides (including neutrophil elastase NE, myeloperoxidase MPO, and so on). Elimination of NETs has been shown to benefit the repair of IRI organs and tissues such as the heart, kidney, liver, intestine, brain, etc., but there are few studies on hindlimb IRI. Our study showed that both DNase I and sivelestat improved the prognosis of hindlimb IRI in rats.

Except that intramuscular injection of DNase I in the IR region improved arterial perfusion of the hindlimbs better than the intravenous injection, the two routes of administration of DNase I showed no significant difference in the clearance of the major components of NETs, the recovery of motor function, the promotion of angiogenesis and the prevention of muscle fibrosis. Albadawi et al found that DNase-I treatment reduced the content of NETs in postischemic muscle, decreased infiltration of inflammatory cells, and improved limb perfusion after ischemia, but did not alter the

Table 1 The Ability of DNase I and Sivelestat to Eliminate NETs and Their Effect on Tissue Repair in Ischemia-Reperfusion Areas

	DNase I 2.5mg iv	DNase I 2.5mg im	Sivelestat 15mg iv	Sivelestat 15mg im	Sivelestat 60mg iv	Sivelestat 60mg im
NETs elimination						
MPO	1	1	1	1	1	1
H3	1	1	1	1	1	1
NE	1	1	1	1	1	1
Total	**3**	**3**	**3**	**3**	**3**	**3**
Organizational and functional recovery						
Blood perfusion	0	1	1	1	0	0
Motor score	1	1	1	1	1	0
Anti-fibrosis	1	1	1	1	1	0
Angiogenesis	1	1	1	1	1	0
Total	**3**	**4**	**4**	**4**	**3**	**0**

Notes: Effective removal of NETs was scored as 1, ineffective as 0; improvement of tissue repair indicators (blood perfusion, motor score, inhibition of fibrosis, and promotion of angiogenesis) was scored as 1, and no change was scored as 0.

Abbreviations: NETs, Neutrophil extracellular traps; MPO, myeloperoxidase; NE, neutrophil elastase; IRI, Hindlimb ischemia-reperfusion injury; IR, Ischemia-reperfusion; ROS, reactive oxygen species; PAD4, protein arginine deiminase 4.

content of pro-inflammatory molecules and skeletal muscle injury.[22] Therefore, they concluded that DNase I treatment cannot prevent IRI, which is different from what we observed. We believe that the reason for this is that DNase I was administered by intraperitoneal injection in the study by Albadawi et al. Due to a large amount of cell-free DNA (cfDNA) present in the peritoneal fluid,[37,38] blood,[32–35] and other body fluids, DNase I was consumed, and the effective concentration eventually distributed in the region of IR was not sufficient to alter the prognosis of IRI. Our experiment also confirmed that local application of DNase I in the ischemic area was more effective than the intravenous injection, as evidenced by better restoration of blood perfusion (Figure 7).

Sivelestat has been shown to be effective in eliminating NETs in acute myocardial infarction, acute renal ischemia, intestinal ischemia-reperfusion injury, etc., but there is no report of improving hindlimb IRI. We used two kinds of doses of sivelestat in our experiments based on the literature of rat studies.[25,26] Sivelestat at a dosage of 15 mg/kg bw/d, whether injected locally into the IR area or infused intravenously, had a good effect on eliminating NETs. It improved regional angiogenesis, limb blood perfusion and motor function, and attenuated skeletal muscle fibrosis after IRI. Intravenous infusion of sivelestat at a dosage of 60 mg/kg bw/d can promote angiogenesis, improve motor function, and combat muscle fibrosis, but is not helpful in improving hindlimb arterial perfusion. Regional injection of sivelestat at a dose of 60 mg/kg bw/d in the IR area was effective in eliminating NETs, but it had no benefit for the prognosis of hindlimb IRI. It even delayed recovery of motor function (Figure 8). We believe that the probable reason is that local administration of high concentrations of sivelestat inhibits neutrophil activation by preventing neutrophil cytoskeletal rearrangement,[39–41] which impairs the function of neutrophil on tissue repair and pro-angiogenesis in ischemic injury.[42–45]

This study has some limitations due to the experimental period and conditions. First, it was not investigated whether administration of DNase I or sivelestat before the onset of ischemia-reperfusion injury had an effect on rat skeletal muscle injury and exercise capacity, There are some studies that have shown that treatment with drugs prior to injury can reduce injury.[31,46] Second, the effect of other methods of administration, such as intraperitoneal injection,[47] on the therapeutic effect was not investigated. Finally, the possibility and efficacy of combined administration of DNase I and sivelestat were not discussed. These deficiencies will be the target of our future research. Despite these limitations, our results provide data to attenuate experimental lower extremity ischemia-reperfusion injury by eliminating NETs and offer a rationale for potential future clinical applications.

Conclusion

In conclusion, we believe that after hindlimb IRI, intramuscular injection of DNase I or intravenous injection of a moderate dose of sivelestat can improve blood perfusion of injured hindlimb by removing NETs, reduce skeletal muscle fibrosis, promote angiogenesis, and then improve hindlimb motor function. Effective removal of NETs may be good strategy to improve ischemia-reperfusion injury of the hindlimb.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This study was supported by National Natural Science Foundation of China (82070288 and 31900813), Sichuan Science and Technology Program (2022YFS0627), Health Commission of Sichuan Province (21PJ100), Office of Science & Technology and Talent Work of Luzhou (2022-JYJ-131), and the Affiliated Stomatological Hospital of Southwest Medical University (2022Y02).

Disclosure

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported or any financial or other potential conflicts of interest.

References

1. Heilmann C, Schmoor C, Siepe M, et al. Controlled reperfusion versus conventional treatment of the acutely ischemic limb: results of a randomized, open-label, multicenter trial. *Circ Cardiovasc Interv*. 2013;6(4):417–427. doi:10.1161/CIRCINTERVENTIONS.112.000371
2. Yang B, Fung A, Pac-Soo C, Ma D. Vascular surgery-related organ injury and protective strategies: update and future prospects. *Br J Anaesth*. 2016;117(Suppl 2):ii32–ii43. doi:10.1093/bja/aew211
3. Leurcharusmee P, Sawaddiruk P, Punjasawadwong Y, Chattipakorn N, Chattipakorn SC. The possible pathophysiological outcomes and mechanisms of tourniquet-induced ischemia-reperfusion injury during total knee arthroplasty. *Oxid Med Cell Longev*. 2018;2018:8087598. doi:10.1155/2018/8087598
4. Yassin MM, Harkin DW, Barros D'Sa AA, Halliday MI, Rowlands BJ. Lower limb ischemia-reperfusion injury triggers a systemic inflammatory response and multiple organ dysfunction. *World J Surg*. 2002;26(1):115–121. doi:10.1007/s00268-001-0169-2
5. Gillani S, Cao J, Suzuki T, Hak DJ. The effect of ischemia reperfusion injury on skeletal muscle. *Injury*. 2012;43(6):670–675. doi:10.1016/j.injury.2011.03.008
6. Jordan JE, Zhao ZQ, Vinten-Johansen J. The role of neutrophils in myocardial ischemia-reperfusion injury. *Cardiovasc Res*. 1999;43(4):860–878. doi:10.1016/S0008-6363(99)00187-X
7. Schofield ZV, Woodruff TM, Halai R, Wu MC, Cooper MA. Neutrophils–a key component of ischemia-reperfusion injury. *Shock*. 2013;40(6):463–470. doi:10.1097/SHK.0000000000000044
8. Doring Y, Soehnlein O, Weber C. Neutrophil extracellular traps in atherosclerosis and atherothrombosis. *Circ Res*. 2017;120(4):736–743. doi:10.1161/CIRCRESAHA.116.309692
9. Sorvillo N, Cherpokova D, Martinod K, Wagner DD. Extracellular DNA NET-works with dire consequences for health. *Circ Res*. 2019;125(4):470–488. doi:10.1161/CIRCRESAHA.119.314581
10. Moschonas IC, Tselepis AD. The pathway of neutrophil extracellular traps towards atherosclerosis and thrombosis. *Atherosclerosis*. 2019;288:9–16. doi:10.1016/j.atherosclerosis.2019.06.919
11. de Boer OJ, Li X, Teeling P, et al. Neutrophils, neutrophil extracellular traps and interleukin-17 associate with the organisation of thrombi in acute myocardial infarction. *Thromb Haemost*. 2013;109(2):290–297. doi:10.1160/TH12-06-0425
12. Doring Y, Libby P, Soehnlein O. Neutrophil extracellular traps participate in cardiovascular diseases: recent experimental and clinical insights. *Circ Res*. 2020;126(9):1228–1241. doi:10.1161/CIRCRESAHA.120.315931
13. Wu X, You D, Cui J, et al. Reduced neutrophil extracellular trap formation during ischemia reperfusion injury in C3 KO mice: C3 requirement for NETs release. *Front Immunol*. 2022;13:781273. doi:10.3389/fimmu.2022.781273
14. Liu J, Dong Z. Neutrophil extracellular traps in ischemic AKI: new way to kill. *Kidney Int*. 2018;93(2):303–305. doi:10.1016/j.kint.2017.09.031
15. Denorme F, Portier I, Rustad JL, et al. Neutrophil extracellular traps regulate ischemic stroke brain injury. *J Clin Invest*. 2022;132(10). doi:10.1172/JCI154225
16. Kang L, Yu H, Yang X, et al. Neutrophil extracellular traps released by neutrophils impair revascularization and vascular remodeling after stroke. *Nat Commun*. 2020;11(1):2488. doi:10.1038/s41467-020-16191-y
17. Oklu R, Albadawi H, Jones JE, Yoo HJ, Watkins MT. Reduced hind limb ischemia-reperfusion injury in Toll-like receptor-4 mutant mice is associated with decreased neutrophil extracellular traps. *J Vasc Surg*. 2013;58(6):1627–1636. doi:10.1016/j.jvs.2013.02.241

18. Santocki M, Kolaczkowska E. On neutrophil extracellular trap (NET) removal: what we know thus far and why so little. *Cells*. 2020;9(9):2079. doi:10.3390/cells9092079
19. Tian Y, Charles EJ, Yan Z, et al. The myocardial infarct-exacerbating effect of cell-free DNA is mediated by the high-mobility group box 1-receptor for advanced glycation end products-Toll-like receptor 9 pathway. *J Thorac Cardiovasc Surg*. 2019;157(6):2256–2269 e2253. doi:10.1016/j.jtcvs.2018.09.043
20. Peer V, Abu Hamad R, Berman S, Efrati S. Renoprotective effects of DNAse-I treatment in a rat model of ischemia/reperfusion-induced acute kidney injury. *Am J Nephrol*. 2016;43(3):195–205. doi:10.1159/000445546
21. Wang S, Xie T, Sun S, et al. DNase-1 treatment exerts protective effects in a rat model of intestinal ischemia-reperfusion injury. *Sci Rep*. 2018;8(1):17788. doi:10.1038/s41598-018-36198-2
22. Albadawi H, Oklu R, Raacke Malley RE, et al. Effect of DNase I treatment and neutrophil depletion on acute limb ischemia-reperfusion injury in mice. *J Vasc Surg*. 2016;64(2):484–493. doi:10.1016/j.jvs.2015.01.031
23. Aune SE, Yeh ST, Kuppusamy P, Kuppusamy ML, Khan M, Angelos MG. Sivelestat attenuates myocardial reperfusion injury during brief low flow postischemic infusion. *Oxid Med Cell Longev*. 2013;2013:279847. doi:10.1155/2013/279847
24. Kambe M, Bessho R, Fujii M, Ochi M, Shimizu K. Sivelestat reduces myocardial ischemia and reperfusion injury in rat hearts even when administered after onset of myocardial ischemia. *Interact Cardiovasc Thorac Surg*. 2009;8(6):629–634. doi:10.1510/icvts.2008.195933
25. Kono T, Okada S, Saito M. Neutrophil elastase inhibitor, sivelestat sodium hydrate prevents ischemia-reperfusion injury in the rat bladder. *Mol Cell Biochem*. 2008;311(1–2):87–92. doi:10.1007/s11010-007-9698-9
26. Tsounapi P, Saito M, Dimitriadis F, et al. Protective effect of sivelestat, a neutrophil elastase inhibitor, on ipsilateral and contralateral testes after unilateral testicular ischaemia-reperfusion injury in rats. *BJU Int*. 2011;107(2):329–336. doi:10.1111/j.1464-410X.2010.09481.x
27. Uchida Y, Freitas MC, Zhao D, Busuttil RW, Kupiec-Weglinski JW. The protective function of neutrophil elastase inhibitor in liver ischemia/reperfusion injury. *Transplantation*. 2010;89(9):1050–1056. doi:10.1097/TP.0b013e3181d45a98
28. Fujimura N, Obara H, Suda K, et al. Neutrophil elastase inhibitor improves survival rate after ischemia reperfusion injury caused by supravisceral aortic clamping in rats. *J Surg Res*. 2013;180(1):e31–36. doi:10.1016/j.jss.2012.04.037
29. Duehrkop C, Banz Y, Spirig R, et al. C1 esterase inhibitor reduces lower extremity ischemia/reperfusion injury and associated lung damage. *PLoS One*. 2013;8(8):e72059. doi:10.1371/journal.pone.0072059
30. Dreyer HC. Tourniquet use during knee replacement surgery may contribute to muscle atrophy in older adults. *Exerc Sport Sci Rev*. 2016;44(2):61–70. doi:10.1249/JES.0000000000000076
31. Herrero de la Parte B, Roa-Esparza J, Cearra I, et al. The prevention of ischemia-reperfusion injury in elderly rats after lower limb tourniquet use. *Antioxidants*. 2022;11(10):1936. doi:10.3390/antiox11101936
32. Butt AN, Swaminathan R. Overview of circulating nucleic acids in plasma/serum. *Ann N Y Acad Sci*. 2008;1137:236–242. doi:10.1196/annals.1448.002
33. Han DSC, Lo YMD. The nexus of cfDNA and nuclease biology. *Trends Genet*. 2021;37(8):758–770. doi:10.1016/j.tig.2021.04.005
34. Lo YM, Rainer TH, Chan LY, Hjelm NM, Cocks RA. Plasma DNA as a prognostic marker in trauma patients. *Clin Chem*. 2000;46(3):319–323. doi:10.1093/clinchem/46.3.319
35. Weber C, Jenke A, Chobanova V, et al. Targeting of cell-free DNA by DNase I diminishes endothelial dysfunction and inflammation in a rat model of cardiopulmonary bypass. *Sci Rep*. 2019;9(1):19249. doi:10.1038/s41598-019-55863-8
36. Hanberg P, Bue M, Kabel J, Jørgensen AR, Søballe K, Stilling M. Tourniquet-induced ischemia and reperfusion in subcutaneous tissue, skeletal muscle, and calcaneal cancellous bone. *APMIS*. 2021;129(4):225–231. doi:10.1111/apm.13121
37. Burnham P, Chen F, Cheng AP, et al. Peritoneal effluent cell-free DNA sequencing in peritoneal dialysis patients with and without peritonitis. *Kidney Med*. 2022;4(1):100383. doi:10.1016/j.xkme.2021.08.017
38. Lopez-Rojo I, Olmedillas-Lopez S, Villarejo Campos P, et al. Liquid biopsy in peritoneal fluid and plasma as a prognostic factor in advanced colorectal and appendiceal tumors after complete cytoreduction and hyperthermic intraperitoneal chemotherapy. *Ther Adv Med Oncol*. 2020;12:1758835920981351. doi:10.1177/1758835920981351
39. Eguchi T, Yoshida K, Kondo R, et al. Sivelestat prevents cytoskeletal rearrangements in neutrophils resulting from lung re-expansion following one-lung ventilation during thoracic surgery. *Inflammation*. 2013;36(6):1479–1484. doi:10.1007/s10753-013-9689-9
40. Goto Y, Hiramatsu Y, Ageyama N, et al. Rolipram plus Sivelestat inhibits bone marrow-derived leukocytic lung recruitment after cardiopulmonary bypass in a primate model. *J Artif Organs*. 2019;22(1):44–52. doi:10.1007/s10047-018-1071-0
41. De Alessandris S, Ferguson GJ, Dodd AJ, et al. Neutrophil GM-CSF receptor dynamics in acute lung injury. *J Leukoc Biol*. 2019;105(6):1183–1194. doi:10.1002/JLB.3MA0918-347R
42. Peiseler M, Kubes P. More friend than foe: the emerging role of neutrophils in tissue repair. *J Clin Invest*. 2019;129(7):2629–2639. doi:10.1172/JCI124616
43. Phillipson M, Kubes P. The healing power of neutrophils. *Trends Immunol*. 2019;40(7):635–647. doi:10.1016/j.it.2019.05.001
44. de Oliveira S, Rosowski EE, Huttenlocher A. Neutrophil migration in infection and wound repair: going forward in reverse. *Nat Rev Immunol*. 2016;16(6):378–391.
45. Wang J. Neutrophils in tissue injury and repair. *Cell Tissue Res*. 2018;371(3):531–539. doi:10.1007/s00441-017-2785-7
46. Leurcharusmee P, Sawaddiruk P, Punjasawadwong Y, et al. CoenzymeQ10 and ischemic preconditioning potentially prevent tourniquet-induced ischemia/reperfusion in knee arthroplasty, but combined pretreatment possibly neutralizes their beneficial effects. *Antioxidants*. 2022;11(2):419. doi:10.3390/antiox11020419
47. Ogura Y, Tajiri K, Murakoshi N, et al. Neutrophil elastase deficiency ameliorates myocardial injury post myocardial infarction in mice. *Int J Mol Sci*. 2021;22(2):722. doi:10.3390/ijms22020722