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Research Article

Inhibition of RIP1/RIP3 Necroptosis Pathway Promote Erectile Function in Cold-Stressed Rat Model

Pei Yang, Lipan Niu, Bingbing Zhu, Siyiti Amuti, Adilijiang Yiming, and Fengxia Liu

Department of Human Anatomy, Basic Medical College, Xinjiang Medical University, Urumqi, Xinjiang, China

Correspondence should be addressed to Adilijiang Yiming; adljym@126.com and Fengxia Liu; liufengxia555@126.com

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Cold stimulation is the most common stressor in cold regions. Continuous cold stimulation can cause a series of pathophysiological changes in the body, such as aggregated neutrophils, macrophage activation, and increased inflammatory factors, which is also a risk factor for erectile function impairment. In addition, necroptosis is an important form of programmed cell death. However, the mechanisms of necroptosis in erectile function impairment due to cold stimulation have been very poorly studied. Therefore, we explored the mechanism of tumor necrosis factor-α (TNF-α)-mediated receptor interacting protein kinase 1 (RIP1)/receptor interacting protein kinase 3 (RIP3) necroptosis pathway on erectile function among cold-stressed rats. First, we established a cold-stressed rat model using cold stimulation and selected those rats that had developed erectile function impairment. Then, we used Necrostatin-1 (RIP1 specific inhibitor, Nec-1), Etanercept (TNF-α inhibitor, Ent), and Sildenafil (Sil) to intervene for 14 days and subsequently assessed their erectile function by apomorphine test and sexual behavioural test. Lastly, we performed various molecular studies and histopathological analyses of penile tissues collected from these rats after the experiments. We found that erectile function was impaired in cold-stressed rats, with increased penile tissue fibrosis and elevated levels of TNF-α and necroptosis. Contrastingly, intervention with Nec-1 and Ent restored erectile function, reduced penile tissue fibrosis, and decreased TNF-α and necroptosis levels, consistent with the results of intervention with Sil. Based on these results, we confirmed that the TNF-α-mediated RIP1/RIP3 necroptosis pathway was significantly altered in cold-stressed rats. In conclusion, inhibition of the TNF-α-mediated RIP1/RIP3 necroptosis pathway improved erectile function, suggesting that the specific downstream mechanisms need to be further explored.

1. Introduction

Erectile dysfunction (ED) refers to a common male sexual dysfunction disorder in which the penis fails to erect normally or to maintain an erection for a prolonged period of time in response to sexual stimulation [1]. According to the review by Lotti and Maggi [2], the global prevalence of ED in infertile men ranged between 6.7% and 61.6%, posing a serious risk to men's health.

Numerous studies have shown that serum levels of proinflammatory factors, such as tumor necrosis factor-α (TNF-α) and interleukin-6, are elevated in ED patients and rats [3, 4]. In addition, stressful conditions such as cold stimulation, unhealthy diet, and stress can induce an inflammatory response in the body, contributing to fibrosis in penile tissue, which in turn leads to ED [5, 6]. Therefore, inflammation and ED are mutually causal, meaning that inflammation usually leads to the development of ED and that chronic inflammation is also present in the penile tissue of ED patients and rats.

Necroptosis is a newly discovered form of programed proinflammatory cell death. The proinflammatory factor TNF-α, a typical inducer of necroptosis, activates the receptor interacting protein kinase 1 (RIP1)/receptor interacting protein kinase 3 (RIP3) signaling pathway. This pathway triggers downstream oligomerization of mixed lineage kinase domain like protein (MLKL) and its transfer to the cell membrane, inducing cell membrane rupture and ultimately cell disintegration and death. The contents released by cell disintegration can trigger an inflammatory cascade, and high levels of inflammation can in turn promote cell death and exacerbate tissue damage [7, 8].

Combined with the close relationship between ED and inflammation, we hypothesize that necroptosis may play an important role in the process of penile erectile function impairment. Therefore, this study investigated the mechanism of the TNF-α-mediated RIP1/RIP3 necroptosis pathway in penile erectile function impairment by establishing a cold-stressed rat model to provide a theoretical basis for the clinical treatment of ED.

2. Materials and Methods

2.1. Animals. This work was approved by the Laboratory Animal Welfare and Ethics Committee of Xinjiang Medical University (the approved number: IACUC-20210405-7). Sixty male Sprague–Dawley rats (6–7 weeks, 180–220 g) with normal sexual function and 20 sexually mature female Sprague–Dawley rats (6–7 weeks, 180–220 g) were purchased from and housed in the SPF animal laboratory of the Animal Experiment Centre of Xinjiang Medical University. The environmental conditions for all rats were controlled at a temperature of 20−24°C, a relative humidity of approximately 55%, with a 12 hr light/12 hr dark cycle, and a standard diet and water were administered simultaneously.

2.2. Establishment of a Cold-Stressed Rat Model. After 7 days of adaptive rearing of 60 male rats, all rats were verified to be sexually normal using apomorphine test and sexual behavioral test. The 60 male rats were randomly divided into normal control (N, $n = 10$) and cold-stimulated modeling ($n = 50$). The rats in N group were fed and watered normally, with constant temperature and humidity; the rats in cold-stimulated modeling group were normally fed and watered and given cold stimulation (10 hr per day in the artificial climate laboratory at 5−9°C and 85% humidity) for 45 days, during which the rats were regularly examined for changes in sexual function. On day 45 of molding, rats without erection and mating were screened using apomorphine test and sexual behavioral test. Twenty-four rats were screened.

2.3. Grouping and Intervention. Twenty-four rats were randomly divided into four groups: cold-stressed model (M, $n = 6$), Nec-1 (RIP1 specific inhibitor, $n = 6$), Ent (TNF-α inhibitor, $n = 6$), and Sil ($n = 6$). The rats in Nec-1 group were injected intraperitoneally with Nec-1 (0.6 mg/kg, IN0910, Solarbio, Beijing, China); the rats in Ent group were injected intraperitoneally with Ent (0.5 mg/kg, S20120006, CP Guojian Pharma, Shanghai, China); the rats in Sil group were injected intraperitoneally with Sil (1 mg/kg, S6070, Solarbio, Beijing, China); the rats in N and M groups were injected intraperitoneally with equal doses of saline. All groups intervened continuously for 14 days.

2.4. Evaluation of Erectile Function. Erectile latency and erectile number were observed using apomorphine test before and after 14 days intervention. Apomorphine test refers to the subcutaneous injection of apomorphine (90 μg/kg, A4393-250MG, Sigma, St. Louis, MO, USA) into the neck of male rats to observe the presence or absence of erection and erectile latency and erectile frequency within 30 min. If there is no erection, erectile latency was recorded as 1,800 s, and erectile frequency was recorded as 0 [9].

Mounting latency and mounting frequency, intromission latency and intromission frequency, and ejaculation latency and ejaculation frequency were observed using sexual behavioral test before and after 14 days intervention. Sexual behavioral test refers to the intramuscular administration of estradiol benzoate (2 μg/kg, B130613, Sansheng Pharma, Zhejiang, China) at 48 and 24 hr, and the intramuscular administration of progesterone (500 μg/kg, 120804, Xianju Pharma, Zhejiang, China) at 4 hr before the start of the experiment to bring female rats into estrus. The male rats were then acclimatized in the same cage with the estrous female rats for 10 min, and the male rats were observed for the presence or absence of copulation and for mounting latency and mounting frequency, intromission latency and intromission frequency, and ejaculation latency and ejaculation frequency. If there was no copulation, latency was recorded as 1,800 s, and frequency was recorded as 0 [10].

After evaluation of erectile function, the rats were fasted for 12 hr, anesthetized by intraperitoneal injection of 3% sodium pentobarbital (30 mg/kg), and serum and penile tissues were harvested for subsequent experiments.

2.5. Hematoxylin-Eosin Staining and Masson Staining. The front third of the penis was cut down to wash with PBS, fixed with 4% paraformaldehyde, and embedded in paraffin. Slices were a thickness of 5 μm and were prepared using hematoxylin–eosin staining and masson staining. After staining, images were observed and acquired using a Nikon E200 microscope (Tokyo, Japan). The images of the smooth muscle (red) and the collagen fibers (blue) of penile corpus cavernosum obtained were analyzed semiquantitatively using the image analysis software Image J (National Institutes of Health, Bethesda, MD, USA). Three slices from each rat were selected for statistical analysis.

2.6. Immunohistochemistry Analysis and Enzyme-Linked Immunosorbnent Assay. To specifically study TNF-α in penile tissue, the paraffin-embedded penile sections of all the rat groups were immunostained with a specific anti-TNF-α antibody (1 : 100, AF7014, Affinity, Jiangsu, China). After staining, images were observed and acquired using a Nikon E200 microscope. The images obtained were analyzed semiquantitatively using the image analysis software Image J. Three slices from each rat were selected for statistical analysis.

Similarly, to specifically study TNF-α in serum, the concentration of TNF-α was determined using a rat TNF-α enzyme-linked immunosorbent assay kit (SEKR-0009, Solarbio, Beijing, China).

2.7. Quantitative Real-Time Polymerase Chain Reaction Analysis. Total RNA was extracted using an animal tissue total RNA extraction kit (DP451, TIANGEN, Beijing, China). RNA was then reverse-transcribed into cDNA using a reverse transcription kit (KR116, TIANGEN, Beijing, China). Quantitative real-time polymerase chain reaction amplification was performed using an SYBR Green kit (FP205, TIANGEN, Beijing, China). The $2^{-\Delta\Delta}$ comparative threshold was calculated from the resulting comparative threshold. The specific

Table 1: Comparison of erectile latency and erectile frequency before and after intervention.

Groups	Erectile latency (s)		Erectile frequency	
	Before intervention	After 14 days intervention	Before intervention	After 14 days intervention
N ($n=10$)	254.10 ± 173.40	362.80 ± 172.46	4.50 ± 2.22	4.20 ± 1.62
M ($n=6$)	$1{,}800.00 \pm 0.00^{b}$	$1{,}800.00 \pm 0.00^{b}$	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
Nec-1 ($n=6$)	$1{,}800.00 \pm 0.00^{b}$	$1{,}029.33 \pm 406.96^{bd}$	0.00 ± 0.00^{b}	1.67 ± 1.03^{bc}
Ent ($n=6$)	$1{,}800.00 \pm 0.00^{b}$	974.50 ± 469.90^{bd}	0.00 ± 0.00^{b}	1.83 ± 1.47^{bc}
Sil ($n=6$)	$1{,}800.00 \pm 0.00^{b}$	892.17 ± 467.25^{bd}	0.00 ± 0.00^{b}	1.50 ± 1.05^{bc}

Note: The data are presented as mean ± standard error of the mean. $^{b}p<0.01$ in comparison with N group; $^{c}p<0.05$, $^{d}p<0.01$ in comparison with M group.

Table 2: Comparison of mounting latency and mounting frequency before and after intervention.

Groups	Mounting latency (s)		Mounting frequency	
	Before intervention	After 14 days intervention	Before intervention	After 14 days intervention
N ($n=10$)	136.10 ± 103.39	327.40 ± 224.77	17.80 ± 4.21	18.50 ± 3.923
M ($n=6$)	$1{,}800.00 \pm 0.00^{b}$	$1{,}800.00 \pm 0.00^{b}$	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
Nec-1 ($n=6$)	$1{,}800.00 \pm 0.00^{b}$	927.50 ± 456.42^{bd}	0.00 ± 0.00^{b}	5.17 ± 3.25^{bd}
Ent ($n=6$)	$1{,}800.00 \pm 0.00^{b}$	950.00 ± 491.50^{bd}	0.00 ± 0.00^{b}	5.17 ± 2.71^{bd}
Sil ($n=6$)	$1{,}800.00 \pm 0.00^{b}$	804.67 ± 256.57^{bd}	0.00 ± 0.00^{b}	4.83 ± 2.32^{bd}

Note: The data are presented as mean ± standard error of the mean. $^{b}p<0.01$ in comparison with N group; $^{d}p<0.01$ in comparison with M group.

primers used were as follows: rat TNF-α forward (5′-TCTCATTCCTGCTCGTGG-3′) and reverse primers (5′-CTCTGCTTGGTGGTTTGC-3′); RIP1 forward (5′-AGGTACAGGAGTTTGGTATGGGC-3′) and reverse primers (5′-GGTGGTGCCAAGGAGATGTATG-3′); RIP3 forward (5′-TAGTTTATGAAATGCTGGACCGC-3′) and reverse primers (5′-GCCAAGGTGTCAGATGATGTCC-3′); MLKL forward (5′-TCTCCCAACATCCTGCGTAT-3′) and reverse primers (5′-TCCCGAGTGGTGTAACCTGTA-3′); and GAPDH forward (5′-CTGGAGAAACCTGCCAAGTATG-3′) and reverse primers (5′-GGTGGAAGAATGGGAGTTGCT-3′).

2.8. Western Blot Analysis. The rat penile tissues were homogenized in Radio Immunoprecipitation Assay lysis buffer containing phenylmethylsulfonyl fluoride (1 : 100, R0010, Solarbio, Beijing, China). The tissues were quickly ground on ice into homogenate and then reacted for 1 hr. The compounds were centrifuged at 12,000 rpm for 15 min at 4°C. Protein concentrations were measured using a bicinchoninic acid kit (BCA, PC0020, Solarbio, Beijing, China). After performing electrophoresis and transferring the membranes to a blocking buffer, these membranes were incubated overnight at 4°C with primary antibodies against TNF-α (1 : 2,000, AF7014, Affinity, Jiangsu, China), RIP1 (1 : 1,000, ab106393, Abcam, Cambridge, UK), RIP3 (1 : 1,000, ab222320, Abcam, Cambridge, UK), MLKL (1 : 3,000, AF7420, Affinity, Jiangsu, China), phosphorylated RIP1 at tyr384 (1 : 1,000, AF7088, Affinity, Jiangsu, China), phosphorylated RIP3 at ser232 (1 : 1,000, AF7443, Affinity, Jiangsu, China), phosphorylated MLKL at ser358 (1 : 1,000, AF7420, Affinity, Jiangsu, China), and GAPDH (1 : 6,000, AF7021, Affinity, Jiangsu, China). This was followed by incubation with a horseradish peroxidase-linked secondary antibody (1 : 10,000, ZB-2301, ZSGB-BIO, Beijing, China) and subsequent visualization with a chemiluminescent detection system (Azure c600, Dublin, CA, USA). Band intensities were quantified using the image analysis software Image J.

2.9. Statistical Analysis. All data were analyzed using SPSS version 23.0 (SPSS Inc., Chicago, USA). Statistical significance was assessed using two independent samples *t*-tests for comparisons between two groups and one-way ANOVA for comparisons between multiple groups. All results are expressed as mean ± standard error of the mean. Significance for all mean comparisons was accepted as $\alpha = 0.05$, with p values < 0.05 being a statistically significant difference between groups.

3. Results

3.1. Inhibition of the Necroptosis Pathway Improves Erectile Function. The results of apomorphine test (Table 1) showed that before intervention, erectile latency was significantly higher, and erectile frequency was significantly lower in M group compared to N group ($p<0.01$). Similarly, the results of sexual behavioral test (Tables 2–4) showed that before intervention, compared with N group, mounting latency, intromission latency, and ejaculation latency were significantly higher, and mounting frequency, intromission frequency, and ejaculation frequency were significantly lower in M group ($p<0.01$).

However, after 14 days intervention, erectile latency was significantly higher and erectile frequency was significantly lower in M group compared to N group ($p<0.01$); erectile latency was lower and erectile frequency was higher in Nec-1, Ent, and Sil groups compared to M group ($p<0.05$); there was no significant difference in erectile latency and erectile frequency in Nec-1 and Ent groups compared to Sil group

Table 3: Comparison of intromission latency and intromission frequency before and after intervention.

Groups	Intromission latency (s)		Intromission frequency	
	Before intervention	After 14 days intervention	Before intervention	After 14 days intervention
N ($n=10$)	284.30 ± 120.94	487.00 ± 219.206	13.10 ± 4.61	15.60 ± 4.248
M ($n=6$)	$1{,}800.00 \pm 0.00^{b}$	$1{,}800.00 \pm 0.00^{b}$	0.00 ± 0.00^{b}	0.00 ± 0.00^{bd}
Nec-1 ($n=6$)	$1{,}800.00 \pm 0.00^{b}$	$1{,}032.33 \pm 434.88^{bd}$	0.00 ± 0.00^{b}	5.17 ± 3.25^{bd}
Ent ($n=6$)	$1{,}800.00 \pm 0.00^{b}$	$1{,}072.67 \pm 422.63^{bd}$	0.00 ± 0.00^{b}	4.83 ± 2.40^{bd}
Sil ($n=6$)	$1{,}800.00 \pm 0.00^{b}$	872.00 ± 267.51^{bd}	0.00 ± 0.00^{b}	4.50 ± 1.64^{bd}

Note: The data are presented as mean ± standard error of the mean. $^{b}p<0.01$ in comparison with N group; $^{d}p<0.01$ in comparison with M group.

Table 4: Comparison of ejaculation latency and ejaculation frequency before and after intervention.

Groups	Ejaculation latency (s)		Ejaculation frequency	
	Before intervention	After 14 days intervention	Before intervention	After 14 days intervention
N ($n=10$)	296.30 ± 127.04	487.00 ± 219.206	12.40 ± 4.65	15.20 ± 4.29
M ($n=6$)	$1{,}800.00 \pm 0.00^{b}$	$1{,}800.00 \pm 0.00^{b}$	0.00 ± 0.00^{bd}	0.00 ± 0.00^{b}
Nec-1 ($n=6$)	$1{,}800.00 \pm 0.00^{b}$	$1{,}032.33 \pm 434.88^{bd}$	0.00 ± 0.00^{bd}	5.17 ± 3.25^{bd}
Ent ($n=6$)	$1{,}800.00 \pm 0.00^{b}$	$1{,}072.67 \pm 422.63^{bd}$	0.00 ± 0.00^{bd}	4.83 ± 2.40^{bd}
Sil ($n=6$)	$1{,}800.00 \pm 0.00^{b}$	872.00 ± 267.51^{bd}	0.00 ± 0.00^{bd}	4.50 ± 1.64^{bd}

Note: The data are presented as mean ± standard error of the mean. $^{b}p<0.01$ in comparison with N group; $^{d}p<0.01$ in comparison with M group.

($p>0.05$). Similarly, after 14 days intervention, compared with N group, mounting latency, intromission latency, and ejaculation latency were significantly higher, and mounting frequency, intromission frequency, and ejaculation frequency were significantly lower in M group ($p<0.01$); compared with M group, mounting latency, intromission latency, and ejaculation latency were significantly lower, and mounting frequency, intromission frequency, and ejaculation frequency were significantly higher in Nec-1, Ent, and Sil groups ($p<0.01$); compared with Sil group, there was no significant difference in mounting latency and mounting frequency, intromission latency and intromission frequency and ejaculation latency and ejaculation frequency in Nec-1 and Ent groups ($p>0.05$).

3.2. Inhibition of the Necroptosis Pathway Improves Penile Tissue Microstructural. The results of hematoxylin–eosin staining (Figure 1(a)) showed that in N group, the muscle fibers and the collagen fibers in penile tissues were neatly arranged, and the sinusoids were intact; in M group, the muscle fibers in penile tissues were broken, the collagen fibers were accumulated and the sinusoids were reduced and the lacuna was enlarged; while compared with M group, the muscle fibers and the sinusoids in penile tissues were restored to different degrees in Nec-1, Ent, and Sil groups.

The results of Masson staining (Figure 1(b)) showed that in penile tissues, the smooth muscle was stained red, and the collagen fibers were stained blue. In N group, the smooth muscle and the collagen fibers in penile tissues were staggered and evenly distributed, and the smooth muscle content was more than that of the collagen fibers; in M group, the smooth muscle and the collagen fibers in penile tissues were disordered and unevenly distributed, and the smooth muscle content was significantly reduced, and that of the collagen fibers was significantly increased; while compared with M group, the smooth muscle content in penile tissue was increased in Nec-1, Ent, and Sil groups, and the content of the collagen fiber was decreased.

By calculating the area ratio of the smooth muscle to the collagen fibers in penile tissue of rats in each group (Figure 1(c)), it can be seen that compared with N group, the area ratio of the smooth muscle to the collagen fibers in M group was significantly lower ($p<0.01$); compared with M group, the area ratio of the smooth muscle to the collagen fibers in Nec-1, Ent, and Sil groups was higher ($p<0.05$); compared with Sil group, the area ratio of the smooth muscle to the collagen fibers in Nec-1 and Ent groups was no significant difference ($p>0.05$).

3.3. Inhibition of the Necroptosis Pathway Reduces TNF-α Levels. The results of immunohistochemistry (Figures 2(a) and 2(b)) showed that compared with N group, the level of TNF-α was significantly higher in penile tissue in M group ($p<0.01$); compared with M group, the level of TNF-α was significantly lower in penile tissue in Nec-1, Ent, and Sil groups ($p<0.01$); and compared with Sil group, the level of TNF-α was no significant difference in penile tissue in Nec-1 and Ent groups ($p>0.05$).

The results of enzyme-linked immunosorbent assay (Figure 2(c)) showed that compared with N group, the levels of TNF-α in serum in M group were significantly higher ($p<0.01$); compared with M group, the levels of TNF-α in serum in Nec-1, Ent, and Sil groups were significantly lower ($p<0.01$); and compared with Sil group, the levels of TNF-α in serum in Nec-1 and Ent groups were no significant difference ($p>0.05$).

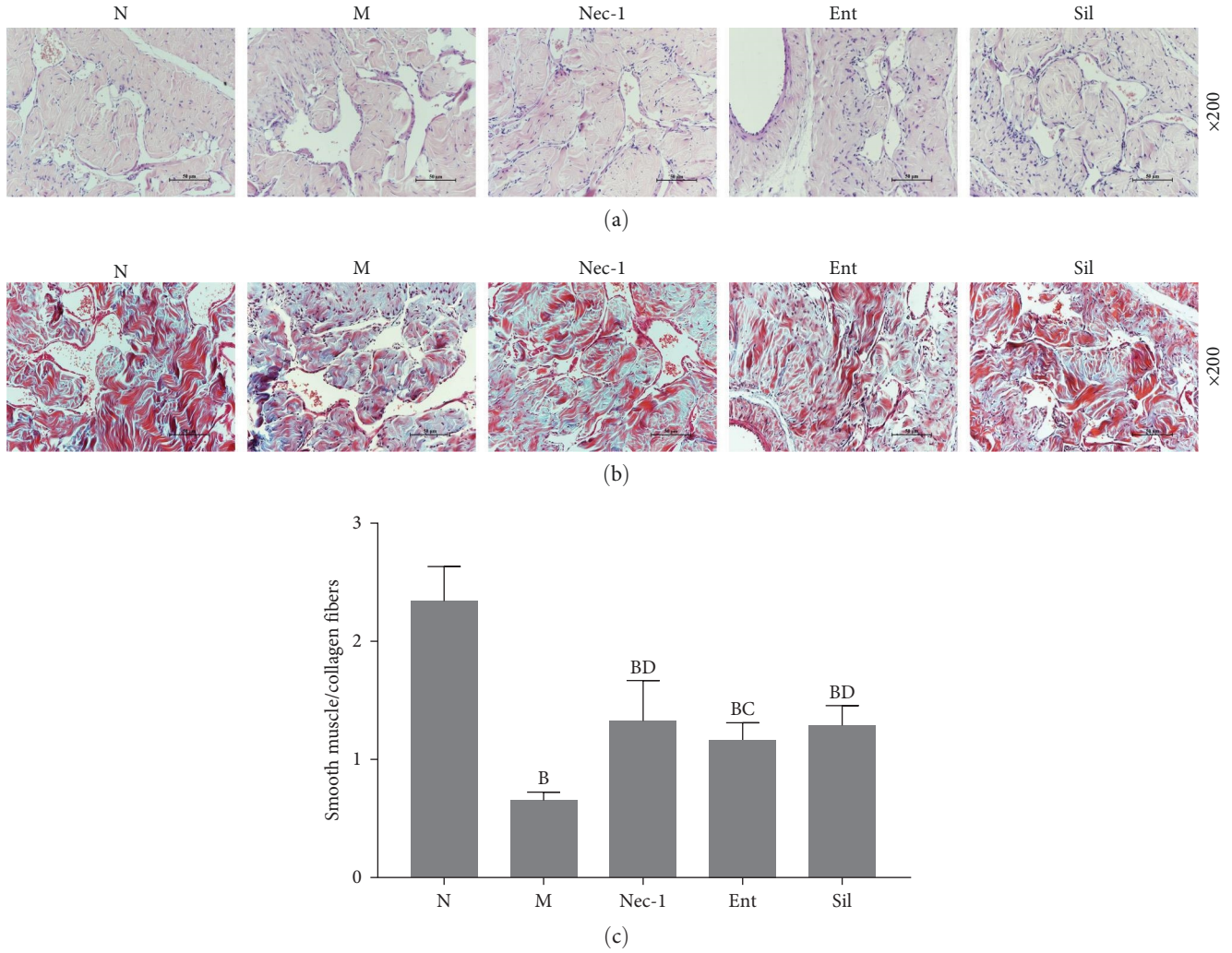

Figure 1: Inhibition of the necroptosis pathway improves penile tissue microstructural changes: (a) representative images of hematoxylin–eosin staining (magnification, ×200); (b) representative images of Masson staining (magnification, ×200). The smooth muscle was stained red, while the collagen fibers were stained blue; and (c) Semiquantitative analysis of the ratio of the smooth muscle to the collagen fibers. Error bars: mean ± standard error of the mean. $^{B}p<0.01$ in comparison with N group; $^{C}p<0.05$, $^{D}p<0.01$ in comparison with M group.

3.4. Inhibition of the Necroptosis Pathway Reduces the mRNA Expression of TNF-α and Factors Related to the Necroptosis Pathway in Penile Tissue. The results of quantitative real-time polymerase chain reaction (Figure 3) showed that compared with N group, the expression of TNF-α, RIP1, RIP3, and MLKL mRNA in penile tissues in M group was significantly higher ($p<0.01$); compared with M group, the expression of TNF-α, RIP3, and MLKL mRNA in penile tissues in Nec-1, Ent, and Sil groups was significantly lower ($p<0.01$); compared with M group, the expression of RIP1 mRNA in penile tissues in Nec-1 and Ent groups was lower ($p<0.05$), and the expression of RIP1 mRNA in penile tissue in Sil group was slightly reduced ($p>0.05$); compared with Sil group, there was no significant difference in the expression of TNF-α, RIP1, and RIP3 mRNA in penile tissues of Nec-1 and Ent groups ($p>0.05$); compared with Sil group, there was no significant difference in the expression of MLKL mRNA in penile tissues of Nec-1 group ($p>0.05$), and the reduction in MLKL mRNA in penile tissues of Ent group was not as significant as that of Sil group ($p<0.05$).

3.5. Inhibition of the Necroptosis Pathway Reduces the Protein Levels of TNF-α and Factors Related to the Necroptosis Pathway in Penile Tissue. The results of western blot (Figure 4) showed that compared with N group, the levels of TNF-α, RIP1, p-RIP1, RIP3, p-RIP3, MLKL, and p-MLKL proteins in penile tissues were increased in M group ($p<0.05$); compared with M group, the levels of TNF-α protein in penile tissues were significantly decreased in Nec-1 and Sil groups ($p<0.01$), and the level of TNF-α protein in penile tissue was slightly reduced in Ent group ($p>0.05$); compared with M group, the levels of RIP1 protein in penile tissues were significantly decreased in Ent and Sil groups ($p<0.01$), and the level of RIP1 protein in penile tissue was slightly reduced in Nec-1 group ($p>0.05$); compared with M group, the levels of p-RIP1 protein in penile tissues were decreased in Nec-1 and Sil groups ($p<0.05$), and the level of

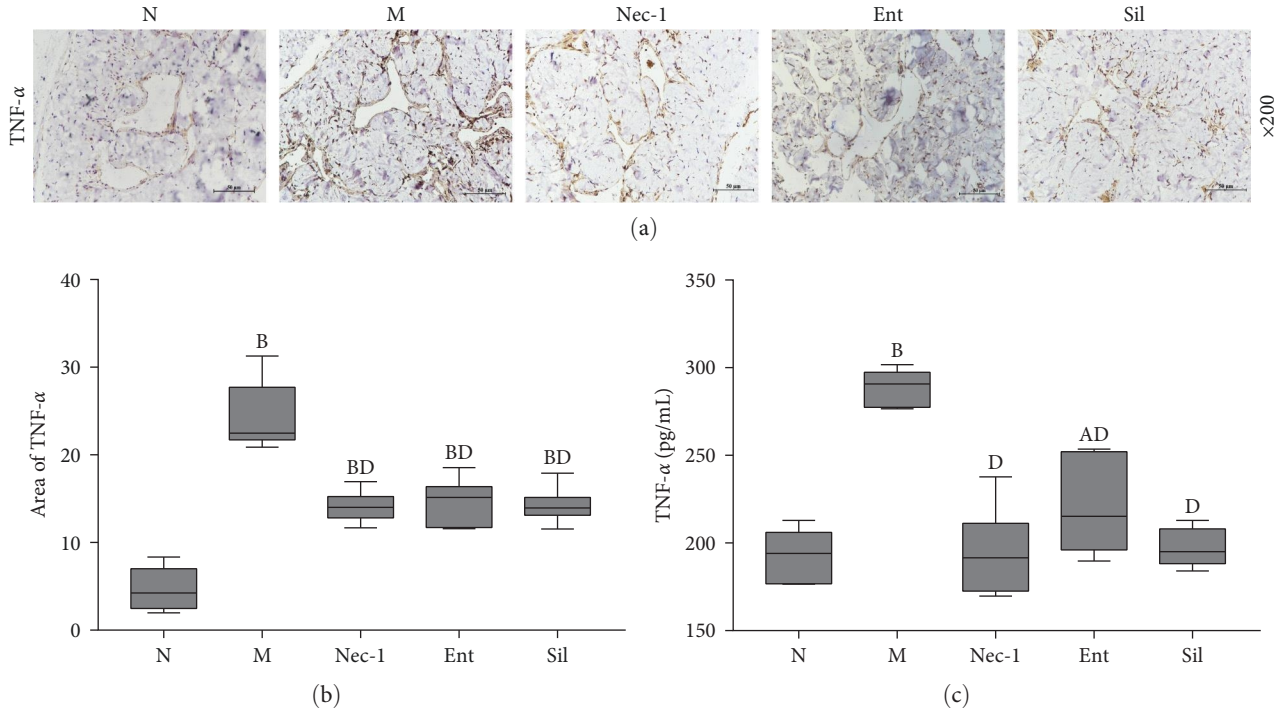

Figure 2: Inhibition of the necroptosis pathway reduces TNF-α levels. (a) Representative images of immunohistochemistry staining of penile tissue with anti-TNF-α antibody in various groups (magnification, ×200). (b) Semi-quantitative analysis of TNF-α expression-positive regions in penile tissues. (c) Levels of TNF-α in serum of different groups. Error bars: mean ± standard error of the mean. $^{A}p<0.05$, $^{B}p<0.01$ in comparison with N group; $^{D}p<0.01$ in comparison with M group.

p-RIP1 protein in penile tissue was slightly reduced in Ent group ($p>0.05$); compared with M group, the levels of RIP3 protein in penile tissues were decreased in Nec-1, Ent and Sil groups ($p<0.05$); compared with M group, the levels of p-RIP3 protein in penile tissues were slightly reduced in Nec-1, Ent and Sil groups ($p>0.05$); compared with M group, the levels of MLKL protein in penile tissues were slightly reduced in Nec-1, Ent and Sil groups ($p>0.05$); compared with M group, the levels of p-MLKL protein in penile tissues were significantly decreased in Nec-1, Ent and Sil groups ($p<0.01$); compared with Sil group, there was no significant difference in the level of TNF-α protein in penile tissue in Nec-1 group ($p>0.05$), and the reduced level of TNF-α protein in penile tissue in Ent group was not as significant as in Sil group ($p<0.05$); compared with Sil group, there was no significant difference in the levels of RIP1 and p-RIP1 proteins in penile tissue in Nec-1 and Ent groups ($p>0.05$); compared with Sil group, there was no significant difference in the levels of RIP3 and p-RIP3 proteins in penile tissue in Nec-1 and Ent groups ($p>0.05$); compared with Sil group, there was no significant difference in the level of MLKL protein in penile tissue in Nec-1 and Ent groups ($p>0.05$); compared with Sil group, there was no significant difference in the level of p-MLKL protein in penile tissue in Nec-1 group ($p>0.05$), and the reduced level of p-MLKL protein in penile tissue in Ent group was not as significant as in Sil group ($p<0.01$).

4. Discussion

It is well known that penile erection is caused by increased arterial blood inflow, active relaxation of penile corpus cavernosal smooth muscle, and decreased venous blood return [11]. The penile corpus cavernosal smooth muscle is central to this hemodynamic change. Any factor that affects the penile corpus cavernosal smooth muscle, which in turn decreases arterial blood flow and increases venous blood return, may lead to the development of ED [12]. There is, therefore, a large amount of study [13,] suggesting that fibrosis of penile corpus cavernosal smooth muscle is an important mechanism in the occurrence and development of ED. In this study, we found that erectile function in cold-stressed rats was impaired and accompanied by fibrosis of penile corpus cavernosal smooth muscle, which is consistent with previous studies [15], and that Sil intervention significantly improved erectile function and could reduce penile tissue fibrosis in rats.

As mentioned earlier, inflammation and ED are mutually causal [9, 16]. The results of this study showed that TNF-α levels were significantly increased in serum and penile tissues of cold-stressed rats and were significantly reduced after intervention with Sil. It is suggested that TNF-α may reduce erectile function by inducing fibrosis of penile corpus cavernosal smooth muscle. However, the specific mechanism of

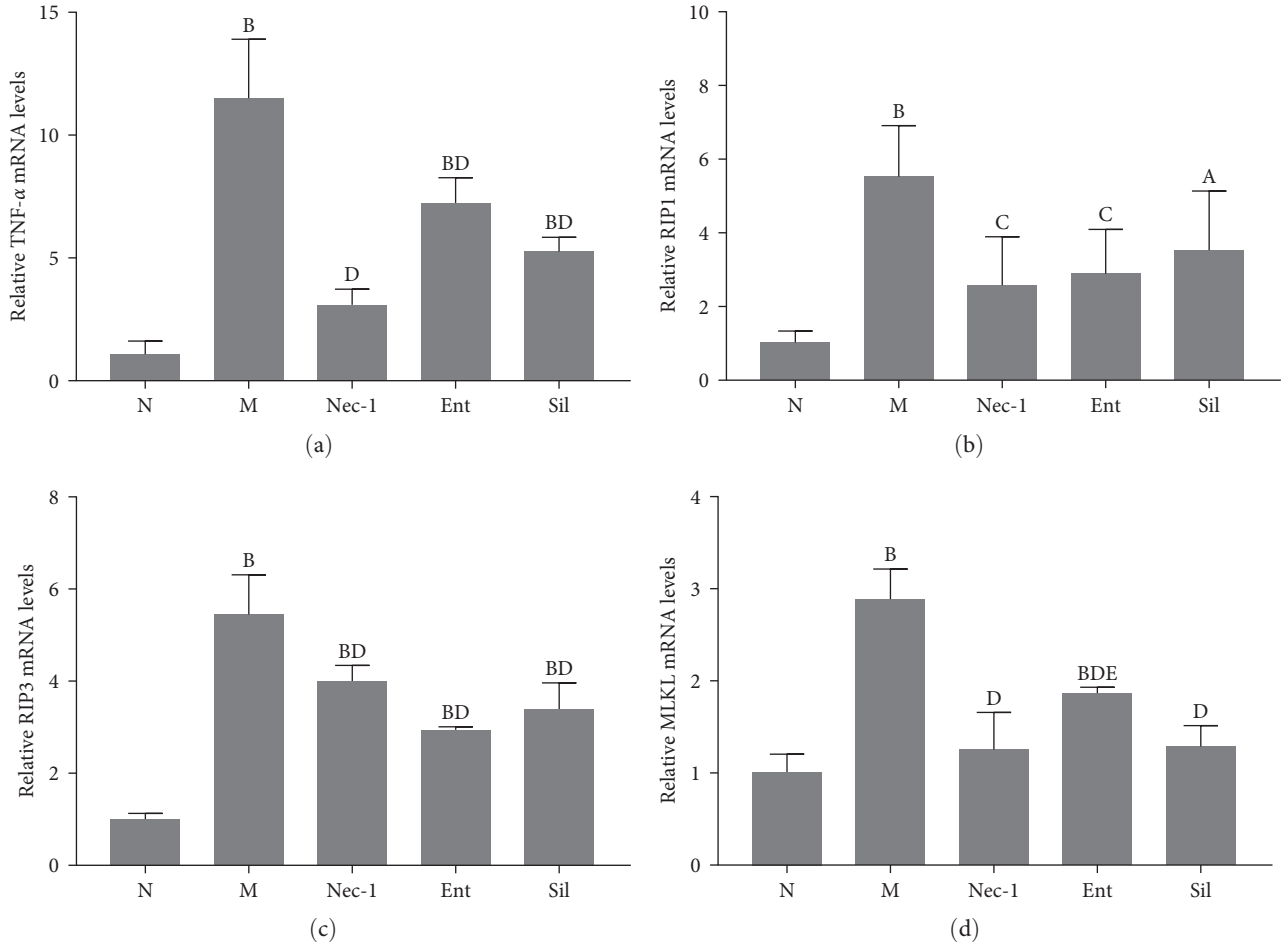

Figure 3: Inhibition of the necroptosis pathway reduces the mRNA expression of TNF-α and factors related to the necroptosis pathway in penile tissue: (a–d) quantitative real-time polymerase chain reaction was used to determine the expression of TNF-α, RIP1, RIP3, and MLKL mRNA in five groups of penile tissues. Error bars: mean $\pm$ standard error of the mean. $^{A}p<0.05$, $^{B}p<0.01$ in comparison with N group; $^{C}p<0.05$, $^{D}p<0.01$ in comparison with M group; $^{E}p<0.05$ in comparison with Sil group.

TNF-α-induced fibrosis of penile corpus cavernosal smooth muscle of cold-stressed rats is still unclear.

Studies have shown that TNF-α is a pleiotropic cytokine that plays a key role in inflammation caused by infection or tissue injury, but in some cases, it can also effectively induce cell death and exacerbate tissue damage [17]. Among these, the TNF-α-mediated RIP1/RIP3 necroptosis pathway is critical in cell death [18]. Numerous studies have demonstrated that the TNF-α-mediated RIP1/RIP3 necroptosis pathway plays a key role in the development of many inflammatory diseases. It has been found that the TNF-α-mediated RIP1/RIP3 necroptosis pathway leads to fibrosis in liver tissue in a rat model of chronic hepatitis [19]. And also, in rats with chronic nephritis, the mechanism of renal interstitial fibrosis was found to be related to the TNF-α-mediated RIP1/RIP3 necroptosis pathway [20]. In addition to this, it has also been found that the male reproductive organs of RIP3 and MLKL knockout mice retain their youthful morphology and function after aging [21]. In this study, we measured the levels of RIP1/RIP3 necroptosis pathway-related factors and their phosphorylation in penile tissues of cold-stressed rats and found that the levels of RIP1, RIP3, MLKL, and their phosphorylation were higher in M group than in N group, and that the levels of these factors were reduced after the intervention with Nec-1 (RIP1-specific inhibitor) and Ent (TNF-α inhibitor). It is suggested that cold stimulation may induce fibrosis of penile corpus cavernosal smooth muscle through activation of the TNF-α-mediated RIP1/RIP3 necroptosis pathway, which in turn reduces erectile function.

In this study, we found that similar to Sil intervention, intervention with Nec-1 and Ent significantly improved erectile function, reduced TNF-α levels, and attenuated penile tissue fibrosis. It is suggested that inhibition of necroptosis may improve erectile function by reducing the level of inflammation and penile tissue fibrosis. Also, this study adequately demonstrated that Sil also inhibited activation of the TNF-α-mediated RIP1/RIP3 necroptosis pathway, suggesting that this may be another molecular mechanism for the treatment of ED with Sil.

MLKL is the most critical factor in necroptosis, and its activation induces cell membrane rupture, leading to tissue damage [22, 23]. It has also been shown that in addition to the classical TNF-α-mediated RIP1/RIP3 pathway, bromodomain-containing protein 4 can also promote the activation of MLKL, which in

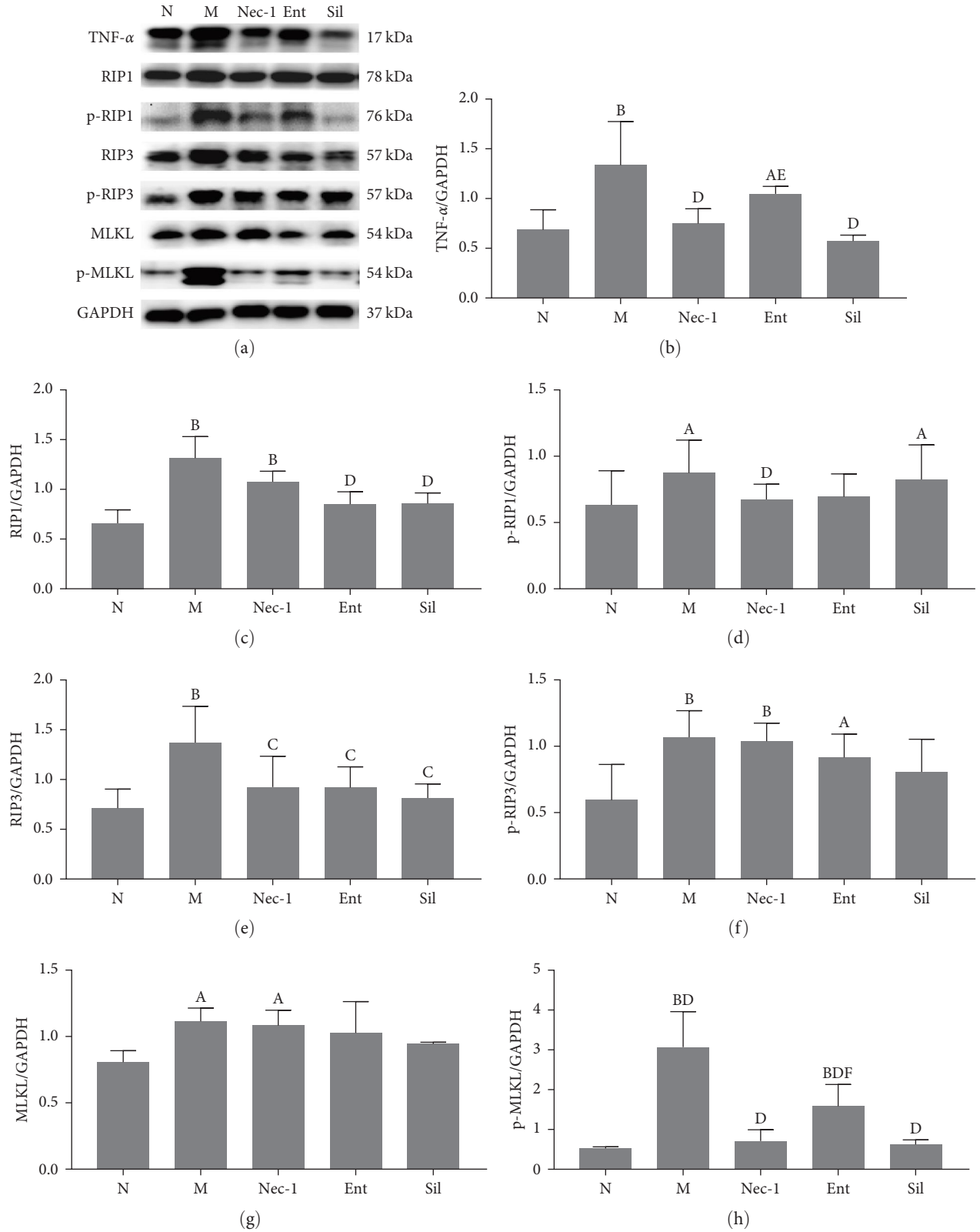

Figure 4: Inhibition of the necroptosis pathway reduces the protein levels of TNF-α and factors related to the necroptosis pathway in penile tissue: (a–h) the levels of TNF-α, RIP1, RIP3, and MLKL proteins in five groups of penile tissues were measured by western blot. Error bars: mean $\pm$ standard error of the mean. $^{A}p<0.05$, $^{B}p<0.01$ in comparison with N group; $^{C}p<0.05$, $^{D}p<0.01$ in comparison with M group; $^{E}p<0.05$, $^{F}p<0.01$ in comparison with Sil group.

turn triggers necroptosis [24]. Similarly, this study found that compared with N group, the level of p-MLKL was 405.81% higher in penile tissues in M group, which was significantly higher than that of p-RIP1 (34.50%) and p-RIP3 (79.97%), suggesting that MLKL, as an executor of necroptosis, has other upstream necroptosis pathways besides the RIP1/RIP3 pathway, which requires follow-up studies. In addition, the exact mechanism of how MLKL induces cell membrane rupture of penile corpus cavernosal smooth muscle cells, ultimately leading to necroptosis, is not yet clear and could be a key direction for future research.

In summary, the TNF-α-mediated RIP1/RIP3 necroptosis pathway was significantly altered in cold-stressed rats, and inhibition of the activation of this pathway could improve erectile function by reducing the level of inflammation and penile tissues fibrosis, which may provide a theoretical reference for the clinical treatment of ED.

Data Availability

All data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

All authors declare that they have no conflicts of interest.

Authors' Contributions

Adilijiang Yiming and Fengxia Liu co-conceived and designed the study. Pei Yang and Lipan Niu wrote the manuscript. Pei Yang and Bingbing Zhu performed the statistical analysis. Siyiti Amuti assisted in some experiments. Pei Yang and Lipan Niu contributed equally to the study and should be considered co-first authors.

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