Protective effect of hypothermia and vitamin E on spermatogenic function after reduction of testicular torsion in rats

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Abstract. This study was designed to investigate the protective effect of hypothermia and vitamin E on spermatogenic function after reduction of testicular torsion in rats. Ninety-six pure inbred male SD rats were divided into group A, B, C and D according to the principle of body weight and birth similarity, with 24 rats in each group. Four groups of rats were respectively twisted on the left testis to establish unilateral testicular torsion rats. Rats in groups A, B, C, D were respectively given normal saline, hypothermia therapy, vitamin E therapy, and hypothermia and vitamin E therapy. The superoxide dismutase (SOD) activity and malondialdehyde (MDA) content of the four groups were detected, and the correlation levels of inflammatory factors IL-1β, hs-CRP and related sex hormones luteinizing hormone (LH), follicle-stimulating hormone (FSH), total testosterone (T) were detected by ELISA. Apoptosis of spermatogenic cells of testis in the four groups was detected by flow cytometry. SOD activity and MDA content in groups B, C and D were significantly higher than those in group A, MDA content was significantly lower than that in group A ($P<0.05$), SOD activity in group D was higher than that in groups B and C, while MDA content was lower than that in groups B and C ($P<0.05$). The levels of IL-1β and hs-CRP in group A were much higher than those in groups B, C and D ($P<0.05$). LH and FSH levels in group A were significantly higher than those in groups B, C and D ($P<0.05$), and in group D were significantly lower than those in groups B and C ($P<0.05$). Apoptosis rate of spermatogenic cells in group A was significantly higher than that in groups B, C and D ($P<0.05$). Hypothermia combined with vitamin E can reverse testicular injury in rats and reduce the apoptosis rate of spermatogenic cells.

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Introduction

Testicular torsion is a common urological emergency. It is defined as the rotation of the spermatic cord's longitudinal axis, which narrows testicular blood flow and causes testicular restriction affected by blood flow, resulting in testicular atrophy. Testicular torsion can occur at any age, but is most common among adolescents (1-3). Experimental studies showed that hemorrhagic infarction of testis caused by testicular torsion began to appear within 2 h after the onset of testicular torsion, irreversible damage occurred after 6 h, and complete infarction occurred before 24 h (4). At the same time, testicular torsion significantly reduced the number of sperm and inhibit sperm motility (5). Therefore, in order to avoid testicular loss and even impaired fertility, timely diagnosis and immediate surgery are the most important issues for the treatment of these patients (6).

Cases of injuries or deaths due to testicular torsion that have not been treated in time occur from time to time. Although blood reperfusion is beneficial to ischemic tissues to some extent, it can also cause a series of adverse reactions and tissue damage, such as testicular atrophy. It will also have certain effects on epididymis and ductus deferens, resulting in increased apoptosis of germ cells, decreased spermatogenic function, and eventually infertility (7,8).

Various methods are used to reduce the damage and apoptosis of spermatogenic cells after torsional reduction, including hypothermia and vitamin E injection. Hypothermia can protect testicular histology and seminiferous tubules from torsion-induced ischemia, resulting in reduction of further damage to seminiferous function after reduction of testicular torsion operation (9). Vitamin E is used for the protection of spermatogenic function after reduction of testicular torsion due to its antioxidant mechanism (10), the effect of inhibiting inflammatory factors (11) and its effect of regulating apoptosis (12). Studies have shown that vitamin E has obvious protective effect on acute testicular torsion and torsion injury. Taking vitamin E can effectively treat testicular torsion and protect sperm activity from torsion (13,14). However, in the past studies, there is very little about combined treatment of vitamin E with hypothermia. Thus, the present study investigated the protective effect of combined treatment of hypothermia and vitamin E on spermatogenic function after reduction of testicular torsion in rats.

Materials and methods

Experimental animals. Ninety-six pure inbred male SD rats (from SJA) of similar age, length and weight, similar size, normal and good in development, and of similar good health were studied. The rats were divided into four groups: Groups A, B, C and D, with 24 rats in each group. Four groups of rats, respectively, were twisted on the left testis to establish a rat model of unilateral testicular torsion. Group A, physiological saline treatment; group B, hypothermia therapy group C, vitamin E therapy; group D, combined treatment of hypothermia with vitamin E. The study was approved by the Ethics Committee of Dongying People's Hospital (Dongying, China).

Animal model. A rat model of testicular torsion was established by Turner *et al* (15). After grouping, rats in each group were anesthetized by intraperitoneal injection with 5% chloral hydrate (350 mg/kg; Tianjin Kemiou Chemical Reagent Co., Ltd.) (16). Then, a 1 cm long incision was made in the left lower abdomen to find the testis, fascia to epididymal head was separated, the gubernaculum testis was ligated and cut off. The left testis was twisted clockwise and fixed for 30 min at 72˚C, then those in groups B and D were placed on sterile saline ice chips for 90 min, the surface temperature of the two groups was controlled at 8-12˚C with ice chips, thermometer was used for detection at all times, and tissue around the exposed testis and its surrounding tissues were wrapped by wet gauze. Groups C and D received 200 mg/kg vitamin E injection from tail vein 30 min before reduction treatment (Shanghai General Pharmaceutical Co., Ltd.; SFDA approval no. H31021260; specification 1 ml: 50 mg x10 branches; batch 2013041253). Groups A and B were injected with the same amount of normal saline slowly from the tail vein 30 min before the reduction treatment. After twisting for 2 h, the left testis was again surgically restored and fixed in the scrotum, and the incision was sutured. Twelve hours after surgery, vitamin E injection of 200 mg/kg was slowly injected from the caudal vein in groups C and D, and the same amount of normal saline was injected from the caudal vein in groups A and B, respectively. No peritonitis was observed when the rats were sacrificed 60 h after surgery and their testicles were taken.

Specimen processing. After all the left testicles were removed, they were washed with normal saline three times and cut along the middle of the longitudinal axis of the testicles. One part was used to make tissue homogenate (Shanghai Medical Instrument Factory), the other part was used to extract primary cells for culture, and then apoptosis was detected.

Detection indicators

i) The superoxide dismutase (SOD) activity and malondialdehyde (MDA) content of the four groups were detected. The homogenate was placed in a centrifuge at 4˚C, centrifuged at 1,500 x g for 10 min, and the supernatant was taken to detect the SOD and MDA content, which was detected by Coomassie brilliant blue protein method (the kits were all from Thermo Fisher Technology, Ltd.).

ii) Four groups of cytokines IL-1β and hs-CRP were detected by ELISA.

iii) The levels of sex hormones in the four groups were detected: The levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and total testosterone (T) in the four groups were detected by ELISA.

iv) Apoptosis detection box was used to detect apoptosis according to the operation instructions. BD FACSCalibur flow cytometer (Shanghai Pudi Biotechnology Co., Ltd.) was used to detect cells transfected for 48 h and stained with Annexin V and propidium iodide (PI) in 6-well plates, and the experiment was repeated 3 times.

Statistical analysis. Data analysis was performed by SPSS 18.0. The measurement data were expressed as mean ± SD, and one-way ANOVA with Least Significant Difference post hoc test were carried out. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Basic data of rats in the four groups. There was no significant difference between group A, B, C and D in basic conditions, such as weight, age, length, and left testis taken from rats in the four groups ($P>0.05$). More details were shown in Table I.

SOD activity and MDA content of the four groups

SOD activity of the four groups. SOD activity in groups A, B, C and D was 26.05±2.46, 54.67±3.87 52.67±3.76 and 67.67±3.57 U/mg, respectively. SOD activity in group A was significantly lower than that in groups B, C and D ($P<0.05$), while that in group D was significantly higher than that in groups B and C ($P<0.05$). There was no difference between group B and C ($P>0.05$) (Fig. 1).

MDA content in the four groups. MDA content in groups A, B, C and D was 4.83±0.07, 2.88±0.05, 2.82±0.06 and 1.49±0.04 nmol/mg, respectively. The MDA content in group A was significantly higher than that in groups B, C and D ($P<0.05$), while the MDA content in group D was significantly lower than that in groups B and C ($P<0.05$). There was no difference between group B and C ($P>0.05$) (Fig. 2).

Levels of IL-1β and hs-CRP in the four groups

i) IL-1β level in the four groups. The levels of IL-1β in groups A, B, C and D were 145.62±5.36, 115.27±3.02, 114.27±3.13 and 61.37±1.83 nmol/ml, respectively. The level of IL-1β in group A was much higher than that in groups B, C and D ($P<0.05$), while that in group D was much lower than that in groups B and C ($P<0.05$). There was no significant difference between group B and C ($P>0.05$) (Fig. 3).

ii) hs-CRP levels of the four groups. Hs-CPR levels in groups A, B, C and D were 65.89±13.05, 40.39±10.37, 41.27±10.20 and 21.57±7.45 mg/l, respectively. Hs-CRP level in group A was much higher than that in groups B, C and D ($P<0.05$), while the activity in group D was much lower than that in groups B and C ($P<0.05$). There was no difference between group B and C ($P>0.05$) (Fig. 4).

LH, FSH and T levels of the four groups. LH levels in groups A, B, C and D were 7.76±1.06, 6.47±0.29, 6.37±0.22 and 5.74±0.15, respectively. LH level in group A was significantly higher than that in groups B, C and D ($P<0.05$), while that in group D

Table I. Basic data of rats in the four groups (mean ± SD) (n=24).

Characteristics	Group A	Group B	Group C	Group D	F-value	P-value
Weight (g)	168.23±11.24	165.94±14.06	167.23±12.78	166.22±13.46	0.156	0.926
Age (days)	37.04±2.02	36.84±2.33	38.04±1.99	37.54±2.41	1.440	0.236
Length (cm)	17.46±1.22	17.52±1.42	17.06±1.72	17.36±1.52	0.457	0.713
Testicular diameter (mm)	14.02±1.03	14.12±0.91	14.07±1.01	14.02±1.03	0.055	0.983
Testicular length (mm)	19.53±1.25	20.02±1.04	19.64±1.16	19.47±1.35	1.007	0.393
Testicular weight (g)	2.11±0.33	2.18±0.27	2.04±0.41	2.21±0.22	1.390	0.251

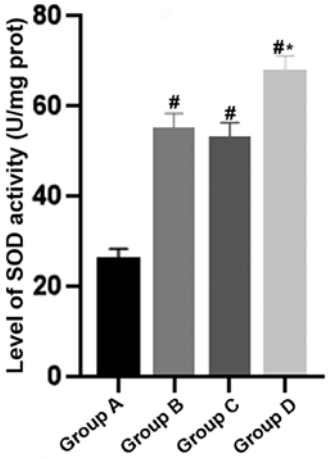

Figure 1. Comparison of the SOD activity of the four groups. The SOD activity of group A was significantly lower than that of groups B, C and D (P<0.05), while the activity of group D was significantly higher than that of groups B and C (P<0.05). There was no significant difference between group B and group C (P>0.05). *P<0.05, comparison with group A; #P<0.05, comparison with groups B and C. SOD, superoxide dismutase.

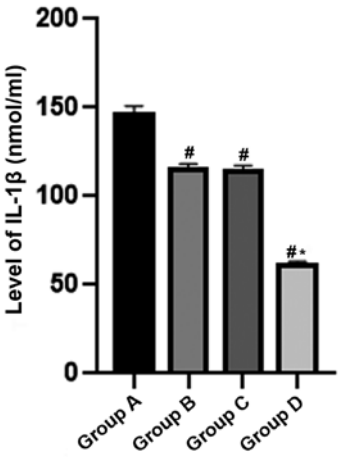

Figure 3. Comparison of the NIHSS scores of patients in the two groups. hs-CRP expression was analyzed by ELISA. The level of IL-1β in group A was much higher than that in groups B, C and D (P<0.05), while the level in group D was much lower than that in groups B and C (P<0.05). There was no significant difference between groups B and C (P>0.05). *P<0.05, comparison with group A; #P<0.05, comparison with groups B and C. IL-1β, interleukin-1β.

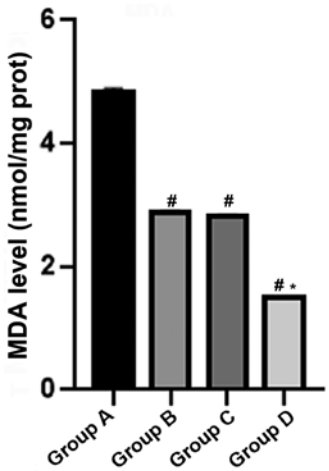

Figure 2. Comparison of the MDA content of the four groups. The MDA content of group A was significantly higher than that of groups B, C and D (P<0.05), while the MDA content of group D was significantly lower than that of groups B and C (P<0.05). There was no significant difference between group B and group C (P>0.05). *P<0.05, comparison with group A; #P<0.05, comparison with groups B and C. MDA, malondialdehyde.

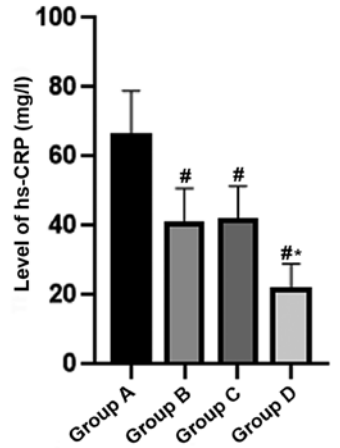

Figure 4. Comparison of the expression level of inflammatory factor hs-CRP in the four groups before and after treatment. ELISA was used to analyze the hs-CRP expression. hs-CRP level in group A was much higher than that in groups B, C and D (P<0.05), while the activity in group D was much lower than that in groups B and C (P<0.05). There was no significant difference between groups B and C (P>0.05). *P<0.05, comparison with group A; #P<0.05, comparison with groups B and C.

was significantly lower than that in groups B and C (P<0.05). There was no significant difference between groups B and C (P>0.05). FSH levels in groups A, B, C and D were 7.96±1.16, 6.97±0.31, 6.89±0.26 and 4.94±0.17, respectively. FSH level

Table II. Levels of LH, FSH and T in groups A, B, C, D (mean ± SD) (n=24).

Index	A	B	C	D	F-value	P-value
LH	7.76±1.06	6.47±0.29[a]	6.37±0.22[a]	5.74±0.15[a,b]	53.02	<0.0001
FSH	7.96±1.16	6.97±0.31[a]	6.89±0.26[a]	4.94±0.17[a,b]	99.73	<0.0001
T	125.76±2.06	137.66±4.05[a]	138.76±3.95[a]	167.76±5.05[a,b]	495.3	<0.0001

[a]P<0.05, comparison with group A; [b]P<0.05 comparison with groups B and C. LH, luteinizing hormone; FSH, follicle-stimulating hormone; T, total testosterone.

Table III. Apoptosis rate of germ cells in groups A, B, C and D (mean ± SD, %) (n=24).

Index	Group A	Group B	Group C	Group D	F-value	P-value
Apoptosis rate (%)	26.03±3.67	5.45±0.53	5.35±0.36	2.35±0.26	1,358	<0.001

in group A was significantly higher than that in groups B, C and D (P<0.05), while that in group D was significantly lower than that in groups B and C (P<0.05). There was no significant difference between group B and C (P>0.05). The T levels in groups A, B, C and D were 125.76±2.06, 137.66±4.05, 138.76±3.95 and 167.76±5.05, respectively. The level of T in group A was much lower than that in groups B, C and D (P<0.05), while that in group D was significantly higher than that in groups B and C (P<0.05). There was no significant difference between group B and group C (P>0.05) (Table II).

Apoptosis rate of the four groups. The apoptosis rates of groups A, B, C and D were 26.03±3.67, 5.45±0.53, 5.35±0.36 and 2.35±0.26%, respectively. The level of apoptosis rate of group A was significantly higher than that of groups B, C and D (P<0.05), while that of group D was significantly lower than that of groups B and C (P<0.05). There was no significant difference between group B and C (P>0.05) (Table III).

Discussion

As an emergency, testicular torsion requires emergency surgical reduction, but even if the ipsilateral testis is rescued, permanent damage will occur, and the contralateral testis may also be damaged, with the risk of infertility (16). Therefore, how to effectively reduce the damage caused by testicular torsion is an important research direction. This study aimed to evaluate the effect of hypothermia and vitamin E on spermatogenic function after reduction of testicular torsion through experiments on the rat model.

SOD is an active substance produced by organisms, which can eliminate harmful substances produced during normal oxygen metabolism, such as free radicals. SOD can react with oxygen radicals to generate hydrogen peroxide, which is converted into water by organisms and excluded. Therefore, SOD activity represents free radical scavenging ability *in vivo*: Higher SOD activity represents higher free radical scavenging ability (17). MDA is an important indicator of lipid peroxidation caused by reactive oxygen species, which reflects the damage degree of free radicals to cells. In the process of vigorous exercise, oxygen consumption has shown a sharp increase. It is also confirmed that the enhancement of mitochondrial activity promotes free radical chain reaction, and the higher the content is, the higher the damage degree of free radicals to cells in testis tissue is (18,19). SOD activity and MDA content will have certain influence on spermatogenic function of testis, SOD level will decrease, and MDA increase will promote apoptosis (20). Vitamin E can inhibit membrane lipid peroxidation by scavenging lipid peroxidation radicals and forming tocopherol radicals (21). Moderate hypothermia can inhibit oxygen metabolism and apoptosis, thus relieving and improving nervous system injury. Related to ischemia-reperfusion injury of rat skeletal muscle, Biwas *et al* (22) found that appropriate hypothermia could reduce MDA and increase SOD, and in myocardial apoptosis, Liu *et al* (23) found that vitamin E reduced the MDA increase and SOD decrease caused by lipid peroxidation and reduce apoptosis. However, in this investigation on spermatogenic function after reduction of testicular torsion, it was discovered that the SOD activity in the testis of rats in groups B, C and D was much higher than that in group A, and the MDA content was significantly lower than that in group A, and the SOD activity in the testis of rats in group D was higher than that in groups B and C, while the MDA content was lower than that in groups B and C. Apoptosis rate of spermatogenic cells was also analyzed. The results showed that the apoptosis rate of groups B, C and D was significantly lower than that of group A, and that of group D was significantly lower than that of groups B and C. Based on the results of SOD, MDA and apoptosis rate, vitamins and hypothermia could improve SOD activity and reduce MDA content after reduction of testicular torsion, thus reducing the apoptosis rate of spermatogenic cells and the combined treatment of the two was better.

Injury caused by ischemia-reperfusion after reduction of testicular torsion led to the production of a large number of pro-inflammatory cytokines and cell adhesion molecules, thus causing severe ischemic tissue damage (24,25), which had a great impact on testicular function. After many testicle torsions and reductions, IL-1β and other cytokines were measured in the treatment of ischemia-reperfusion. These experimental results

all indicated that these inflammatory factors increased after reduction of testicular torsion and ischemia-reperfusion (1,26). Therefore, we evaluated the severity of reduction of testicular torsion and ischemia-reperfusion through inflammatory factors. Vitamin E promoted anti-inflammatory response by binding to the regulatory domain of protein kinase Cα (PKCα) (regulator and antagonist of heart failure), thus reducing various inflammatory factors (27). It was found that vitamin E plays an anti-inflammatory role by reducing the production of IL-1β, IL-6 and TNF-α in colon tissue (28). Hypothermia reduces the level of inflammatory factors and promote anti-inflammatory reaction by reducing the speed of inflammatory reaction and organizing the transportation and combination of some inflammatory factors (29). Wassink *et al* (30) reported on lung transplantation, it was found that moderate hypothermia could reduce the level of inflammatory factors. However, Wassink *et al* (30) and Hsin *et al* (31) reported on cardiovascular injury caused by PM2.5, vitamin E had an inhibitory effect on inflammatory factors such as IL-1β. After referring to similar research results, this study also analyzed the effects of vitamins and hypothermia on IL-1β, hs-CRP and other cytokines after reduction of testicular torsion. We found that the inflammatory factors in the testes of rats in groups B, C and D were lower than those of rats treated with saline, and the two inflammatory factors in the testes of rats in group D were lower than those in groups B and C. Based on the results of our invetigation, we conclude that vitamin E combined with hypothermia treatment reversed the damage after reduction of testicular torsion and had a good inhibitory effect on ischemia-reperfusion.

Sex hormones such as LH, FSH and T play an important role in male reproductive function (32). T is the most important androgen, which can promote meiosis of spermatocytes, differentiation and maturation of germ cells, and enable sperm to mature and release. FSH and LH play a regulatory role on T (33). Therefore, LH, FSH, T and other hormones are important measurement factors in the related research on the effect of testicular torsion reduction on spermatogenic function. Some studies found that vitamin E played an effective part in balancing hormones and fertility by neutralizing free radicals in animals (34). However, the effect of hypothermia on sex hormones is still uncertain. In this investigation, the effects of vitamin E and hypothermia on sex hormones after reduction of testicular torsion were tested simultaneously. LH and FSH levels in groups B, C and D were significantly lower than those in group A, and T levels were significantly higher than those in group A. However, LH and FSH levels in group D were significantly lower than those in groups B and C, and T levels were significantly higher than those in groups B and C. The results of this study showed, FSH and LH levels increased after reduction of testicular torsion was damaged. It regulates T, reduces its secretion, further reducing its effect on spermatocytes, and further weaken its spermatogenic function. Under the treatment of hypothermia and vitamin E, the levels of FSH and LH decreased, and the inhibitory effect on T decreased. Then the level of T increased, promoting spermatogenesis. Therefore, hypothermia and vitamins could reduce the apoptosis rate of spermatogenic cells by promoting the increase of testosterone level.

In conclusion, hypothermia and vitamins can reduce the apoptosis rate of spermatogenic cells by reversing testicular injury in rats, and the combined treatment of the two has better effect and is worthy of promotion.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XB wrote the manuscript. PW and YN conceived and designed the study. RL and JL were responsible for the collection and analysis of the experimental data. XB and HW interpreted the data and drafted the manuscript. XB and PW revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Dongying People's Hospital (Dongying, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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