ORIGINAL RESEARCH

Change and significance of the expression of *c-kit* and SCF following recovery from unilateral testicular torsion in rats

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Manuscript submitted 24th November, 2007
Manuscript accepted 24th April, 2007

Clin Invest Med 2008; 31 (3): E98-E105.

Abstract

Purpose: To investigate the change in expression levels of *c-kit* and SCF, and the protective effects of FSH on ischemia-reperfusion injury due to testicular torsion-detorsion.

Methods: 24 adult male SD rats were divided into three groups of 8: control group, testicular torsion group and FSH-treated group. The control group was treated with sham-operation. Animals in the testicular torsion and FSH-treated groups were subjected to unilateral 720° counter-clockwise testicular torsion for 2 hours and then reperfusion was allowed after detorsion. The FSH-treated group received intraperitoneal injection of FSH 15min before detorsion. Then, the rats were sacrificed and the testes were harvested. Histopathological changes were observed by light microscope, and the expression levels of *c-kit*, SCF in testicular tissue in the different groups were detected by Immunohistochemical assay and Quantitative Real-time RT-PCR analysis. Finally, the relative proportions of germ cells were measured by FCM.

Results: *c-kit* and SCF were positive expressed in 52.58% and 61.16% of testicular cells of control tissues, respectively. Decreases of *c-kit* and SCF positive cells (15.01% and 9.18%) were found in the testicular torsion group. After being treated by FSH, the number of positive cells increased (31.25% and 20.01%). Moreover, the *c-kit* and SCF mRNA expression was increased dramatically ($P<0.01$) in response to FSH stimulation. Furthermore, the

number of haploid, diploid and tetraploid cells has also increased significantly in drug-treated testes ($P<0.01$).

Conclusion: The mechanism of tissue damage in the testicular torsion model, includes changes in the expression of *c-kit* and SCF following torsion. Also, FSH has a protective effect on germ cells after unilateral testicular torsion, which was reflected by increased *c-kit* and SCF levels.

List of Abbreviations

SCF	Stem cell factor
FSH	Follicle stimulating hormone
RT-PCR	Reverse transcription-PCR
RTK	Receptor tyrosine kinase
PBS	Phosphate-buffered saline
FCM	Flow cytometry

The susceptibility of the mammalian testis to ischemic damage has been well documented and ischemia has been considered as a causative factor in some forms of male infertility, the reason for which is the seminiferous tubule is permanently on the brink of hypoxia and transient ischemia causes focal damage of the seminiferous tubules.^{1, 2} Testicular torsion, a form of ischemic injury, is a serious urological emergency that

occurs in youngsters with high incidence and requires prompt surgical intervention to prevent testicular damage. The function of the testis may be influenced from the ischemia-reperfusion injury following testicular torsion and detorsion. The degree of injury is determined by the severity of arterial compression and the interval between the onset of symptoms and surgical intervention.³ Testicular torsion in laboratory animals causes permanent aspermatogenesis by germ cell-specific apoptosis.

Ischemia and reperfusion of the testis stimulates an intracellular signaling cascade in the testicular endothelial cells. *c-Kit* encodes for the receptor tyrosine kinase (RTK) and belongs to type III receptor family.⁴ The RTKs activate several signaling pathways within the cells, such as *c-kit*/SCF. In the testis, stem cell factor (SCF), which is produced by Sertoli cells and a progenitor cell growth factor, binds to and activates the *c-Kit* receptor tyrosine kinase, which is critical for migration, adhesion, proliferation, survival of primordial germ cells and spermatogonia during testicular development.^{5, 6} Several reports have shown the decreasing expression of *c-Kit* and SCF mRNA in testicular biopsies of azoospermic patients.⁷ However, there are no reports available in any of the public databases concerning their expression in ischemia-reperfusion testis tissues. We have used a unilateral testicular torsion model of rats to examine the characteristic changes in *c-Kit* and SCF gene expression occurring during recovery of the testis from ischemic damage. On the basis of the previous reports that FSH prevented germ cells from undergoing apoptosis *in vitro* and that SCF expression is highly dependent on FSH stimulation in the rat seminiferous tubules cultured *in vitro*^{8, 9}, we used testicular torsion rat models to investigate the differences in *c-Kit* and SCF gene expression between torsioned testes and FSH treated torsioned testes to evaluate the protective effects of FSH to ischemia-reperfusion injury following testicular torsion.

Materials & methods

Experimental Animals

The study group consisted of 24 adult male Sprague-Dawley (SD) rats weighing 240 - 260g, obtained from the Department of Laboratory Animal Science, Wuhan University, Hubei province of P.R.China. All animals were treated humanely and the Medical Laboratory Animal Management Committee of Wuhan University approved all animal procedures. The rats were housed in a temperature-controlled room ($24^{\circ}\pm 1^{\circ}\text{C}$) on a 12-hour light and dark cycle, with free access to food and water.

Animal preparation and surgical procedure

Surgical torsion was carried out as described by Turner¹⁰ with modifications. 24 adult male Sprague-Dawley rats were divided into three groups of 8, control group, testicular torsion group and FSH-treated group. The surgical procedures were performed under general anesthesia induced by intraperitoneal one-shot injection of ketamine (8 mg/kg) and xylazine (10 mg/kg), and maintained using inhaled 0.5% halothane in air. The skin of the scrotal area was shaved and prepared with 10% povidone iodine solution. A mid-scrotal vertical incision was performed for access to both testes. Torsion was created by twisting the right testis 720° in a counterclockwise direction and maintained by fixing the testis to the scrotum with a 6-0 nylon suture passing through the tunica albuginea and dartos. The testis was left on top of the incised region, covered with a sterile gauze pad kept moist with normal saline, while the rat was kept under continuous anesthesia. After 2 hours of ischemia, the suture was removed, and the right testis was untwisted and replaced in the scrotum for reperfusion. During the sham operation in the control group, the left testis was brought through the incision and then replaced without twisting, and a nylon suture was placed through the tunica albuginea. After each surgical intervention, the incision was closed using Michel sutures. The

animals were left to recover for four days. After that, they were anesthetised, the testes were removed and the animals were euthanised using an intracardiac barbiturate overdose injection.

Groups

The sham-operated group served as the control group. The testicular torsion group underwent 2 hours of testicular torsion and then detorsion. The FSH treated group received treatment with intraperitoneal injection of 20mg/kg recombinant human FSH (10 ng/ml, Org 32489, Organon, Oss, The Netherlands) 15 min before detorsion. The control group and the testicular torsion group received injection of normal saline (NS) in the same manner.

Histopathologic assessment of testicular damage

A four-level grading scale, similar to that of Cosentino et al.¹¹, was used to evaluate histologic injury of seminiferous tubules after testicular torsion and detorsion. Representative pieces of testes were immersed in Bouin's fixative, kept at 4°C for 5 days, and then embedded in paraffin. Blocks were trimmed, and 5µm thick sections were stained with haematoxylin and eosin. The slides were evaluated by two pathologists in a blind, randomly numbered fashion and each given a grade as following: Grade 1 showed normal testicular architecture with an orderly arrangement of germinal cells; Grade 2 showed less orderly, noncohesive germinal cells and closely packed seminiferous tubules; Grade 3 injury exhibited disordered, sloughed germinal cells with shrunken, pyknotic nuclei and less distinct seminiferous tubule borders; Grade 4 injury defined seminiferous tubules that were closely packed with coagulative necrosis of the germinal cells.

Immunohistochemistry Assay

Expression levels of c-Kit and SCF in testicular tissue were analyzed by immunohistochemical staining. Tissues were fixed in 10% buffered formalin and embed-

ded in paraffin. Commercially available monoclonal antibodies to c-Kit and SCF were used. Immunohistochemical staining was carried out on TMA sections using the avidin-biotin method and a commercially available kit. One paraffin-embedded block of testicular tissue was selected from each case and cut into 4µm sections. Deparaffinized sections were treated with methanol containing 3% hydrogen peroxide for 10 min before conducting antigen retrieval using a microwave oven at 95°C for 5 min and cooling at 25°C for 2 hours. After washing with phosphate-buffered saline (PBS), blocking serum was applied for 10 min. The sections were incubated with an anti- c-Kit monoclonal antibody and anti- SCF monoclonal antibody overnight at 4°C. Negative control sections were incubated with PBS instead of the primary antibody. After washing in PBS, a biotin-marked secondary antibody was applied for 10 min followed by a peroxidase-marked streptavidin for an additional 10 min. The reaction was visualized by using 3, 3'-diaminobenzidine tetrahydrochloride. The nuclei were counterstained with hematoxylin. Positive and negative immunohistochemistry controls were routinely used. Reproducibility of staining was confirmed by reimmunostaining via the same method in multiple, randomly selected specimens. Two experienced pathologists independently examined staining while blind to the description of slices. The number of positive cells that showed immunoreactivity on the cell membrane and cytoplasm in the representative ten microscopic fields was counted and the percentage of positive cells was calculated.

RNA isolation

RNA was extracted from 1g of fresh testicular tissue with Trizol reagent (Gibco-BRL). Extraction and centrifugation steps were carried out according to the manufacturer's instructions. The mRNA was purified from 1 mg of total RNA using oligo(dT)-cellulose columns by standard protocols. The concentrations of total RNA and mRNA were estimated using a spectro-

photometer at 260 nm wavelength, and the purity of RNA was determined by calculating the ratio of absorbance at 260/280 nm.

Quantitative Real-time RT-PCR

Single-stranded cDNAs were generated using Superscript II reverse transcriptase (Invitrogen) following the manufacturer's directions. Briefly, reverse transcription of 2 µg of DNase-I treated RNA was performed in a 20 µl reaction mixture containing 0.75 µl oligodT, 1 µl Primer Mix (10mM, reaction concentration was 0.4mM), 0.25 µl random hexamer, 4 µl 5×Reaction Buffer, 0.5 µl RNase Inhibitor (Takara Biotechnology), 4 µl dNTP (10mM), 1 µl MMLV reverse transcriptase (Promega, Madison, USA).

Quantitative Real-time PCR experiments were performed on a RotorGene 2000 system (Corbett Research, Mortlake, Australia), using SYBR Green PCR Master Mix according to the manufacturer's protocol (Shanghai Kaifang S&T Company, Shanghai, China). Using β-actin plasmid DNA constructed by ourselves as a positive control and based on 10 times concentration gradients, fluorimetric quantitative PCR was performed between 2 to 20000 µg/L concentration gradients. The amount of target gene expression was extrapolated from the standard curve and normalized to β-actin. Data were analyzed using Rotor-Gene v5.0 (Corbett Research).

PCR was carried out in 25 µl reaction mixtures containing 1 µl cDNA, 2 µl primers, 1.25 µl 20 × SYBR Green I, 0.5 µl 50 × Rox Dye (Invitrogen), 2.5 µl 10 × PCR Buffer (mM MgCl₂), 1 µl Mg²⁺ (25 mmol/L), 2 µl dNTP Mix (2.5 mmol/L per nucleotide), 0.125 µl Hot Star Taq E (Takara Biotechnology). The following pairs of primers were used: *c-kit*, sense 5'-AGA CAG GCT CTT CTC AAC CAT-3' and antisense 5'-GCT TGG CAG GAT CTC TAA CA-3' (288bp); *SCF*, sense 5'- ACT TGG ATT ATC ACT TGC ATT TAT C -3' and antisense 5'- CTT CCA GTA TAA GGC TCC AAA AGC -3' (689 and 605bp); β-

actin, sense 5'-GTG GGG CGC CCC AGG CAC CA-3' and antisense 5'-CTC CTT AAT GTC ACG CAC GA-3' (660bp). All primers were synthesized by Sangon Co. (Shanghai, China). The thermal cycling conditions were as follows: an initial melting step of 5 min at 94 °C, followed by 35 cycles of 60 s at 94 °C, 60 s at 58 °C and 60 s at 72 °C, and a final elongation step of 1 min at 72 °C. Duplicate PCR amplifications were performed for each sample.

Flow Cytometry

The DNA distribution histograms of testicular cells were obtained by FCM as reported by Koji Shiraishi, et al.¹² In brief, the testis was dispersed with 0.1% collagenase (type Ia; Sigma) at 37°C for 1 h, fixed in 70% ethanol, and stored at 4°C until the assay. The samples were treated with ribonuclease (type IIIa; Sigma) in PBS at 37°C for 30 min and 0.5% pepsin A (Sigma) at 37°C for 15 min before being stained with 2.5 mg/ml of propidium iodide (Sigma) for FCM (FACSC alibur, BD Ltd., USA). The relative proportions of haploid (N), diploid (2N), and tetraploid (4N) cells were calculated, and the percentage of haploid cells was used as an index of spermatogenesis.

Statistical analysis

Data obtained were expressed as mean±S.D. and analysed by one-way ANOVA with the post-hoc Tukey's test applied for paired comparisons. A difference between means was considered significant if *P* was < 0.05.

Results

Histopathological assessment of testicular damage

The results of morphological examination as shown in Fig.1A demonstrated that the surgical procedure was effective in inducing the required level of testicular damage. The contralateral testes with sham operation were not damaged in the modeling procedure, as evidenced by grading of 1. In contrast, the testes that un-

FIGURE 1. Histopathological changes of testes tissues in rats of different groups observed by light microscope (HE staining, original magnification $\times 100$). A: Normal testes in the control group show active spermatogenesis (grade 1) and orderly arrangement of germinal cells; B: In the testicular torsion group, injury exhibited disordered, sloughed germinal cells with shrunken, pyknotic nuclei and less distinct seminiferous tubule borders (grade 3); C: In the FSH treated group, seminiferous tubules packed closely with noncohesive and less orderly germinal cells (grade 2).

derwent testicular torsion in the testicular torsion group showed similar histopathological changes based on counting ten tubules for each sample and were in grade 3 (Fig.1B). After being treated by FSH, the pathological changes of testicular tissue decreased to grade 2 compared with the testicular torsion group (Fig.1C). This indicates that, at the stage of FSH protection, destruction of testes has partially disappeared.

Expression Levels of c-kit and SCF Protein in testicular tissue

Expression of *c-kit* and SCF protein in testicular tissue was examined immunohistochemically. The number of positive cells in the three groups is shown in Table.1. *c-kit* and SCF were expressed in 52.58% and 61.16% of control testicular cells, respectively. Decreases of *c-kit* and SCF positive cells (15.01% and 9.18%) were found in the testicular torsion group. After treatment with FSH, the positive cells increased

(31.25% and 20.01%) indicating that FSH has some protective effects on testes with ischemia injury.

Quantitative expression of c-kit and SCF in testis Tissues

Expression levels of *c-kit* and SCF gene in testicular tissue were examined by Quantitative Real-time RT-PCR method. The expression of β -actin mRNA was detected as an internal standard. Triplicate experiments were carried out to test any variability in this step, noting that the RNA was isolated from a different set of rats each time. Mean *c-kit* and SCF mRNA levels in tissues from Testicular Torsion group and FSH treated group are shown in Table 2. All values are expressed relative to those in tissues from control group. The quantity expression of *c-kit* mRNA and SCF in control, Testicular Torsion and FSH treated groups were 4.08 ± 0.03 , 2.56 ± 0.75 , 3.45 ± 0.36 , and 4.27 ± 0.11 , 2.78 ± 0.52 , 3.68 ± 0.23 , respectively, a

TABLE 1. Percentage *c-kit* and SCF positive cells in testis tissues of in different groups

Groups	No. of rats	<i>c-kit</i>	SCF
Control	8	52.58 ± 1.26	61.16 ± 3.25
Testicular Torsion	8	$15.01 \pm 2.45^{\Delta}$	$9.18 \pm 0.12^{\Delta}$
FSH treated	8	$31.25 \pm 1.31^{*#}$	$20.01 \pm 5.07^{*#}$

All results were expressed as mean $\pm$ SD. (n=8)

$^{\Delta}P < 0.05$, $^{\Delta}P < 0.01$ compared with the control group

$^{*}P < 0.05$ compared with the testicular torsion group

TABLE 2. *c-kit* and SCF mRNA expression levels in testicular tissue

Groups	No. of rats	<i>c-kit</i>	SCF
Control	8	4.08 ± 0.03	4.27 ± 0.11
Testicular Torsion	8	$2.56 \pm 0.75^{\Delta}$	$2.78 \pm 0.52^{\Delta}$
FSH treated	8	$3.45 \pm 0.36^{*#}$	$3.68 \pm 0.23^{*#}$

All results were expressed as mean $\pm$ SD. (n=8)

$^{\Delta}P < 0.05$, $^{\Delta}P < 0.01$ compared with the control group

$^{*}P < 0.05$ compared with the testicular torsion group

($P < 0.01$ among groups). This suggests that FSH can increase the quantity of *c-kit* and SCF in injured testicular tissue.

Protective Effects of FSH on Spermatogenesis

Distribution histograms of testicular cells in the groups obtained by FCM are shown in Table 3. Haploid cells accounted for $66.7 \pm 1.08\%$ in the control group, decreasing to $23.8 \pm 2.5\%$ after 2h of torsion followed by reperfusion in the testicular torsion group ($P < 0.05$). FSH had some therapeutic effect on the decrease of haploid cells, the percentage of haploid cells in the FSH treated group increased to $45.3 \pm 2.3\%$ ($P < 0.05$) (Figure 2).

Discussion

It has been estimated that the male is the major cause of conception failure in about 22~26% of infertile couples.^{13, 14} Male infertility is a contributing element in as many as half of the couples attending an infertility clinic. There are many causative factors¹⁵, one of which is ischemia-reperfusion damage due to testicular torsion and detorsion. This damage resembles ischemia-reperfusion observed in other tissues, the main phenomenon of which is hypoxia. The seminiferous tubules are on the brink of hypoxia and the sensitivity of the testis may bring about ischemia damage.^{16, 17} Many molecules may be involved in this process including the *c-kit*/SCF signal pathway. SCF exerts its biological effects by binding to the tyrosine kinase receptor *c-kit*, which is located on the cell surface.^{18, 19} Signal transduction by this receptor plays a critical role in hematopoiesis, in mast cell development and function, and in the development of melano-

FIGURE 2. Protective effects of FSH on the percentage of haploid cells after 4 days of recovery from 2h of ischemia.

cytes and germ cells. SCF exists in soluble or transmembrane forms, and it has been reported that membrane bound SCF may induce more persistent tyrosine kinase activation than the soluble version.^{20, 21} There are two forms of SCF, mSCF and sSCF, causing differential effects on primordial germ cells have been reported.²² During testicular development, SCF/*c-kit* interaction plays an important role in primordial germ cell migration and survival, and in spermatogonial adhesion, proliferation and survival.²³ Few previous reports have paid attention to their influence in torsion/detorsion ischemia-reperfusion damage.

The extent of the ischemia-reperfusion injury induced by torsion in rat models can be controlled by the degree and duration of the torsion. This was selected from previous studies that optimized these parameters as assessed by histological and various biochemical parameters. We chose the protocol that induced moderate damage relevant to clinical situations. Our histopathological findings showed clear ischemia-

TABLE 3. The number of spermatogenic cells in testes of rats in different groups

Groups	No. of rats	Haploid cells	Diploid	Tetraploid
Control	8	5829.13 ± 180.24	1621.16 ± 131.25	1927.19 ± 207.28
Testicular Torsion	8	1695.01 ± 112.45 [▲]	698.17 ± 50.13 [▲]	512.32 ± 60.08 [▲]
FSH treated	8	3401.25 ± 129.31 ^{*#}	1202.00 ± 65.31 ^{*#}	867.38 ± 290.29 ^{*#}

All results were expressed as mean ± SD. (n=8)

[#] $P < 0.05$, [▲] $P < 0.01$ compared with the control group

^{*} $P < 0.05$ compared with the testicular torsion group

reperfusion injury of germinal cells with shrunken, pyknotic nuclei and less distinct seminiferous tubule borders in the testis tissues of the untreated torsion/detorsion group. After being treated by FSH, the pathological damage in torsion testes recovered to a certain extent.

Several chemicals, drugs, and physical methods have been investigated to protect organs against ischemia-reperfusion injury.²⁴ FSH upregulates SCF expression in both immature and mature testis. In the present study, we tested the protective effects of FSH on torsion/detorsion injury according to the results of immunohistochemical assay and Quantitative Real-time RT-PCR analysis. As *c-kit* and SCF acts on the germ cells to stimulate spermatogenesis, decrease of mRNA levels in spermatogenic failure, coincident with the pathological changes in testes in the untreated torsion/detorsion group, suggests that the paracrine control of Sertoli cells on germ cells was decreased in many cases of human spermatogenic failure. On the other hand, FSH administration led some sequential recovery of impaired testes from torsion-detorsion damage, followed by an increase of *c-kit* and SCF expressed in FSH treated group. Previous reports indicated that FCM could determine the percentage of haploid cells, including spermatozoon and spermatids, which is useful in analyzing germ cell differentiation.²⁵ The protective effect of FSH on spermatogenesis of testes was also demonstrated by FCM.

In conclusion, these results show the change of SCF and *c-kit*, and the protective effects of FSH during the ischemia-reperfusion injury in the torsion/detorsion rat model. Taken together, FSH appears to be a likely candidate drug for modulating SCF/*c-kit* complex-induced cell proliferation in the acute ischemia-reperfusion injury model. These findings provide new insight into the potential action of FSH in testicular torsion-detorsion damage and may generate novel strategies for therapeutic drug development.

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