



Protective Effects of Uncultured Adipose-Derived Stromal Vascular Fraction on Testicular Injury Induced by Torsion-Detorsion in Rats

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Received March 18, 2018;
accepted for publication
November 16, 2018; first
published December 19, 2018.

[http://dx.doi.org/
10.1002/sctm.18-0063](http://dx.doi.org/10.1002/sctm.18-0063)

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Key Words. Stem cell therapy • Ischemia-reperfusion injury • Spermatogenesis •
Torsion-detorsion • Stromal vascular fraction • Adipose stem cells

ABSTRACT

Torsion-detorsion (T/D)-induced testicular injury may lead to male subfertility and even infertility. Stem cell therapy provides an alternative to attenuate testicular injury and promote spermatogenesis. Adipose-derived stromal vascular fraction (SVF) can be acquired conveniently without in vitro expansion, which may avoid the potential risks of microbial contamination, xenogenic nutritional sources, etc., during cell culture. In this study, we investigate the protective effects of autologous uncultured SVF on testicular injury and spermatogenesis in a rat model of T/D. Animals were randomly divided into sham, T/D+ phosphate-buffered saline, and T/D + SVF groups (18 rats in each group). SVF was isolated, labeled with lipophilic fluorochrome chloromethylbenzamido dialkylcarbocyanine, and transplanted into T/D testis by local injection. At 3, 7, 14, and 28 days F surgery, testicular tissue and serum samples were harvested for histopathological, immunohistochemical, Western blot, and enzyme-linked immunosorbent assays. Histopathological findings demonstrated severe injury in the testis with decreased Johnsen's score led by T/D, while uncultured SVF reduced testicular injury and elevated the decreased score. Injected SVF cells were mainly integrated into interstitial region and seminiferous tubules, enhanced the secretion of basic fibroblast growth factor and stem cell factor in the testis, contributed to the declining level of malondialdehyde and restoration of hormonal homeostasis, and then reduced the injury of Leydig cells and germ cells, as well as promoting spermatogenesis. Our findings demonstrated that autologous uncultured SVF could protect the testis from testicular ischemia-reperfusion injury and promote spermatogenesis, which provide significant clinical implications for the prevention of infertility induced by testicular T/D. *STEM CELLS TRANSLATIONAL MEDICINE* 2019;8:383–391

SIGNIFICANCE STATEMENT

Testicular torsion is a urological emergency that can result in severe acute ischemia injury of the testis. Even though detorsion operation is performed, the torsion-detorsion (T/D) may eventually lead to male subfertility and even infertility because of ischemia-reperfusion (I/R) injury of the testis. Stem cell therapy is viewed as a promising option to rescue I/R injury of organs. Uncultured adipose-derived stromal vascular fraction (SVF) has emerged as an attractive cell source for cell-based therapy. The study on a rat model of testicular T/D demonstrated that autologous uncultured SVF could protect the testis from testicular injury and promote spermatogenesis in testicular T/D rats.

INTRODUCTION

Testicular torsion is a urological emergency that can result in severe acute ischemia injury of the testis, thus an immediate surgical intervention is required to prevent testicular injury [1]. However, although detorsion operation is performed immediately after diagnosis to restore testicular blood flow, ischemia-reperfusion (I/R) injury is unavoidable during the whole process of

testicular torsion-detorsion (T/D). Testicular I/R injury induced by T/D can result in germ cell apoptosis and decreases impair spermatogenesis, and then ultimately lead to male subfertility and even infertility. Previous study in animal models demonstrated that, despite the blood flow of the testis was returned to normal without the occurrence of infarction, T/D could still lead to the loss of spermatogenesis [2]. Another

study indicated that reduced sperm counts could be found in patients who experienced testicular T/D after a long-term follow-up [3]. The possible mechanism of testicular I/R injury may account for the altered hormone production resulted from torsion and generation of oxygen-derived free radicals led by detorsion [4].

Up to now, researchers have attempted various candidate to attenuate T/D-induced testicular I/R injury and facilitate spermatogenesis [5–7]. More recently, adipose tissue-derived mesenchymal stem cells (AdMSCs) have been explored to be able to rescue testicular torsion-induced germ cell injury and induce spermatogenesis in seminiferous tubules of busulfan-induced azoospermic rat [8, 9]. Transplanted AdMSCs through local injection have been demonstrated to prevent I/R-induced intrinsic apoptosis, reduce the oxidative stress of the injured testicular tissue, and transdifferentiate into spermatogenic cells [8–10]. Adipose-derived stromal vascular fraction (SVF) is a rich source of AdMSCs [11]. Studies have reported that administration of uncultured SVF could attenuate injury and improve the function of various tissues and organs, such as the heart [12], kidney [13], urethral sphincter [14], etc. Recently, both our team and other groups have demonstrated that, compared with AdMSCs, uncultured SVF exhibited equal effect on attenuating acute renal I/R injury and recovering penile erection [15, 16]. Uncultured SVF has emerged as an attractive cell source for cell-based therapy because of the real-time isolation with a sufficient quantity and avoidance of the potential risks of microbial contamination, xenogenic nutritional sources, etc., during the process of cell culture [17]. It seemed that uncultured SVF may be particularly suitable for the treatment of acute organ injury, including T/D-induced testicular I/R injury. However, to the best of our knowledge, no study has so far attempted to investigate the effect of uncultured SVF on testicular I/R injury and repair of spermatogenesis.

We hypothesize that uncultured SVF may contribute to the repair of spermatogenesis after T/D-induced testicular injury. Therefore, the present study has been performed to explore the potential protective effects of uncultured SVF in testicular I/R injury in a rat model of testicular T/D.

MATERIALS AND METHODS

Animals and Study Design

A total of 54 male Sprague-Dawley rats at the age of 8–10 weeks weighing 220–250 g were used in this study. Animals were fed in a standard room with controlled temperature and humidity, on a 12 hours light/dark cycle, and provided with food and water ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Nanjing First Hospital, Nanjing Medical University. This work was performed in accordance with the institutional and national guidelines for laboratory animals.

The rats were randomly divided into three groups (12 in each): sham-operated group (sham), T/D + phosphate-buffered saline (PBS) group, and T/D + SVF group. Before transplantation, the uncultured SVF cells were detected by flow cytometric analysis. Animals were sacrificed at 3 and 7 days after surgery. The blood and testicular tissue samples were

harvested and used for the following experiments. Another 18 rats were randomly divided into sham, T/D + PBS, and T/D + SVF groups (6 in each) and sacrificed at 14 and 28 days after surgery for histopathological examination to further observe the protective effect of uncultured SVF on testicular I/R injury. All surgical procedures were carried out by sterile techniques under anesthesia with intraperitoneal injection of ketamine (100 mg/kg).

Isolation and Characterization of SVF

Autologous SVF was isolated from epididymal adipose tissue according to our previously published protocol [13, 18]. Briefly, rat adipose tissue was washed thrice with ice-cold sterile PBS, minced into small pieces, and digested with 0.075% type I collagenase at 37°C for 30–40 minutes with gentle shaking. After processing and purification, including centrifugation, repetitive wash steps, and treatment with Red Blood Cell Lysis Buffer, the cell pellet was resuspended with PBS and counted with an automated cell counter.

Cell markers of uncultured SVF were determined by flow cytometric analysis with the following antibodies: fluorescein isothiocyanate (FITC) conjugated anti-CD31 (Bioss Antibodies Inc., Woburn, MA, USA), FITC conjugated anti-vascular endothelial growth factor receptor-2 (VEGFR-2; Bioss Antibodies Inc.), FITC conjugated anti-CD90 (Bioss Antibodies Inc.), FITC conjugated anti-CD45 (BioLegend, San Diego, CA, USA), phycoerythrin (PE) conjugated anti-CD11b/c (BioLegend, San Diego, CA, USA), PE conjugated anti-CD34 (Bioss Inc.), PE conjugated anti-CD106 (Bioss Inc.), PE conjugated anti-CD133 (Novus Biologicals, Colorado, USA). The labeled SVF cells were analyzed with FACSCaliber (BD Biosciences, San Diego, CA, USA). An isotype-matched IgG was set for each procedure.

Cell Labeling

SVF cells were labeled with the lipophilic fluorochrome chloromethylbenzamido dialkylcarbocyanine (CM-Dil; Molecular Probes, Grand Island, NY, USA) according to the manufacturer's instruction. Briefly, SVF cells were incubated with CM-Dil at 37°C for 5 minutes (1 μ g CM-Dil per 10^6 cells), followed by incubation at 4°C for 15 minutes, and finally washed twice for the subsequent studies.

Testicular T/D and Cell Transplantation

Testicular T/D model was established as described previously with modification [7]. In brief, the tunica vaginalis was opened under a mid-scrotal incision. After the right testis was removed, the left testis was rotated 720° clockwise and fixed for 4 hours. Then, the left testis was untwisted. In the sham group, the rats were treated identically without the torsion of the left testis. During the 4 hours of torsion, autologous SVF was isolated and labeled with CM-Dil described above. After the establishment of testicular T/D model, the labeled SVF was suspended in 100 μ l PBS at the dosage of 2×10^6 cells and transplanted into the left testis through local injection in the T/D + SVF group, while equal volume of PBS was injected instead in the T/D + PBS group.

Histopathology

Testicular tissues were fixed with 10% formaldehyde, dehydrated, embedded in paraffin, sectioned at 5- μ m, and stained with H&E. Slides were evaluated by a pathologist under

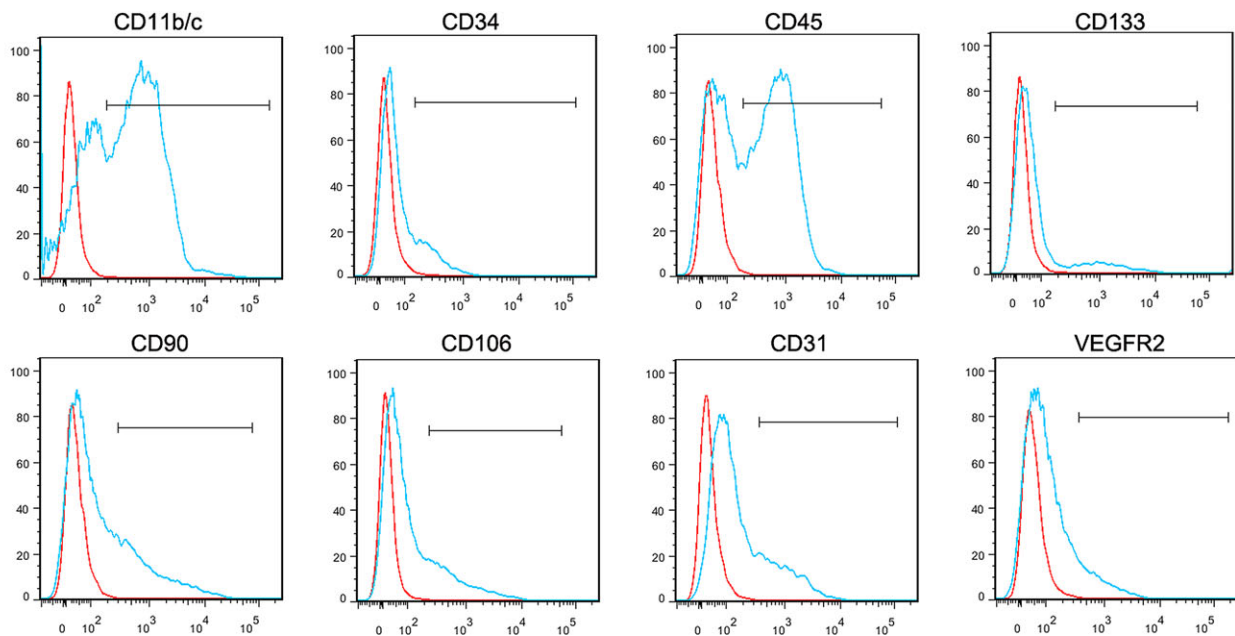


Figure 1. Representative flow cytometry histograms of uncultured adipose derived stromal vascular fraction. Abbreviation: VEGFR2, vascular endothelial growth factor receptor.

blinded conditions by a standard light microscopy (Olympus BX53, Tokyo, Japan). The severity of germ cell injury was determined and graded according to the Johnsen's scoring system [19]. Briefly, at least 10 random fields in each slide were evaluated systematically at $\times 200$ magnification. Each tubule was given a Johnsen's score from 1 to 10. Complete spermatogenesis with many spermatozoa present was evaluated as score 10. Testicular spermatogenesis in each group was assessed by measuring mean score from all evaluated tubules.

Immunohistochemistry

Tissue sections were made as described above and immunohistochemically stained by using the anti-DEAD (Asp-Glu-Ala-Asp)-box helicase 4/MVH (VASA) antibody (Abcam, Cambridge, MA, USA), anti-SOX9 antibody (Abcam), and anti-Cytochrome P450 1A2 antibody (Abcam) to detect germ cell, Sertoli cell, and Leydig cell, respectively. Immunohistochemical assay was conducted according to our previous published protocol [20].

Cell Tracking

Cell tracking of SVF was carried out by detecting CM-Dil-labeled cells according to our previous protocol [13]. In brief, testicular tissues retrieved at 3 and 7 days after T/D were froze, cut into 5- μ m sections, and mounted with 4'-6-diamidino-2-phenylindole. CM-Dil-labeled cells were detected under a fluorescence microscope.

Enzyme-Linked Immunosorbent Assay

Serum levels of malondialdehyde (MDA) and hormones, such as testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), at 7 days after T/D were analyzed by enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (USCN Life Science, Inc., Wuhan, Hubei,

People's Republic of China). The absorbance was tested at 450 nm on a microplate reader (Tecan, Männedorf, Switzerland). The concentrations of MDA and hormones were determined on the basis of standard curve.

Western Blot Analysis

Local expression of growth factors in the testis at 7 days after T/D was determined by Western blot analysis according to our previously described protocol [13]. Total protein was extracted from retrieved testis specimens with RIPA lysis buffer that contained a cocktail of protease inhibitor (Roche). Primary antibodies against basic fibroblast growth factor (bFGF, Bioss Inc., Woburn, MA, USA) and stem cell factor (SCF, Bioss Inc.) were used in this study. Anti-glyceraldehyde-3-phosphate dehydrogenase antibody was used as loading control. The density of immunoreactive proteins was assessed by using the Gel-Pro32 software (Media Cybernetics).

Statistical Analysis

All data were expressed as means \pm SD. Statistical analyses for multiple comparisons among all groups were conducted by one-way analysis of variance and the post hoc Tukey test. $p < .05$ was considered statistically significant.

RESULTS

Characterization of SVF

Flow cytometric analysis showed that uncultured SVF cells were a heterogeneous population that expressed hematopoietic (CD11b/c [37.44% \pm 4.56%], CD34 [15.42% \pm 3.51%], CD45 [63.2% \pm 7.96%], and CD133 [18.04% \pm 2.94%]), mesenchymal (CD90 [34.38% \pm 6.60%] and CD106 [21.62% \pm 7.24%]), and endothelial (CD31 [21.08% \pm 2.62%], CD34 [15.42% \pm 3.51%], and VEGFR-2 [21.22% \pm 6.64%]) markers (Fig. 1).

Histopathological Findings

T/D-induced testis at both 3 and 7 days after surgery showed severe injury changes, compared to the testis in sham group, including irregular and atrophic seminiferous tubules morphology, as well as germinal cell sloughing. Progressive injury was observed over time. At 28 days, spermatogonia were the only germ cells that were present in most tubular section. SVF-treated rats exhibited reduced atrophic and degenerative changes of the tubular epithelium. In the T/D + PBS group, Johnsen's score was significantly lower than that in the sham group ($p < .05$). Administration of SVF contributed to an elevated score, which was still lower than that of sham group ($p < .05$). Testicular tissue in the T/D + SVF group at 7 days showed an improved histological appearance in parallel with elevated score compared to that at 3 days. However, more severe injury and reduced score were found in testicular tissue of the T/D + SVF group at 28 days compared to that at 7 days (Fig. 2).

Immunohistochemical Findings

Immunohistochemical findings showed that there was no statistical difference on the number of SOX9-positive cells among the three groups. T/D led to a significant decrease in the number of both VASA and P450-positive cells in the testis ($p < .01$ for both VASA and P450), whereas SVF injection contributed to a significant increase in the number of both cells ($p < .01$ for both VASA and P450), however, which was still less than that in the sham group ($p < .01$ for both VASA and P450; Fig. 3).

Cell Locating in the Testis

Cell locating of SVF was determined by the detection of CM-Dil staining in the testis after T/D (Fig. 4). At 3 days after SVF therapy, red labeling of CM-Dil was mostly located in testicular interstitial region. At 7 days after SVF transplantation, CM-Dil-positive SVF integrated into both interstitial region and seminiferous tubules. Interestingly, the fluorescence of CM-Dil could also be detected in vascular wall of the testis.

Expression of MDA and Hormones

Serum level of MDA in the T/D + PBS group was significantly higher than in the sham group, while SVF therapy significantly decreased the level of MDA in testicular T/D rats ($p < .05$). Serum level of testosterone in the T/D + PBS group was lower than in the sham group, whereas SVF injection contributed to an elevation of serum testosterone level in testicular T/D rats ($p < .05$). The level of FSH and LH significantly increased in the T/D + PBS group compared to the sham group, whereas SVF treatment significantly decreased the level of FSH and LH in rats that underwent testicular T/D ($p < .05$; Fig. 5).

Expression of Growth Factors in the Testis

Both SCF and bFGF were detectable in the testis of sham group. However, the amount of SCF and bFGF significantly decreased in T/D-induced testis ($p < .01$ for both SCF and bFGF), whereas SVF injection could significantly improve the expression of both factors ($p < .01$ for both SCF and bFGF), which were still lower than that in sham group ($p < .01$ for both SCF and bFGF; Fig. 6).

DISCUSSION

This study first demonstrated that local injection of uncultured adipose-derived SVF could prevent testicular tissue injury induced by T/D in rats. After injection, autologous SVF cells could integrate into interstitial region and seminiferous tubules, as well as vascular wall of the testis, secrete various growth factors (e.g., bFGF and SCF), decrease the oxidative stress, regulate the hormone levels, and then promote the regeneration of Leydig cell and germ cell, thus contributing to the improved histological appearance and Johnsen's score in the testis. As the safety and effects of SVF injection for functional recovery of diseased organs have been confirmed in various clinical practice [12, 21–23], our data provide significant clinical implications for the prevention of infertility induced by testicular T/D.

Testicular T/D is considered as a typical I/R injury for the testis, associating with the overgeneration of reactive oxygen species (ROS), which has destructive effects on a series of cellular functions [24]. MDA, a stable end product of lipid peroxidation that is generated by ROS, is widely used as an indirect indicator of ROS, which has high reactivity and short half-life. Study has reported that AdMSCs could reduce the MDA level elevated by testicular T/D [8]. Our study showed that administration of uncultured SVF, which is a rich source of AdMSCs, was also able to eliminate the serum level of MDA elevated by testicular T/D, indicating the potential effect of SVF on neutralizing ROS and ameliorating oxidative stress. However, as the expression level of MDA in tissue section is also an important indicator of oxidative stress, it is necessary to detect the expression level of MDA in testicular tissue in the future.

It is shown that T/D definitely caused testicular damage that led to the unbalance of sexual hormones homeostasis [25], which is essential for spermatogenesis. In the presence of LH, Leydig cells produce testosterone that is required for adult fertility [26]. By acting with FSH, testosterone can stimulate Sertoli cell activity and spermatogenesis [26]. In this study, Leydig cells were damaged and reduced after T/D, resulting in the decreased testosterone level, after which the LH and FSH levels were increased on basis of the positive feedback of the "hypothalamus-pituitarium-testis" axis. Such results were similar to previous study [8]. Administration of SVF contributed to the increased P450-positive Leydig cells and testosterone level in T/D rats, as well as the decreased LH and FSH level. In addition, SVF injection also resulted in increased VASA-positive sperm cells in T/D rats, suggesting the promoting effect of SVF on spermatogenesis. Cell tracking showed that injected SVF cells were mainly integrated into interstitial region and seminiferous tubules, which indicated that SVF cells might play a major role on the repair and regeneration of Leydig cells and germ cells. However, it needs further investigation whether SVF cells could differentiate into the above two kinds of cells, in spite that AdMSCs were reported to be capable of differentiating into sperm cells [9, 10].

SCF is known as steel factor or kit ligand that binds to the c-Kit receptor located on the membrane of germ cells and Leydig cells [27, 28]. The SCF/c-Kit signaling pathway plays a vital role in proliferation, differentiation, and survival of germ cells [29]. As a member of FGFs, bFGF, also named FGF2, is demonstrated to be a survival factor for Sertoli cells as well as a mitogenic factor for gonocytes [30]. The production of bFGF

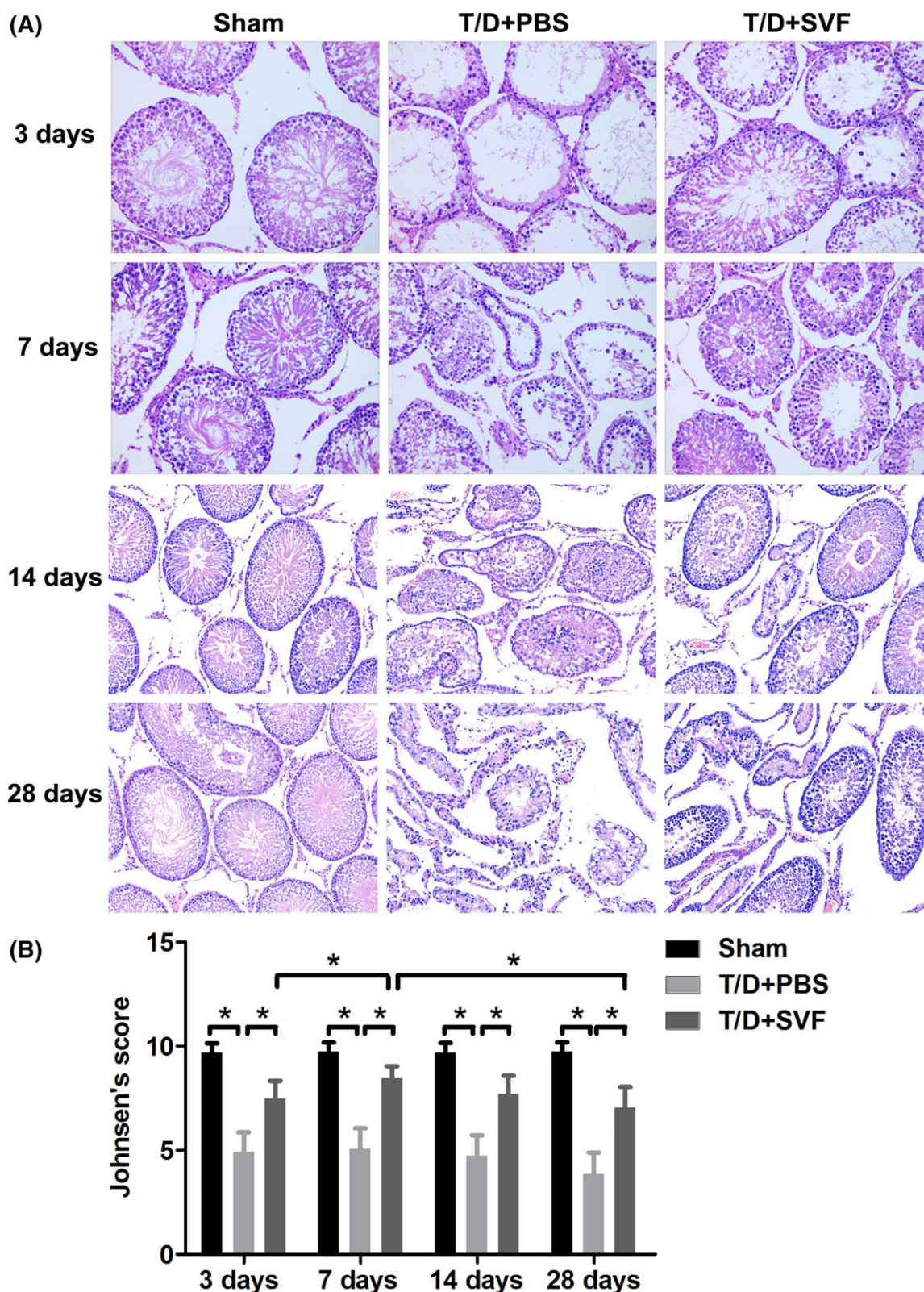


Figure 2. Representative microscopic findings of rat testicular tissue in sham, T/D + PBS, and T/D + SVF groups at 3, 7, 14, and 28 days after surgery. Tissue sections were stained with H&E, reduced from $\times 200$. Compared to the sham group, the testis in T/D + PBS group showed severe injury changes, including irregular and atrophic seminiferous tubules morphology, as well as germinal cell sloughing, whereas the testis in T/D + SVF group showed reduced atrophic and degenerative changes of the tubular epithelium, at each time period after surgery. Progressive injury was observed over time. At 28 days, spermatogonia were the only germ cells that were present in most tubular section (A). Johnsen's score was determined in each group at each time period after surgery (B). *, $p < .05$. Abbreviations: PBS, phosphate-buffered saline; SVF, stromal vascular fraction; T/D, torsion-detorsion.

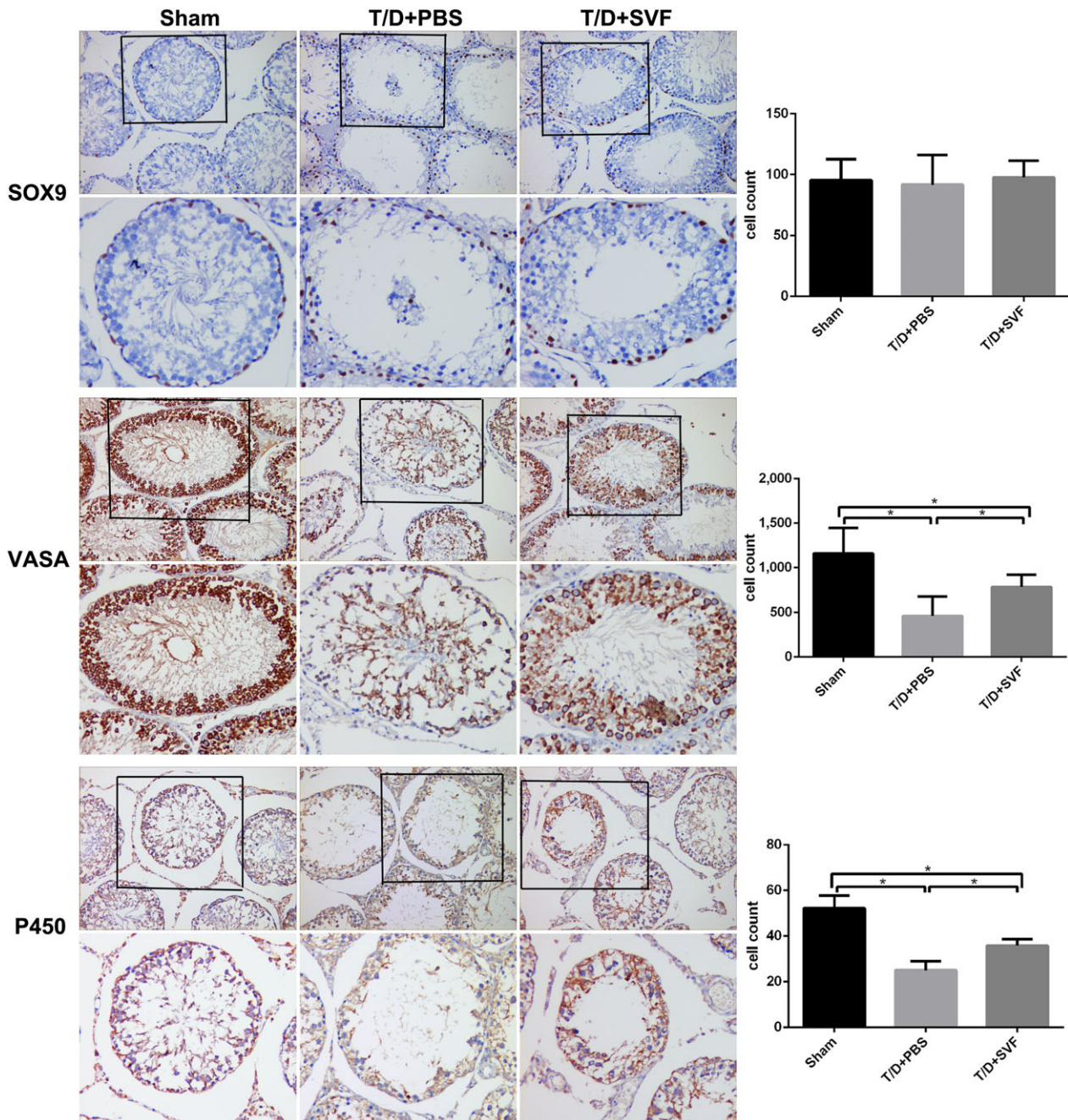


Figure 3. Representative photomicrographs of rat testicular immunostaining for SOX9, VASA, and P450 in sham, T/D + PBS, and T/D + SVF groups at 7 days after surgery, reduced from $\times 200$. Sertoli cells were positive for SOX9 in the three groups. Germ cells were positive for VASA. Both Leydig cells and spermatocytes were positive for P450. P450-positive Leydig cells could be found in interstitial region. Semi-quantitative analysis of the cell count of germ cells, Sertoli cells, and Leydig cells was performed in each group on the basis of immunohistochemical staining against VASA, SOX9, and P450, respectively. *, $p < .05$. Abbreviations: PBS, phosphate-buffered saline; SVF, stromal vascular fraction; T/D, torsion-detorsion.

by germ cells is responsible for the regulation of spermatogenesis [31]. Research has demonstrated that SVF cells could secrete a list of growth factors including bFGF [32]. Furthermore, stem cells cultured from adipose-derived SVF are also reported to be able to produce SCF [8]. In our present study, the expression of both bFGF and SCF was enhanced in SVF-treated T/D testis, suggesting that transplanted SVF might attenuate testicular injury and promote spermatogenesis

through paracrine of these two kinds of growth factors. Although there was no statistical difference on the quantity of Sertoli cells in T/D testis with or without SVF transplantation, the secreted bFGF might have an important role in regulating the function of Sertoli cells [33].

Generally, stem cells can be transplanted through local injection or systematical administration for the repair of injured tissue. However, because of the physiological blood-

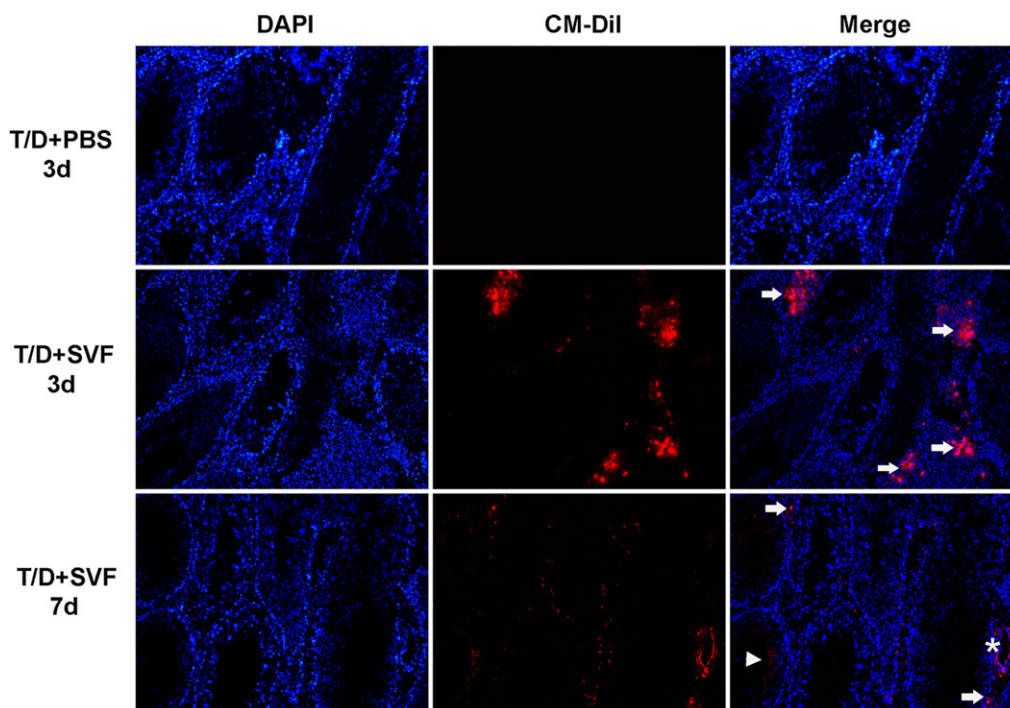


Figure 4. Cell tracking for CM-Dil-labeled SVF in testicular tissue of T/D + PBS and T/D + SVF group at 3 and 7 days after T/D, reduced from $\times 200$. Arrow implies that SVF cells located in interstitial region. Triangle means that SVF cells migrated into seminiferous tubules. Asterisk suggests that SVF cells integrated into vascular wall of the testis. Abbreviations: CM-Dil, chloromethylbenzamido dialkylcarbocyanine; DAPI, 4',6-diamidino-2-phenylindole; PBS, phosphate-buffered saline; SVF, stromal vascular fraction; T/D, torsion-detorsion.

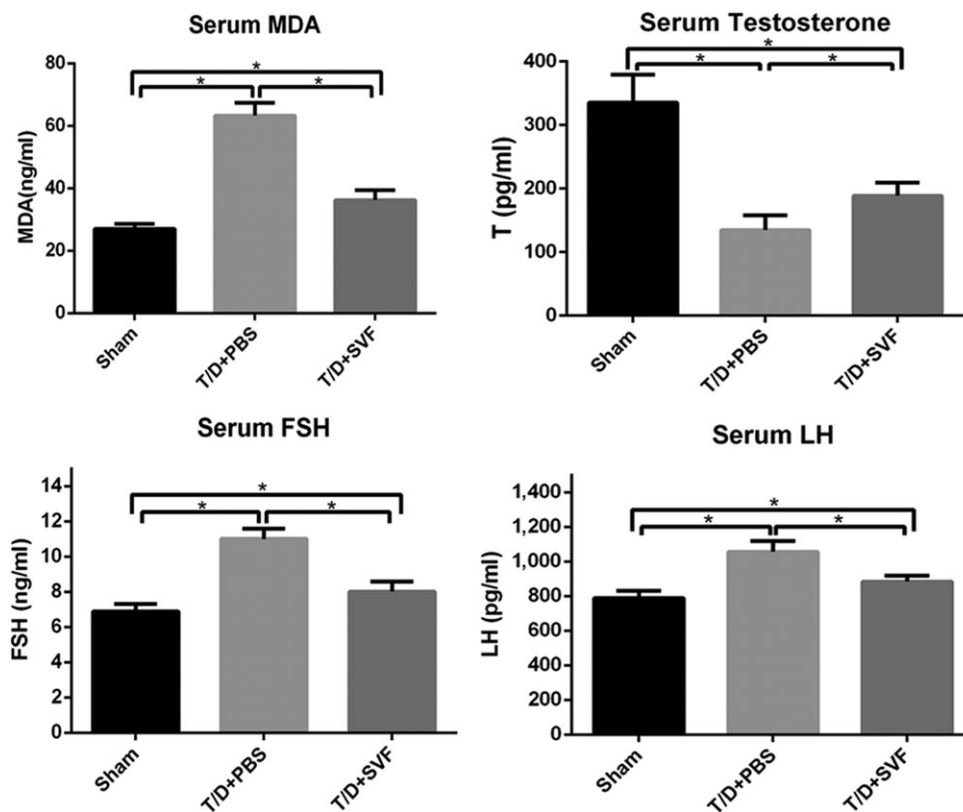


Figure 5. Serum MDA, T, FSH, and LH was evaluated by enzyme-linked immunosorbent assay in sham, T/D + PBS, and T/D + SVF groups at 7 days after surgery. *, $p < .05$. Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone; MDA, malondialdehyde; PBS, phosphate-buffered saline; SVF, stromal vascular fraction; T, testosterone; T/D, torsion-detorsion.

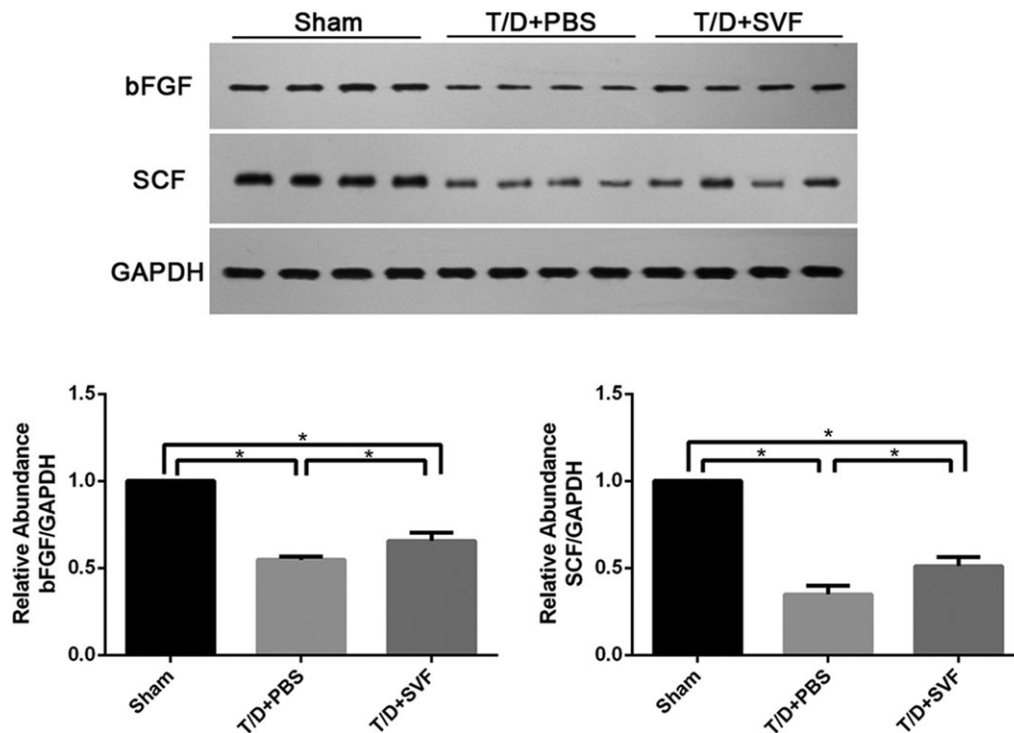


Figure 6. Expression of bFGF and SCF in testicular tissues was measured by Western blot analysis in sham, T/D + PBS, and T/D + SVF groups at 7 days after surgery. Western blots were quantified with data expressed as relative abundance of bFGF/GAPDH and SCF/GAPDH. *, $p < .05$. Abbreviations: bFGF, basic fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; SCF, stem cell factor; SVF, stromal vascular fraction; T/D, torsion-detorsion.

testis barrier, transplanted stem cells by intravenous injection may not be able to enter the testis via the circulation. Therefore, local administration of stem cells through intratesticular injection has emerged as a preferred method for the repair of testicular injury. Previous studies have demonstrated that local injection of stem cells provides a useful therapeutic strategy to rescue infertility [8–10]. Local injection of stem cells contributed to the restoration of male fertility and birth of offspring in the rats with busulfan-treated testes [10]. In this study, intratesticular administration of SVF significantly protected the testis from testicular injury and promoted spermatogenesis after testicular injury. However, intratesticular injection procedure might result in the production of anti-sperm antibody, which may lead to infertility. Long-term follow-up study is needed to evaluate antisperm antibody-induced secondary infertility in the future.

Even though uncultured SVF-treated testicular tissue showed more severe injury and reduced Johnsen's score at 28 days compared to that at 7 days, SVF administration still exhibit protective effect on injured testis and promoting effect on spermatogenesis after T/D. However, long-term effects of SVF on protecting the testis against T/D-induced testicular injury remain to be explored. In addition, long-term fate of SVF is also needed to be elucidated by cell tracking technology to observe whether SVF cells differentiate into germ cells or other cells in the testis. As CM-Dil may only be useful for short-term tracking of cells in vivo because of the transient labeling, future studies may consider long-acting labeling technique to track SVF cells and assess their fate, localization, and behavior in vivo. Finally, we have found that SVF could also integrate into vascular wall of the testis after injected and

produce bFGF, which was also an angiogenic factor. Further study is needed to explore whether SVF cells promote angiogenesis in the damaged testis induced by T/D.

CONCLUSION

This study demonstrated that autologous uncultured SVF was capable of protecting the testis from testicular injury and promoting spermatogenesis in testicular T/D rats, through reducing oxidative stress, restoring sexual hormone homeostasis, and producing growth factors. The present study raises the feasibility of uncultured SVF therapy for rescuing testicular injury induced by T/D, whereas further clinical studies are needed.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (81570613 and 81370853 to R.J.), Jiangsu Provincial Social Development Project (BE2017615 to R.J.), Jiangsu Provincial Medical Youth Talent (QNRC2016071 to L.Z.), and Nanjing Medical Science and Technology Development Foundation (ZDX16006 to R.J. and JQX17007 to L.Z.).

AUTHOR CONTRIBUTIONS

L.Z.: conception/design, data analysis, financial support, manuscript writing, final approval of manuscript; K.S.: conception/design, collection and/or assembly of data, data analysis,

manuscript writing, final approval of manuscript; L.X.: administrative support, manuscript writing, final approval of manuscript; F.Z.: collection and/or assembly of data, data analysis, final approval of manuscript; H.T.: collection and/or assembly of data, final approval of manuscript; C.Z.: data analysis, final approval of manuscript; Z.X. and Y.G.: provision of study material, final approval of manuscript; R.W.: data interpretation,

final approval of manuscript; R.J.: conception/design, financial support, administrative support, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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