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ORIGINAL ARTICLE

The impact of low-intensity extracorporeal shock waves on testicular spermatogenesis demonstrated in a rat model

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Abstract

Background: In rodent models, low-intensity extracorporeal shock wave therapy has been shown to negatively impact semen concentration after treatment on the penis, implying that the reproductive system in close proximity may be indirectly affected by this modality. We hypothesized that shock waves are detrimental to spermatogenesis, and the aim of this study was to evaluate the effect of shock waves on spermatogenesis after direct shockwave treatment on testes using different energy settings.

Methods: Twenty-five male Sprague Dawley rats, 8 weeks old, were divided into five groups, including one control group and four treatment groups each treated using shock waves of different intensities. All rats in the treatment groups received 2000 shocks on the left testis twice a week for 4 weeks, with shock wave intensity and frequency varied by treatment group: 0.1 mJ/mm^2 at 4 Hz for Group A, 0.15 mJ/mm^2 at 4 Hz for Group B, 0.35 mJ/mm^2 at 4 Hz for Group C, and 0.55mJ/mm^2 at 3 Hz for Group D. At the end of the experiment, sperm collected from the epididymis was evaluated for concentration and motility. Testicular spermatogenesis, the apoptotic index of germ cells, and the expression of a meiotic-specific gene were also analyzed.

Results: The treatment group receiving shock wave intensity at 0.55 mJ/mm^2 showed a significant decrease in sperm concentration, motility, and Johnsen score as compared to other groups. The apoptotic index of spermatogenic cells increased as the intensity of the shock wave treatment escalated, and reach a statistically significant difference at 4 weeks posttreatment. Treating testes with intensity levels of 0.55 mJ/mm^2 at 3 Hz interfere with the quality or quantity of spermatogenesis and also increases in spermatogenic cell apoptosis, whereas the expression of the SYCP3 gene significantly decreased after treatment with intensity levels of 0.10 mJ/mm^2, 0.15 mJ/mm^2, and 0.35 mJ/mm^2 at 4 Hz.

Conclusion: Treating testes with intensity levels of 0.55 mJ/mm^2 at 3 Hz interfere with the quality or quantity of spermatogenesis and also increases spermatogenic cell apoptosis, whereas the expression of the SYCP3 gene significantly decreased after treatment with intensity levels of 0.10 mJ/mm^2, 0.15 mJ/mm^2, and 0.35 mJ/mm^2 at 4 Hz.

Keywords: Apoptosis; Low-intensity extracorporeal shock waves; Spermatogenesis; SYCP3; Testis

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1. INTRODUCTION

Bulk semen parameters, such as sperm motility and concentration, have been proven to be affected by extracorporeal shock wave treatment used for management of urolithiasis.[1–3] In contrast to the high-intensity shock wave lithotripsy used in urolithiasis, low-intensity extracorporeal shock wave therapy (Li-ESWT) has recently been introduced as a novel modality to treat erectile dysfunction (ED).[4] It is postulated that the improvement of erectile function after Li-ESWT is related to the upregulation of vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase, and nerve nitric oxide synthase expression, along with restoration of endothelial and smooth muscle content.[5–8] Unlike the commonly used oral phosphodiesterase type 5 inhibitor offered as first-line ED therapy with a reported 6-36 hours effectiveness after dosing, Li-ESWT may exert positive clinical effects on erectile function for a period of up to 6 months, or as long as 24 months in some cases.[4,9] The persistent beneficial effects are possibly due to the amelioration

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of impaired penile hemodynamics, which is considered a pathophysiological mechanism underlying vasculogenic ED.

While ED becomes increasingly prevalent with age (the majority of men treated for ED are over the age of 40), it is not impossible for young men to experience ED during sexual intercourse. It has been reported that 11% of men in their 30s and 8% of men in their 20s encounter varying degrees of ED.[10] Considering that these men may select Li-ESWT as their first-line treatment option as recommended by the European Association of Urology for their reproductive age,[11] the potential impact of this treatment on fertility is a major concern, and, accordingly, should be addressed before Li-ESWT is commenced. In rodent models, Li-ESWT has been shown to negatively impact semen concentration after 9 sessions of treatment on the penis, implying that the reproductive system in close proximity may be indirectly affected by this modality.[12] We hypothesized that shock waves are detrimental to spermatogenesis, and the aim of this study was to evaluate the effect of shock waves on spermatogenesis after direct shockwave treatment on testes using different energy settings.

2. METHODS

2.1. Animals

Male Sprague Dawley (SD) rats (8 weeks old) weighing 250 to 300 g were purchased from the Animal Center and housed at 22 ± 1°C, with a light cycle between 08:00h and 20:00h, and fed with standard pellets and water ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committee of our institution (Approval number IACUC 2016-134).

2.2. Experimental design and treatment

A total of 25 healthy adult male SD rats, 8 weeks old, were divided into five groups comprising a control group (of n = 5 rats) and four treatment groups. While put under general anesthesia using a mixture of Zoletil (20 mg/kg) and xylazine (10 mg/kg), the four treatment groups (denoted A, B, C, and D) received different intensities of shock waves as generated by Duolith SD1 ultra (Storz Medical AG, Tägerwilen, Switzerland). The shock wave probe provides a tissue penetration depth of 0 to 30 mm. Each rat in each treatment group received 2000 shocks on the left testis twice a week for 4 weeks, with a fixed intensity and frequency based on the treatment group (Fig. 1): 0.1 mJ/mm^2 (n = 5) at 4 Hz, 0.15 mJ/mm^2 (n = 5) at 4 Hz, 0.35 mJ/mm^2 (n = 5) at 4 Hz, 0.55mJ/mm^2 (n = 5) at 3 Hz for groups A, B, C, and D, respectively. Rats in the control group received general anesthesia for an equivalent amount of time twice a week for 4 weeks during the experimental period but without shock wave treatment.

2.3. Ultrasonographic follow-up

Conventional grey-scale and color Doppler ultrasonography examinations of the testes were performed after the last course of LI-ESWT with a LOGIQ E9 ultrasound scanner equipped with an 11 to 15 Hz linear transducer (GE Healthcare, Milwaukee, WI, USA). Both testicles were examined in orthogonal transverse and longitudinal planes for detection of diffuse heterogeneity or hypoechoic lesions in the testicular parenchyma and the presence of arterial flow.

2.4. Hormone evaluation

Blood samplings were performed between 8 and 11 AM. After collecting the blood via cardiac puncture and held at room temperature for 30 minutes, the serum was obtained following centrifugation at 3000 rpm at 4°C for 15 minutes and stored at −20°C until analysis of testosterone using routine assay (electrochemiluminescence immunoassay).

2.5. Testis preparation

At the end of the experiment, all animals were sacrificed and bilateral testes were removed following termination of shock wave treatment, and their weights were measured. The testes were fixed with 4% paraformaldehyde in phosphate buffer saline (PBS) (pH 7.4) for 24 hours. After PBS washing, tissues were dehydrated and embedded in paraffin at 60°C. Transverse tissue sections at 4-μm thickness were obtained.

2.6. Sperm characteristics

Bilateral epididymides were dissected using a razor blade and separated, followed by mincing into small fragments with scissors in a Petri dish filled with 10 mL Dulbecco Modified Eagle Medium (DMEM) in order the separate the sperm from the epididymis. Sperm concentration was evaluated after diluting samples with DMEM (1/10) using a Neubauer chamber (Paul Marienfeld GmbH & Co. KG, Germany). Sperm motility indicated the percentage of sperm that exhibited forward and progressive motility.

2.7. Johnsen score

The testicular sections were deparaffinized, gradually hydrated, and examined after hematoxylin and eosin (HE) staining were performed. Each section was viewed systematically under light microscopy at ×400 magnification. The Johnsen score was used to evaluate spermatogenesis in the seminiferous tubules. To minimize potential observer biases, the Johnsen score is reviewed by two blinded investigators (W.J.C. and Y.K.C) A total of 20 seminiferous tubules were examined in each cross-section, and a score of 1 to 10 was assigned to each cross-section as follows[13]:

10: Complete spermatogenesis and perfect tubules
9: Many spermatozoa present but irregular germinal epithelium
8: Only a few spermatozoa present
7: No spermatozoa but many spermatids present
6: No spermatozoa, only a few spermatids present
5: No spermatozoa or spermatids present but many spermatocytes present
4: Only a few spermatocytes (<5) and no spermatids or spermatozoa present
3: Only spermatogonia present
2: No germ cells but Sertoli cells are present
1: Neither germ cells nor Sertoli cells present

2.8. TUNEL assay

We used the DeadEnd colorimetric TUNEL System (Promega G7360; Madison, WI, USA) to determine the proportion of apoptosis in spermatogenic cells according to the manufacturer's instructions. As per protocol, the 4% paraformaldehyde-fixed testis sections from each group were dewaxed, rehydrated, and kept in PBS. Proteinase K solution was then added to permeabilize tissue sections, which were then washed with PBS, and added to an incubation buffer containing equilibration buffer, biotinylated nucleotide mix, and rTdT (recombinant terminal deoxynucleotidyl transferase). The mixture was then incubated for 60 minutes at 37°C in a humidity chamber. The sections were immersed in 2X saline-sodium citrate buffer, washed in PBS, then incubated with horseradish peroxidase-labeled streptavidin (HRP-Streptavidin) at room temperature for 30 minutes. The biotin-tagged nucleotides that are bound by HRP-Streptavidin are further detected using the stable chromogen,

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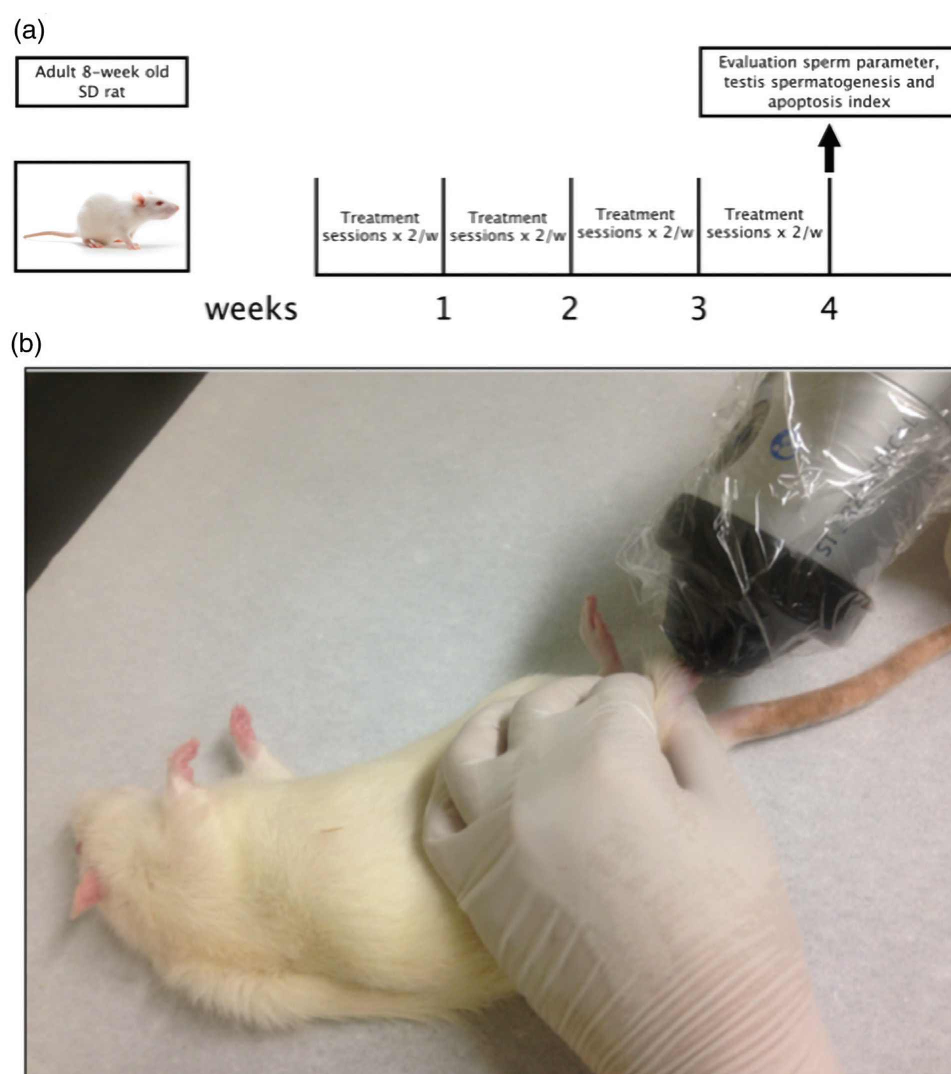

Fig. 1 Experimental protocol and shock wave application. A, SD rats were treated with various energy setting of Li-ESWT twice a week for 4 weeks. Sperm parameter, testis spermatogenesis evaluation, and apoptosis index were carried out on the day following the last Li-ESWT session. B, Photograph of shock wave application on the left testis of SD rats using Duolith SD1 ultra shock wave machine (Storz Medical AG). SD = Sprague Dawley.

diaminobenzidine (DAB). After washing in deionized water and draining off excess water from the slide, the section containing the treated specimen was mounted by glass coverslips and the edges were sealed with clear nail polish. For positive control, slides were treated with DNase I (10 U/L) before TUNEL staining. Detection of fragmented DNA was then observed with a Leica DM2500 upright light microscope (Wetzlar, Germany). Apoptotic index (AI) is defined as a percentage of the TUNEL-positive germ cells divided by the total number of germ cells in 50 randomly selected focused seminiferous tubules. The numbers of spermatogenic cells per seminiferous tubule were counted using ImageJ software (NIH; Bethesda, MD; http://imagej.nih.gov/ij/).

2.9. Quantitative polymerase chain reaction

Quantitative real-time PCR was performed to determine the mRNA expression levels of synaptonemal complex protein 3 (SYCP3) in the testes. Total cellular RNAs were extracted from the harvested tissues and treated cells using Tri-reagent according to the manufacturer's instructions (Sigma, St. Louis, MO, USA). The purified RNA samples were dissolved in RNase-free water and each sample underwent quantitative RT-PCR to measure the levels of mRNAs of various genes. The real-time PCR System (ABI StepOne Plus, ABI QuanStudio 3) was used for the PCR reaction. The temperature was set at 95°C for 2 minutes for denaturation, followed by the PCR cycle: 2 minutes at 95°C for denaturing, 10 seconds at 60°C for annealing, and 20 seconds at 72°C for elongating. The PCR cycle would repeat 40 times to monitor the fluorescence signal of SYBR Green. The threshold cycle (Ct) values for the target genes were normalized with the Ct value of the housekeeping gene GAPDH. Normalization was performed based on the following formula: relative mRNA expression = $2^{-\Delta Ct}$ ($\Delta Ct = Ct^{target\ gene} - Ct^{GAPDH}$).

2.10. Statistical analysis

Analysis was performed by using SAS 9.2 statistics software (SAS Institute Inc., Cary, NC, USA). Continuous variables were expressed as mean ± SD. All data were analyzed with the Kruskal-Wallis test to check for significant differences among the groups and Mann-Whitney test to evaluate continuous variables between two means. Statistical significance was deemed to be determined if the conventional *p*-value <0.05.

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3. RESULTS

3.1. Ultrasonographic evaluation of the testis

Bilateral testis condition was assessed by sonography for each rat after the last course of LI-ESWT. While nearly all ultrasonographic examinations showed no testicular abnormalities, a heterogeneous echo pattern area with a well-defined border but without inner vascularity was depicted in a rat's left (treated) testicle after eight courses of LI-ESWT at the energy 0.55 mJ/mm^2 (Fig. 2).

3.2. Changes of sperm concentration and motility

The left epididymal sperm concentration was found to be 160.4 ± 42.4 million/mL in the control group; and 156.2 ± 61.2, 236.4 ± 48.7, 211.4 ± 29.8, and 28.8 ± 12.9 million/ml in groups A, B, C, and D, respectively (Table 1, Fig. 3A). The left epididymal sperm motility was 70.6 ± 8.6% in the control; and 70.4 ± 12.5%, 72.6 ± 5.5%, 67.4 ± 9.3%, and 16.2 ± 7.8% in groups A, B, C, and D, respectively (Table 1, Fig. 3B). Sperm analysis of the left epididymal sample revealed that sperm concentration and motility in group D were significantly decreased (Fig. 3), whereas there was no significant statistical difference in right epididymal sperm concentration or motility found amongst the groups. In addition, sperm concentration and motility did not differ between the left and right sides of epididymis in control group, groups A, B, and C, but decreased significantly in group D at the left side in comparison to the right ($p < 0.05$).

3.3. Effect of shock waves on hormone levels

The serum testosterone levels were 4.3 ± 2.3 ng/mL in the control; and 4.8 ± 3.4 ng/mL, 3.9 ± 0.9 ng/mL, 3.3 ± 1.8 ng/mL, and 5.1 ± 3.1 ng/mL in groups A, B, C, and D, respectively The dispersion of the serum testosterone was found to be similar across the control and treatment groups ($p > 0.05$) (Table 1).

3.4. Histopathological effects and measurement of spermatogenesis with Johnsen score

HE stain of the left testicular tissue showed normal testicular architecture with an orderly arrangement of differentiating spermatogenic cells, Sertoli cells, and Leydig cells without histopathological lesions in groups A, B, and C. Whereas in group D, degeneration of Sertoli cells as well as disorganization of germ cell layers within affected seminiferous tubules was observed (Fig. 4). The Johnsen score of left testes was significantly lower in group D, compared with control and other treatment groups (p=0.011). In particular, scores in the control were 9.2 (right) and 9.1 (left), whereas scores were 9.1 (right) and 9.3 (left) in group A, 9.2 (right) and 9.2 (left) in group B, 9.2 (right) and 9.1 (left) in group C, and 9.2 (right) and 7.5 (left) in group D, respectively. It can be noted that the average scores were above nine for each group except the left side (treated side) in group D, and showed no difference between sides within the same group in control, groups A, B, and C, indicating the presence of spermatogenesis in average seminiferous tubules in groups receiving low-intensity shock wave treatment (Fig. 4).

3.5. Assessment of apoptosis

The spermatogenic cell apoptosis was assayed using the TUNEL method. As shown in Fig. 5A, TUNEL-positive cells were observed as dark brown spots. The AI of the left testis in the control group was 1.04 ± 0.18 % (left). There was a statistically significant increase in AI from 1.04 ± 0.25%, 1.86 ± 0.38%, 2.18 ± 0.22% to 9.38 ± 1.98% with increasing intensity levels across groups A, B, C, and D, respectively ($p < 0.001$) (Fig. 5B).

3.6. mRNA expression of the spermatogenesis marker

The mRNA expression of the spermatogenesis-related gene, SYCP3, required for homologous centromere pairing during meiosis, was selected to indicate spermatogenic activity.[12] Fig. 6 demonstrated that exposure to ESWT at an intensity of 0.1 mJ/mm^2, 0.15 mJ/mm^2, and 0.35 mJ/mm^2 to the left testis led to down-regulation of SYCP3 mRNA levels in the testicular tissue, whereas testicular tissue receiving energy intensity at 0.55 mJ/mm^2 retained its SYCP3 mRNA expression.

4. DISCUSSION

This pilot study was conducted to investigate the effects of low-intensity shock wave exposure on spermatogenic activity. Sperm density and sperm motility following exposure to shock wave treatment with different intensity levels were also evaluated and compared. In the present study, we found no difference in sperm count or motility and no alteration in testicular spermatogenesis activity across different energy settings of 0.1, 0.15, and 0.35 mJ/mm^2 after eight courses of Li-ESWT, but a notable deterioration in sperm parameters and spermatogenesis activity after treatment energy settings with 0.55 mJ/mm^2. On the other hand, the expression of early meiotic markers, SYCP3, was retained after treatment energy settings with 0.55 mJ/mm^2, but exceptionally suppressed across energy settings from 0.1, 0.15, to 0.35 mJ/

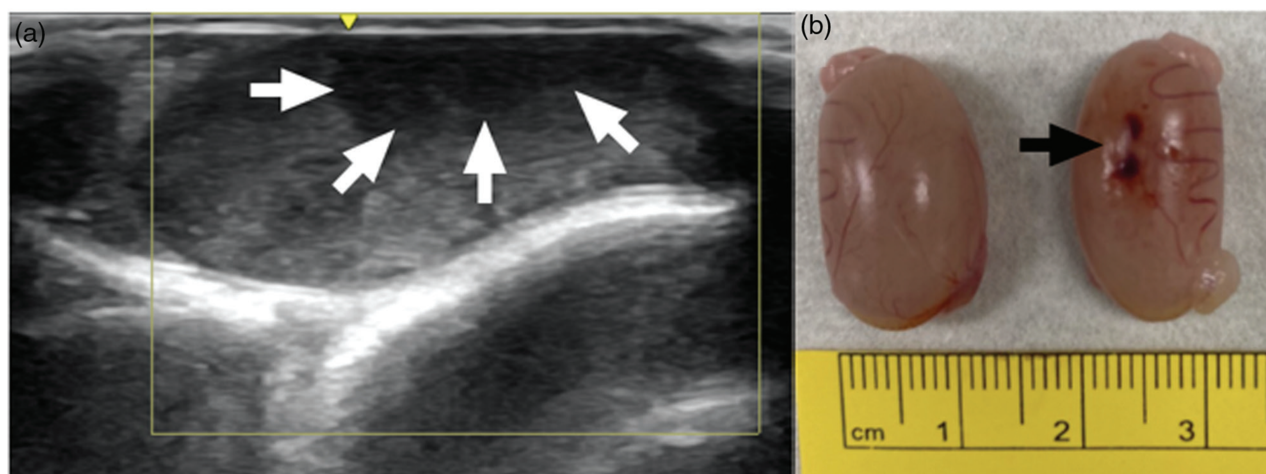

Fig. 2 Grey-scale ultrasound image in sagittal and testis specimen appearance. A, Ultrasound findings in a rat's left testicle showing heterogenous avascular lesion (white arrows). B, Photograph of the left testis specimen shows a small area of hemorrhage (black arrow).

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Table 1

Effects of shock waves on testicular weight, epididymal sperm count/motility, serum testosterone levels, Johnsen score of testicular histopathology, and apoptotic index of testicular germ cells

Parameters	Control		Group A (0.1 mJ/mm²)		Group B (0.15 mJ/mm²)		Group C (0.35 mJ/mm²)		Group D (0.55 mJ/mm²)	
	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left
Testis weight (g)	1.80 ± 0.06	1.83 ± 0.09	1.69 ± 0.18	1.70 ± 0.17	1.78 ± 0.14	1.77 ± 0.13	1.67 ± 0.05	1.69 ± 0.07	1.69 ± 0.08	1.68 ± 0.10
Sperm concentration (10^6/mL)	174.8 ± 44.5	160.4 ± 42.4	199.4 ± 58.1	156.2 ± 61.2	249.6 ± 103.1	236.4 ± 48.7	235.6 ± 57.5	211.4 ± 29.8	249.8 ± 70.3	28.8 ± 12.9*
Sperm motility (%)	67.6 ± 5.7	70.6 ± 8.6	68.2 ± 9.4	70.4 ± 12.5	65.0 ± 10.6	72.6 ± 5.5	65.6 ± 8.4	67.4 ± 9.3	75.0 ± 7.5	16.2 ± 7.8*
Testosterone (ng/mL)	4.3 ± 2.3		4.8 ± 3.4		3.9 ± 0.9		3.3 ± 1.8		5.1 ± 3.1	
Johnsen score	9.2 ± 0.2	9.1 ± 0.2	9.1 ± 0.1	9.2 ± 0.2	9.2 ± 0.2	9.2 ± 0.3	9.2 ± 0.3	9.1 ± 0.1	9.2 ± 0.2	7.5 ± 0.7*
Apoptotic index (%)	1.08 ± 0.15	1.04 ± 0.18	1.06 ± 0.32	1.04 ± 0.25	1.08 ± 0.24	1.86 ± 0.38	1.30 ± 0.12	2.18 ± 0.22	1.12 ± 0.13	9.38 ± 1.98*

*$p < 0·05$ vs contralateral side and corresponding control, groups A, B, and C.

mm². To our knowledge, this report is the first assessment of Li-ESWT impact on testicular spermatogenesis and epididymal sperm parameters as compared with high-intensity ESWT after direct testicular shock wave exposure.

ESWT was introduced in the field of urolithiasis, orthopedics, and cardiology decades ago. However, only recently has the application of Li-ESWT to treating vasculogenic disorders started to gain widespread attention.[14] An in vitro study wherein human umbilical vein endothelial cells were treated with various energy levels of shock waves from 0.02 to 0.35 mJ/mm² disclosed significantly enhanced expression of VEGF at a low-intensity setting of 0.09 mJ/mm².[15] The observed neoangiogenesis effects may further enable improvement in penile hemodynamics after certain courses of Li-ESWT.[4] Its therapeutic effect on the penis has been evaluated in several single-arm trials and proven to be a beneficial means for managing vasculogenic ED, yet data from randomized trials are conflicting.[16] While Li-ESWT is currently regarded as a new standard of care for men with vasculogenic ED, no study has ever evaluated the fertility outcome affected by direct low-intensity shock waves on testes. Two previous studies have investigated the effects of shock waves on Wistar rats' testes at energy settings of 19 kV at 1 Hz and 15 keV at 1 Hz generated by two different lithotripters (JDPN-IV and Direx Tripter X1).[17,18] Fertility potential, as evaluated by means of female rat pregnancy rates or fetal numbers, did not differ from a control group at 1, 3, and 6 weeks after 1000 applied shocks (19 kv, 1 Hz).[17] However, the complete absence of spermatogenesis occurring 5 weeks after 500- or 1000-shock treatments (15 keV, 1 Hz) has been reported, even when no depletion of Sertoli cells was observed.

As the reproductive tracts and organs lie in close anatomic proximity to the shock wave pathway during urolithiasis treatment, a number of studies have examined the impact of extracorporeal shock wave lithotripsy on semen parameters after management for lower ureteric urolithiasis, in an effort to evaluate possible gonadotoxic effects of the shock wave. Most studies confirm a transient deterioration in semen parameters in the 10 days following treatment and a return to baseline levels by 90 days, indicating that shock wave energy does not have a long-term detrimental effect on spermatogenesis and appears not to interfere with sperm maturation in testis.[19] Nonetheless, shock wave lithotripsy has been shown to cause short-term DNA damage and increase oxidative stress, both of which have been implicated as contributing factors to infertility.[20]

The testicular function of adult rats after penile low-intensity shock wave treatment has been evaluated by a recent study conducted by Zang et al.[12] Results have demonstrated that sperm concentration declined significantly after treatment at a shock wave pressure level of 3.2 BAR (equivalent to 0.18 mJ/mm²) applied three times a week for 3 consecutive weeks (300 shocks per session, 2 Hz), along with decreased expression of SYCP3, a component required for meiosis during spermatogenesis. Testosterone biosynthesis, on the other hand, was not influenced with respect to the serum testosterone concentration and the expression levels of steroidogenic acute regulatory protein and 3-beta-hydroxysteroid dehydrogenase enzyme. Therefore the authors concluded that impotent patients who desire fertility preservation should consider treatment modalities other than Li-ESWT, to circumvent ED.[12] Interestingly, the kinetic energy device employed in this animal research generated radial waves, whereas focused or linear pressure waves are generally adopted in the urological field for ED treatment at the present time.[16] Based on their propagation patterns, compressing waves can be divided into two categories: focused and unfocused. Radial or unfocused pressure waves differ from focused waves in that the wavelet propagates radially from the transmission point to distribute its less focused energy over a larger surface area, which is

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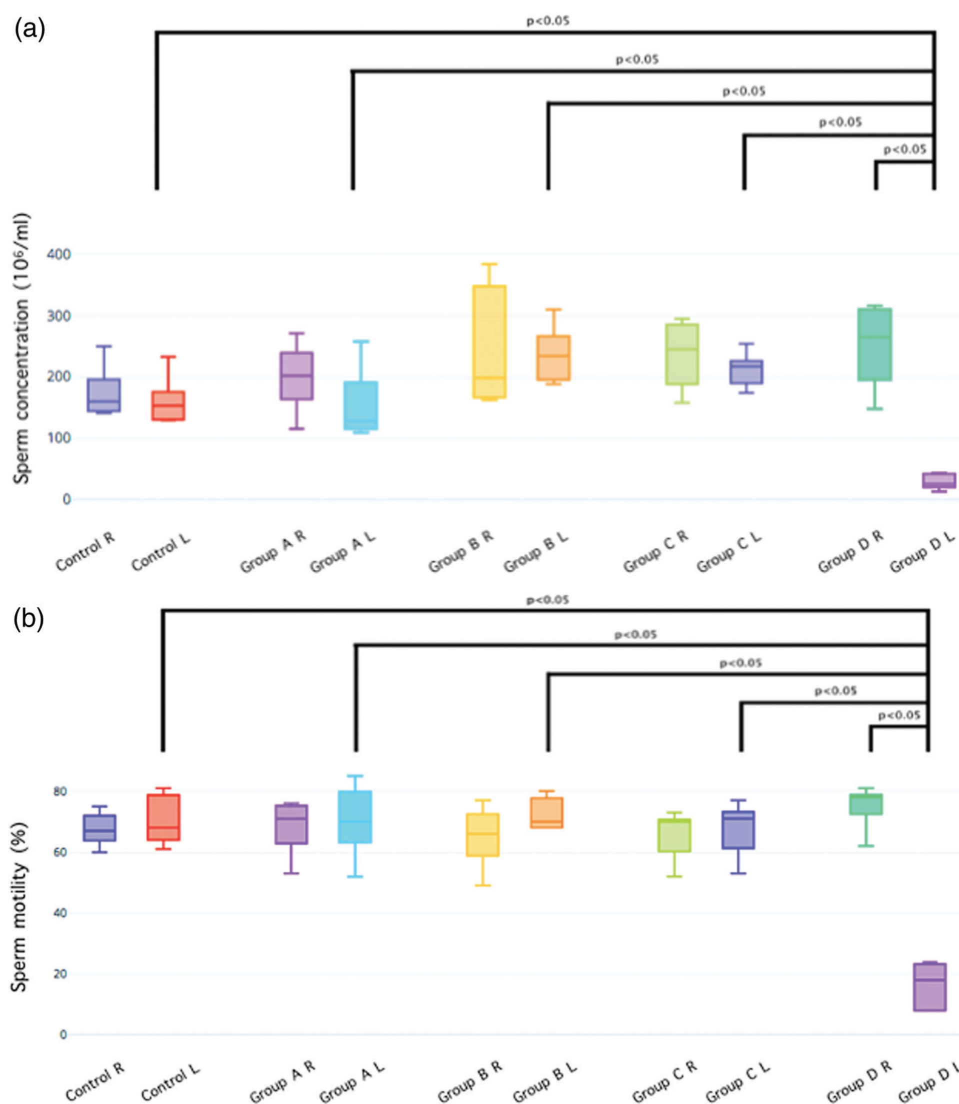

Fig. 3 Changes of epididymal sperm concentration and motility after shock wave treatment. A, Sperm concentration of Control, Group A, Group B, Group C, and Group D. B, Sperm motility of Control, Group A, Group B, Group C, and Group D. The line in the center of each box represent the median of the sample. Group A: 0.1 mJ/mm^2, Group B: 0.15 mJ/mm^2, Group C: 0.35 mJ/mm^2, and Group D: 0.55 mJ/mm^2.

in contrast to the focused shock wave that directs each shock to a defined point. After it disperses eccentrically from the applicator tip, the radial wave pressure rapidly attenuates upon entering the human body, making its therapeutic effectiveness relatively superficial, since the applied energy decreases with the square of the penetration depth.[21] The distinct physical characteristics of the radial wave may explain why spermatogenesis and sperm concentration are impacted, even though neither the testis nor reproductive tract is targeted during the whole treatment course.

One previous animal study has assessed sperm parameters and testicular histology alterations after exposure to Li-ESWT from 0.02 mJ/mm^2, 0.04 mJ/mm^2, to 0.06 mJ/mm^2, with a purpose to determine a relatively safe energy density.[22] After a total of 8 courses of shock wave treatment given once every 2 days, the Johnsen score significantly decreased in rats receiving energy flux density of 0.06 mJ/mm^2 at 2 weeks follow-up. This finding is in contrast to ours in that changes in sperm parameters and testicular histology were only noticed in rats treated with high-intensity shock waves (0.55 mJ/ mm^2) in lieu of in a low-intensity setting (0.1 mJ/ mm^2 or 0.15 mJ/ mm^2). The potential impact on serum testosterone levels was also studied. Notably, and similar to our study, shock waves did not suppress testosterone levels at short-term follow-up, however, in their animal study, the levels appear to be adversely affected in rats receiving repetitive low-energy ESWT with an energy flux density of 0.06 mJ/ mm^2 at 8 weeks follow-up.[22]

It was estimated that the duration of the cycle of the seminiferous epithelium is 12.9 days for SD rats, and their spermatogenesis cycle is about 51.6 days.[23] In our experiment, we tested sperm parameters, testis spermatogenesis, and apoptosis index 4 weeks after completion of Li-ESWT, which is approximately the duration of two cycles of the seminiferous epithelium. Accordingly, only short-term effect on semen parameters and spermatogenesis could be detected. Nevertheless, we did observe overt scrotal hematoma formation in one of our study rats, and also, noticed a dose-dependent AI change (increase in the proportion of apoptotic cells) in testicular samples after exposure to shock waves. Furthermore, the quantitative changes (decrease in sperm density and motility) or morphological responses (reflected by a lower Johnsen's score) were well demonstrated in epididymal sperm and testicular tissues following high-intensity ESWT (0.55 mJ/mm^2). Surprisingly, however, the mRNA expression of

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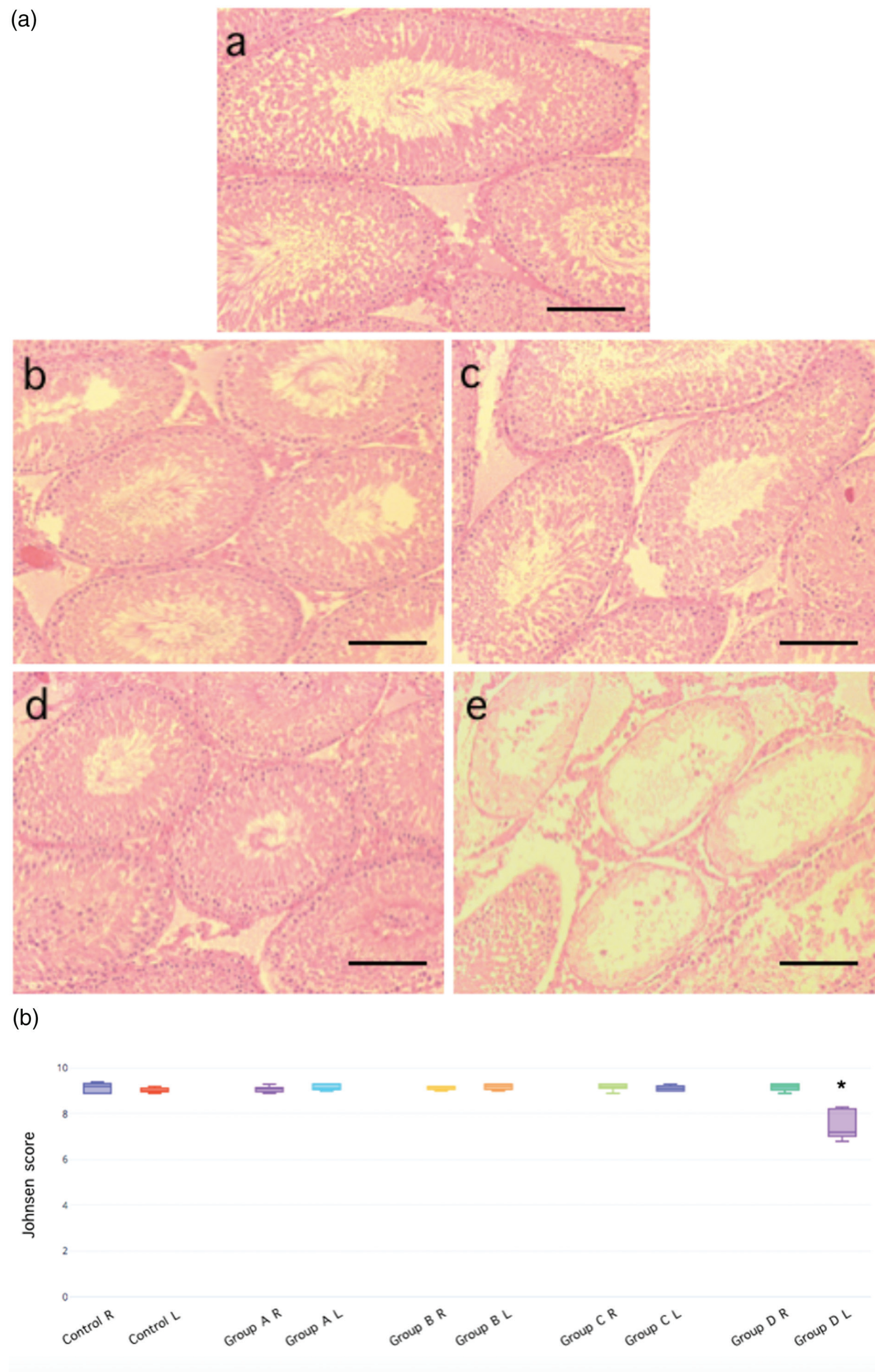

Fig. 4 Analysis of testis micrograph and Johnsen score in control group and treatment groups. A, Hematoxylin and eosin stain of left testis tissue in Control (a), Group A (b), Group B (c), Group C (d), and Group D (e). B, A comparison of the testicular Johnsen score between Control, Group A, Group B, Group C, and Group D. The line in the center of each box represent the median of the sample. Scale bar: 100 μm. Group A: 0.1 mJ/mm^2, Group B: 0.15 mJ/mm^2, Group C: 0.35 mJ/mm^2, and Group D: 0.55 mJ/mm^2. **p* < 0.05 vs contralateral side and corresponding control, group A, B, and C.

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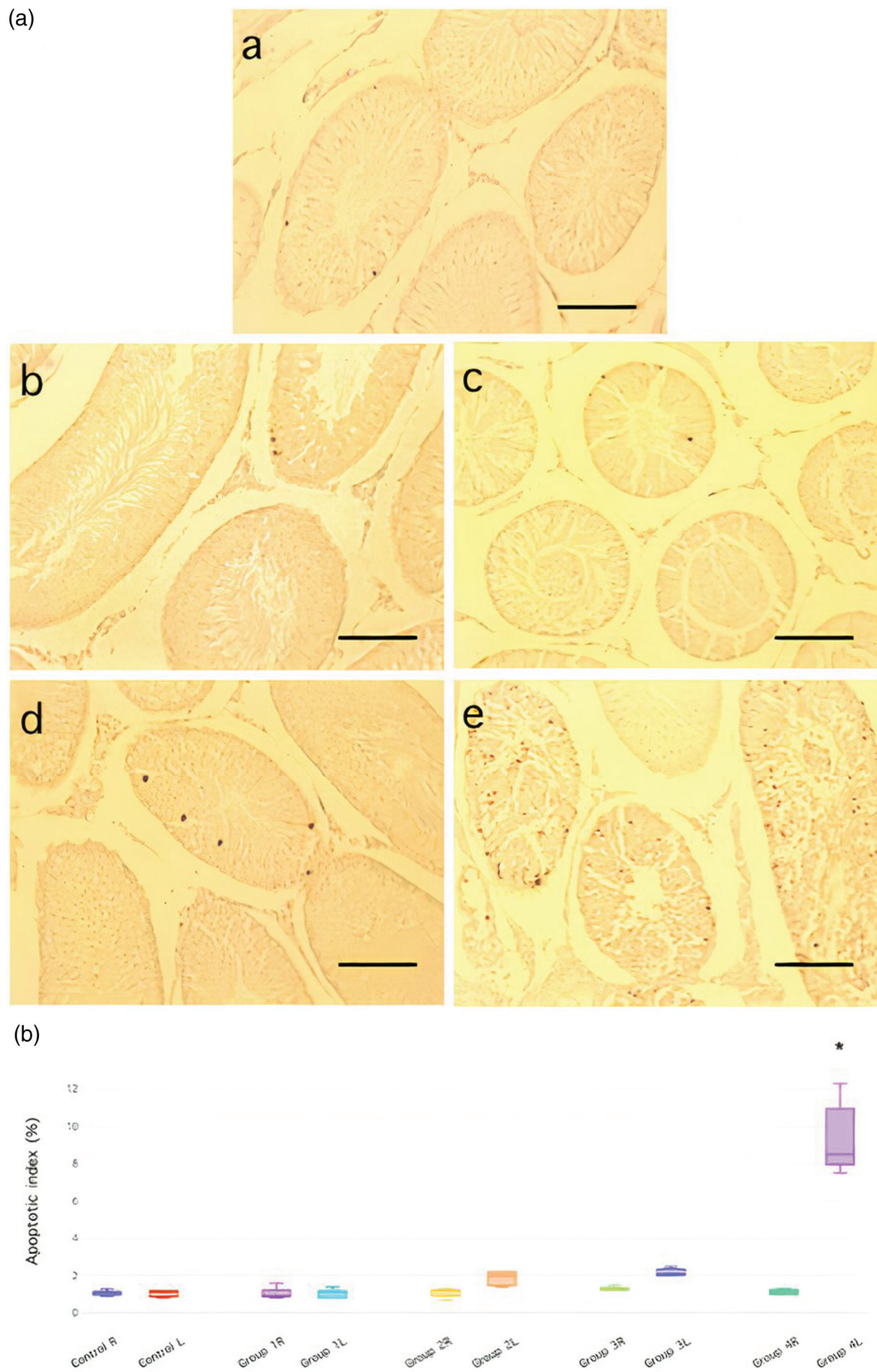

Fig. 5 Detection of apoptotic cells in TUNEL assay in controls and extracorporeal shock wave groups. A, Representative micrographs of cell apoptosis in left testis tissue detected by TUNEL (dark brown spots represents apoptotic cells) in Control (a), Group A (b), Group B (c), Group C (d), and Group D (e). B, Quantification of percentage of apoptotic cells. The line in the center of each box represent the median of the sample. Scale bar: 100 μm. Group A: 0.1 mJ/mm^2, Group B: 0.15 mJ/mm^2, Group C: 0.35 mJ/mm^2, and Group D: 0.55 mJ/mm^2.

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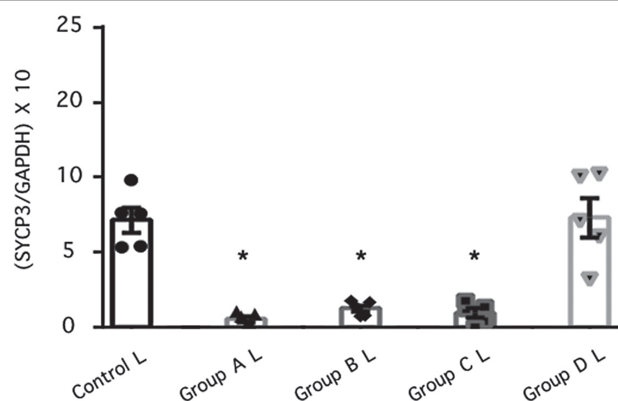

Fig. 6 The relative expression of SYCP3 mRNA of left testicular tissue in controls and extracorporeal shock wave groups. The relative expression levels were normalized to the expression amount of GAPDH. Data are expressed as mean ± standard error of the mean; *$p < 0.05$ vs control and group D. Group A: 0.1 mJ/mm^2, Group B: 0.15 mJ/mm^2, Group C: 0.35 mJ/mm^2, and Group D: 0.55 mJ/mm^2.

an early meiotic marker, SYCP3, was markedly reduced after treatment with low (0.10 mJ/mm^2, 0.15 mJ/mm^2) and medium-intensity (0.35 mJ/mm^2) extracorporeal shock waves instead of in high-energy settings, indicating that spermatogenic process is evidently affected though not detected in epididymal sperm parameters as well as by testicular histological analysis. Further research will be needed to investigate the impact of Li-ESWL on long-term spermatogenesis and fertility alterations in animal models. Alternatively, serum testosterone biosynthesis was not affected across different treatment groups, but other bioeffects, such as regulation of gonadotropin secretion and production of reactive oxygen species, were not evaluated in this current study, and will require further research to determine the impact of Li-SWT on hormonal milieu and oxidative stress.

Using the 4 Hz frequency and energy setting from 0.09 to 0.15 mJ/mm^2 have been adopted in previous clinical studies to improve erectile function in men with ED.[4,9,24] High-energy session using an energy level of 0.55 mJ/mm^2 is widely used in treating musculoskeletal disorder,[25] and the reason why we applied the energy setting of 0.55 mJ/mm^2 and “3 Hz” is because it is the maximal energy level of the shock wave machine (Duolith SD1 ultra) used in the present study.

The main limitations of the present study are listed as follows. First and foremost, outcomes were measured by investigating sperm parameters and testicular spermatogenesis but not fecundity (ie, fetal number), therefore we cannot conclude whether reproductive potential was negatively affected after Li-ESWT. Besides, the follow-up time was limited to 1 month, and therefore, this study cannot reflect the effect on long-term results and the recovery time of spermatogenesis impairment after Li-SWT. Furthermore, we did not measure the sperm nuclear DNA fragmentation after treatment, of which the degree to some extent reflect the integrity of the genetic material of the gamete. Last but not least, the expression of VEGF was not examined, as its level has been shown in the previous study to be negatively correlated with germ cell damage.[26] Regarding the effects of Li-ESWT upon individual cell groups in the testis, another experiment specifically designed for this purpose is ongoing.

While our data cannot be directly used to draw conclusions about potential harm to spermatogenesis or semen quality parameters for men treated for ED with Li-ESWT, we did establish that there is a statistically significant increase in germ cell apoptosis and deterioration of sperm density and sperm motility observed after applying focused shock waves on rat testis using high-intensity settings, whereas low to medium-intensity ESWT lead to downregulation of meiosis-specific gene. Based on our findings, it seems likely that shock waves of intensity ranging from 0.1 mJ/mm^2 to 0.55 mJ/mm^2 causes a various detrimental effect on spermatogenesis. Furthermore, further studies are warranted to evaluate the potential beneficial effects of Li-ESWT to testicular disorders at a feasible range of shock wave intensity.

In conclusion, treating testes with intensity levels of 0.55 mJ/mm^2 at 3 Hz interfere with the quality or quantity of spermatogenesis and also increases spermatogenic cell apoptosis, whereas the expression of the SYCP3 gene significantly decreased after treatment with intensity levels of 0.10 mJ/mm^2, 0.15 mJ/mm^2, and 0.35 mJ/mm^2 at 4 Hz.

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