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Restraint stress of male mice triggers apoptosis in spermatozoa and spermatogenic cells via activating the TNF-α system

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Research Article

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Summary

Studies have indicated that psychological stress impairs human fertility and that various stressors can induce apoptosis of testicular cells. However, the mechanisms by which psychological stress on males reduces semen quality and stressors induce apoptosis in testicular cells are largely unclear. Using a psychological (restraint) stress mouse model, we tested whether male psychological stress triggers apoptosis of spermatozoa and spermatogenic cells through activating tumour necrosis factor (TNF)-α signalling. Wild-type or TNF-$\alpha^{-/-}$ male mice were restrained for 48 h before examination for apoptosis and expression of TNF-α and TNF receptor 1 (TNFR1) in spermatozoa, epididymis, seminiferous tubules and spermatogenic cells. The results showed that male restraint significantly decreased fertilization rate and mitochondrial membrane potential, while increasing levels of malondialdehyde, active caspase-3, TNF-α and TNFR1 in spermatozoa. Male restraint also increased apoptosis and expression of TNF-α and TNFR1 in caudae epididymides, seminiferous tubules and spermatogenic cells. Sperm quality was also significantly impaired when spermatozoa were recovered 35 days after male restraint. The restraint-induced damage to spermatozoa, epididymis and seminiferous tubules was significantly ameliorated in TNF-$\alpha^{-/-}$ mice. Furthermore, incubation with soluble TNF-α significantly reduced sperm motility and fertilizing potential. Taken together, the results demonstrated that male psychological stress induces apoptosis in spermatozoa and spermatogenic cells through activating the TNF-α system and that the stress-induced apoptosis in spermatogenic cells can be translated into impaired quality in future spermatozoa.

Introduction

Although it has been reported that psychological stress in men affects fertility with impaired semen quality (Fenster *et al.*, 1997; Collodel *et al.*, 2008; Gollenberg *et al.*, 2010; Janevic *et al.*, 2014; Nargund, 2015), the mechanisms by which psychological stress impairs semen quality have yet to be explored. Researches using rodent models have suggested that stressors such as chronic immobilization (Yazawa *et al.*, 1999; Sasagawa *et al.*, 2001), heat stress (Miura *et al.*, 2002) or reperfusion of testes after ischaemia (Koji *et al.*, 2001) could cause testicular germ cell apoptosis. However, although Rahman *et al.* (2018) reported that spermatozoa at post-meiotic stages of development were more susceptible to stress than meiotic spermatogenic cells, reports on the direct effect of stress on spermatozoa maturing in epididymis and vas deferens are limited (Pérez-Crespo *et al.*, 2008). Furthermore, although previous studies have suggested that the pro-apoptotic effects of male stress on spermatogenic cells might be translated into impaired quality of the future spermatozoa, no experiment has been reported to verify this expectation.

Members in the tumour necrosis factor (TNF) receptor (TNFR) superfamily show strong ability to bind TNFs and induce cell apoptosis (Kavurma *et al.*, 2008). Studies have shown the presence of TNF-α in the semen of infertile men (Estrada *et al.*, 1997). Expression of TNF-α mRNA was detected in pachytene spermatocytes, round spermatids and interstitial macrophages of mouse testes (De *et al.*, 1993). In the dog, TNF immunolabeling was observed in seminiferous tubules, in mature spermatozoa during the epididymal transit and in ejaculated spermatozoa (Payan-Carreira *et al.*, 2012). TNF-α significantly reduced progressive motility of human spermatozoa *in vitro* at higher concentrations in a dose- and time-dependent manner (Lampiao and du Plessis, 2008). Furthermore, Xiao *et al.* (2019) observed that although the Fas/FasL system played an important role in psychological stress-induced apoptosis of spermatozoa and spermatogenic cells, it triggered sperm apoptosis dependently through promoting TNF-α and TNF-related apoptosis-inducing ligand (TRAIL) secretion. While the above data suggested that the TNF-α system might be active in mammalian spermatogenic cells and spermatozoa, its

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role in the stress-induced apoptosis of spermatogenic cells and spermatozoa remains to be verified by systematic studies.

In this study, we tested by using a novel mouse restraint system that can best mimic psychological stress whether psychological stress in male mice triggers apoptosis in spermatogenic cells and mature spermatozoa through activating the TNF-α system, and whether the pro-apoptotic effect of male stress on spermatogenic cells can be translated into impaired quality of the future spermatozoa, by using a novel mouse restraint system that can best mimic psychological stress (Paré and Glavin, 1986; Glavin *et al.*, 1994; Zhang *et al.*, 2011; Zhao *et al.*, 2013; Wu *et al.*, 2015).

Materials and methods

Ethics statement

Care and use of mice were conducted exactly in accordance with the guidelines and approved by the Animal Care and Use Committee of the Shandong Agricultural University, China (Permit number: SDAUA-2001-0510). According to the guidelines of the committee, the animal handling staff (including each post-doctoral, doctoral or masters student) must be trained before using animals. Mice must be housed in a temperature-controlled room with proper darkness–light cycles, fed with a regular diet, and maintained under the care of the Experimental Animal Center, Shandong Agricultural University College of Animal Science and Veterinary Medicine. In total, 456 mice were used in this study, including 348 males and 36 females of Kunming strain, 36 males of C57BL/6 J breed and 36 males of TNF-α knockout mice. The mice were sacrificed without pain by cervical dislocation. The only procedure performed on the dead animals was the collection of testes with epididymis and vasa deferentia or ovaries.

Unless otherwise specified, all chemicals and reagents used in this study were bought from the Sigma Chemical Co. (St. Louis, MO, USA).

Animals and their restraint treatment

Mice of Kunming breed, which were used for most experiments in this study, were bred in this laboratory. Wild-type C57BL/6 J and TNF-$\alpha^{-/-}$ mice with a C57BL/6 J genomic background were purchased from Shandong University Center for Laboratory Animals and Model Animal Research Center of Nanjing University, respectively. All mice were raised in a room under 14 h light:10 h dark cycles, the dark cycle starting at 20:00 h. Male mice were used at the age of 10–12 weeks. For restraint treatment, an individual mouse was placed in a microcage constructed by the authors (Zhang *et al.*, 2011) and restrained for 48 h. Within the microcage, while a mouse could move back and forth and take food and water freely, it could not turn around. The microcage was placed in an ordinary home cage, which was offered the same photoperiod and controlled temperature as the home cage containing control mice.

Measurement of malondialdehyde (MDA) level

Spermatozoa were collected from caudae epididymides and vasa deferentia in RIPA buffer (R0010, Solarbio, Beijing) supplemented with 10 µl phenylmethanesulfonyl fluoride (PMSF). Total protein concentration in spermatozoa samples was determined using a BCA Protein Assay Kit (P0012; Beyotime, China). A MDA Detection Kit purchased from Nanjing Jiancheng Bioengineering Institute was used to determine the MDA level in spermatozoa. Briefly, MDA were allowed to react with thiobarbituric acid (TBA) for 40 min at 95°C in acidic conditions. The MDA-TBA conjugate formed was then measured at 532 nm using a plate reader (Infinite 50, TECAN). All the data were normalized to nM/mg protein.

Western blot

We isolated seminiferous tubules and caudae epididymides using tweezers. The caudae epididymides were carefully cleaned off spermatozoa before further treatment. Spermatozoa were recovered from caudae epididymides and vas deferens and were washed by centrifugation (200 *g*). One sperm sample from one mouse or 0.1 g of seminiferous tubules or caudae epididymides were washed twice in cool PBS, placed in a homogenizer containing 1 ml RIPA buffer (R0010, Solarbio) and 10 µl PMSF, and homogenized on ice for 20 min. Then, we centrifuged the homogenates (14,000 *g*) at 4°C for 30 min and collected supernatant containing the cell lysate. We then determined the total protein concentration of the supernatant using a BCA Protein Assay Kit (P0012; Beyotime) and adjusted the concentration to 1 µg/µl for further treatment. Then, we placed 20 µl of sample in a 0.5-ml microfuge tube and stored it frozen at −80°C until use. To extract protein, we added 5 µl of 5× sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer to each tube, and heated the tubes to 100°C for 5 min. We ran SDS-PAGE on polyacrylamide gel to separate total proteins, and transferred the proteins obtained onto polyvinylidene fluoride membranes electrophoretically. Then, we washed the membranes in TBST (150 mM NaCl, 2 mM KCl, 25 mM Tris and 0.05% Tween 20; pH 7.4), blocked them with TBST containing 3% BSA at 37°C for 2 h, and incubated them at 4°C overnight with mouse anti-GAPDH polyclonal antibodies (1:1000, cw0100A, CWBio), rabbit anti-TNFR1 polyclonal antibodies (1:1000, ab19139, Abcam), or rabbit anti-active caspase-3 polyclonal antibodies (1:500, 9664S, CST). Afterwards we washed the membranes in TBST, and then incubated them for 1.5 h at 37°C with goat anti-mouse IgG AP conjugated (1:2000, CW0110, CWBio) or goat anti-rabbit IgG AP conjugated (1:2000, CW0111, CWBio) secondary antibodies. Then, we washed the membranes in TBST and detected them using a BCIP/NBT alkaline phosphatase colour development kit (C3206, Beyotime). We determined the relative quantities of proteins by analyzing the sum density of each protein band image using an ImagePro Plus software.

Analysis of mitochondrial membrane potential by JC-1 staining and flow cytometry

Spermatozoa recovered from epididymis and vas deferens were washed and the sperm concentration was adjusted to $1–9 \times 10^6$ in D-PBS before JC-1 staining. A mitochondrial membrane potential (MMP) detection (JC-1) kit (C2006, Beyotime) was used to detect MMP. Briefly, 1 ml D-PBS containing $1–9 \times 10^6$ spermatozoa was mixed with 1 ml of JC-1 dye working solution. The spermatozoa were then incubated in the dark at 37°C for 20 min. After being washed three times with a JC-1 staining buffer, the stained spermatozoa were subjected to flow cytometry (BD LSR Fortessa™) to evaluate green and red fluorescence of the JC-1 dye. Data obtained were analyzed using FlowJo software (FlowJo 7.6 LLC, OR, USA).

Immunofluorescence microscopy

Spermatozoa were recovered from caudae epididymides and vasa deferentia. To isolate spermatogenic cells, using tweezers we tore

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seminiferous tubules in a digestive solution containing trypsin and EDTA (T1300, Solarbio) and digested them for 20 min at 37 °C. We performed all the procedures for immunostaining at room temperature unless otherwise mentioned. We washed spermatogenic cells or spermatozoa in PBS and centrifuged (300 *g*) them for 5 min between treatments. Our immunostaining procedures were as follows: (a) fixation at 37°C for 30 min in 4% paraformaldehyde; (b) permeabilization for 10 min in 0.1% Triton X-100; (c) block for 1 h with 3% BSA; (d) overnight incubation at 4°C with rabbit anti-TNFR1 polyclonal antibodies (1:100, ab19139, Abcam), rabbit anti-active caspase-3 polyclonal antibodies (1:100, 9664S, CST) or goat anti-TNF-α polyclonal antibodies (1:100, AF-410-NA, R&D Systems); (e) incubation for 1 h with Cy3-conjugated goat-anti-rabbit IgG (1:400, 111–165–144, Jackson Immuno Research) or donkey anti-goat IgG H&L (Alexa Fluor® 488) (1:200, ab150129, Abcam); and (f) incubation for 5 min with 10 μg/ml Hoechst 33342. Then, we mounted the stained cells on a glass slide and observed them under a Leica laser scanning confocal microscope (TCS SP2). The microscope detected fluorescence with bandpass emission filters (Hoechst 33342, 420–480 nm; Alexa Fluor® 488, 505–540 nm; and Cy3, 560–605 nm), and it recorded the captured signals for Hoechst, Alexa Fluor® 488, and Cy3 as blue, green and red, respectively. We analyzed fluorescence intensity using the ImagePro software under fixed thresholds across all slides to quantify caspase-3, TNFR1 and TNF-α. The averages of relative fluorescence from stressed mice were expressed relative to the averages from the control mice that was set to one.

Enzyme-linked immunosorbent assay (ELISA) for TNF-α

We used a Mouse Tumour Necrosis Factor α (TNF-α) ELISA kit (BLUE GENE) to measure TNF-α in seminiferous tubules and caudae epididymides. We minced 0.3–0.5 g tissues in 0.5 ml PBS with a glass homogenizer on ice, and then, froze and thawed the resulting suspension several times to break the cell membranes further. We then centrifuged the suspension at 800 *g* for 15 min, and added 100 μl supernatant or standards in the coated wells. After adding 50 μl of conjugate to each well, we incubated plates for 1 h at 37 °C. After we washed the coated wells with washing solution and dried them using paper towels, we added 50 μl of substrate A and B to each well and incubated the plate at 37°C for 15 min. Then, we terminated the reaction by adding 50 μl stop solution, and measured the optical density at 450 nm using a Infinite50 TECAN plate reader. We calculated TNF-α concentrations against the standard curves.

Quantitative real-time polymerase chain reaction

To isolate RNA, we placed 0.1 g seminiferous tubules in a homogenizer with 1 ml TRIzol reagent and homogenized on ice for 10 min. We then centrifuged the homogenates (20,000 *g*) at 4°C for 10 min. Following resuspended in diethylpyrocarbonate-treated MilliQ water (DEPC-dH_2O), we dissolved the purified RNA in DEPC-dH_2O and quantified it spectroscopically at 260 nm. We then assessed purity and integrity of the RNA by determining the A_{260}/A_{280} ratio (1.8–2.0) and by 1% agarose electrophoresis.

We performed reverse transcription in a total volume of 20 μl using a Transcriptor Reverse Transcriptase (Roche). We mixed 2 μl of each RNA sample, 1 μl oligo(dT_{18}) (Fermentas), and 10 μl of DEPC-dH_2O in a 0.2 ml reaction tube, and incubated the mixture with a PCR instrument at 65°C for 10 min. We then cooled the tube on ice for 2 min and centrifuged it (200 *g*, 4°C) for a few seconds. Then, we added 4 μl of 5× RT buffer, 0.5 μl RNase inhibitor (Roche), 2 μl dNTP (Fermentas) and 0.5 μl Transcriptor Reverse Transcriptase to the reaction tube. Finally, we incubated the mixture at 55°C for 30 min, at 85°C for 5 min, and then stored it at −20°C before use.

We used a Mx3005P real-time PCR instrument (Stratagene, Valencia, CA) to quantify TNF-α and TNFR1 mRNAs. The gene-specific primers for Gapdh (NM_008084.2): forward 5′-AAGGTGGTGAAGCAGGCAT-3′, reverse 5′-GGTCCAGGGTTTCTTACTCCT-3′; for Tnf-α (M13049.1): forward: TAGCCCACGTCGTAGCA, reverse: GCAGCCTTGTCCCTTGA; for Tnfr1 (NM_011609.4): forward CATCTTACTTCATTCACGAGCGTTGTC, reverse AGCAGAGCCAGGAGCACCAG. We performed amplification reactions in a 10 μl reaction volume with 1 μl cDNA, 5 μl 2× SYBR Green Master Mix (Agilent), 0.15 μl ROX™ (reference dye), 3.25 μl RNase-free water and 0.3 μl each of forward and reverse gene-specific primers (10 μM). We adopted cycle amplification conditions with an initial denaturation step at 95°C for 3 min, 40 cycles at 95°C for 20 s and 60°C for 20 s. We performed sequencing, dissociation curve analysis and gel electrophoresis of the PCR products to determine specificity of the reaction. We normalized gene expression to the internal control of glyceraldehyde-3-phosphate dehydrogenase (gapdh). By using the $2^{-(\Delta\Delta CT)}$ method, we expressed all values relative to the calibrator samples.

Incubation of spermatozoa with sTNF-α

We mixed semen collected from caudae epididymides and vas deferens of four mice in 1.6 ml M2 medium, and then allowed the spermatozoa to swim up for 10 min at room temperature. Afterwards we recovered the supernatants and adjusted their sperm concentration to $2–4 \times 10^7$/ml, then we placed 200 μl semen in a 0.5-ml centrifuge tube with M2 alone (control) or M2 containing 0, 25, 50, 100 or 150 ng/ml of soluble TNF-α (410-MT, R&D Systems). Then, we covered the semen with paraffin oil, and stored it at 4°C for 24 h. At the end of the storage, we incubated semen for 10 min in a CO_2 incubator at 37.5°C and assessed for sperm motility and fertilization potential.

Assessment for sperm motility

We assessed sperm motility using a computer-assisted sperm analyzer system (Sperm Class Analyzer; Microptic SL, Barcelona, Spain). The system performs sperm motility classification in accordance with the World Health Organization (WHO) standard parameters for human spermatozoa. This classification identified the spermatozoa as either type a = rapid progressive, type b = slow progressive, type c = non-progressive; or type d = immotile. We designated sperm motility as percentages of both type a and type b spermatozoa.

In vitro *fertilization*

We collected *in vivo* sperm masses from caudae epididymides and vasa deferentia, and transferred them to 1 ml of T6 medium containing 10 mg/ml BSA. We centrifuged (250 *g*, 5 min) the preserved semen, and resuspended them with T6 medium with 10 mg/ml BSA. We incubated both *in vivo* and preserved semen for 1.5 h for capacitation. We superovulated female Kunming mice (8–12 weeks after birth) with eCG (10 IU, i.p.) and hCG (10 IU, i.p.) at a 48-h interval. We recovered oocytes 13 h after hCG injection, and placed 25–30 oocytes in a fertilization drop of 150-μl fertilization medium (T6 with 20 mg/ml BSA). Then, we added capacitated sperm to the fertilization drops to give a final

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sperm concentration of 1–9 × 10^6/ml. After incubation for 6 h, we fixed oocytes in ethanol: acetic acid (3:1, vol/vol) for at least 24 h. We stained the fixed oocytes with 1% aceto-orcein and observed them under a phase contrast microscope. Our criteria for fertilized oocytes were that they must contain two pronuclei and a sperm tail in the ooplasm.

Data analysis

Each treatment contained at least three replicates. Percentage data were analyzed using analysis of variance (ANOVA) when each measure contained more than two groups or with independent-sample *t*-test, when each measure had only two groups after they were arcsine transformed. The differences were located by performing a Duncan multiple-comparison test during ANOVA. All the data were analyzed using the SPSS (Statistics Package for Social Sciences) software (SPSS 11.5, SPSS Inc. Chicago, IL, USA), and were expressed as mean ± standard error of the mean (SEM). A difference was considered significant only when the *P*-value was less than 0.05.

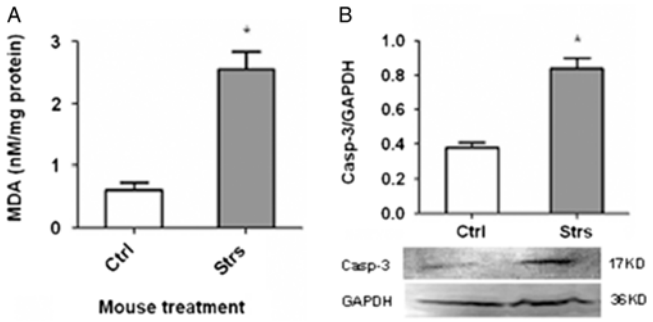

Figure 1. Effects of male restraint stress on contents of malondialdehyde (MDA) and expression of active caspase-3 in spermatozoa. Stressed (Strs) mice were restrained for 48 h before spermatozoa were recovered from caudae epididymides and vas deferens for measurements. Spermatozoa from unstressed control (Ctrl) mice were also examined as controls. (*A*) MDA contents (nM/mg protein) as assayed by spectrophotometrical detection of 2-thiobarbituric acid (TBA)-MDA adduct. (*B*) Ratio of active caspase-3/GAPDH as measured by western blotting. Each treatment was repeated three times with each replicate containing spermatozoa from three mice. *Significant difference ($P < 0.05$) from values in the control group.

Results

Effects of male restraint stress on the redox status and active caspase-3 level of spermatozoa

To evaluate the status of oxidative stress and apoptosis in spermatozoa after restraint stress, contents of malondialdehyde (MDA) and active caspase-3 were measured by spectrophotometry and western blotting, respectively, in spermatozoa from caudae epididymides and vas deferens of control and stressed mice. The results showed that both levels of sperm MDA (Fig. 1A) and active caspase-3 (Fig. 1B) were significantly higher in stressed mice than in control mice, suggesting that restraint of male mice induced oxidative stress and apoptosis in mature spermatozoa.

Effects of male restraint on mitochondrial membrane potential of spermatozoa

As it has been reported that MMP decreased at the early stage of apoptosis, leading to release of the pro-apoptotic factor cytochrome *c* (Heiskanen *et al.*, 1999), we examined sperm MMP. Under normal conditions with the mitochondria membrane intact, JC-1 forms J-aggregates that gives out red fluorescence at 585 nm in the mitochondrial inner membrane, but when MMP is destructed, JC-1 cannot be transported into the mitochondria and is present as monomers that gives out green fluorescence at 530 nm in the cytoplasm (Smiley *et al.*, 1991). Therefore, JC-1 staining is often used to measure MMP (Cottet-Rousselle *et al.*, 2011). Our flow cytometry after JC-1 staining indicated that, relative to control mice, semen from stressed mice contained significantly less JC-1 red-positive but more JC-1 green-positive spermatozoa (Fig. 2A, B). Furthermore, the ratio of red/green spermatozoa in stressed mice was significantly lower than that in control mice. The results further confirmed that restraint of male mice induced apoptosis of mature spermatozoa.

Effects of male restraint stress on expression of TNFR and TNF-α in mouse spermatozoa

To explore the role of the TNF-α signalling in the stress-induced apoptosis of mature spermatozoa, expression of TNF-α and TNFR1 was localized and quantified by immunostaining with respective antibodies; TNFR1 was also detected by western blotting. The results demonstrated that all the spermatozoa showed TNF-α (Fig. 2C) and TNFR1 (Fig. 2D) staining on both heads and middle pieces. The expression levels of both TNF-α (Fig. 2E) and TNFR1 (Fig. 2F, G) were significantly higher in stressed than in unstressed control spermatozoa, suggesting that restraint of male mice triggered sperm apoptosis by activating TNF-α signalling.

Effects of male restraint stress on expression of TNFR1, TNF-α and caspase-3 in caudae epididymides

To specify whether the male stress damages maturing spermatozoa by inducing apoptosis and facilitating TNF-α production in epididymis, expression of active caspase-3 and TNFR1 was analyzed by western blotting, and TNF-α content was measured by ELISA, in the caudae epididymides from control and stressed mice. The expression levels of active caspase-3, TNF-α and TNFR1 in caudae epididymides were all significantly higher in stressed than in control mice (Fig. 3), suggesting that the restraint stress of male mice triggered apoptosis of the epididymis and increased their secretion of TNF-α, which induced apoptosis in maturing spermatozoa through interaction with TNFR on the spermatozoon.

Effects of male restraint stress on apoptosis and TNF-α signalling of seminiferous tubules

To determine the effect of male restraint on apoptosis of spermatogenic cells, expression of active caspase-3, TNF-α and TNFR1 in seminiferous tubules was assayed by western blot analysis, real-time PCR or ELISA. Compared with control mice, male restraint significantly increased levels of active caspase-3, TNF-α and TNFR1 in seminiferous tubules (Fig. 4).

Effects of male restraint stress on expression of TNF-α and TNFR1 in spermatogenic cells

When localization and quantification of TNF-α and TNFR1 were performed by immunofluorescence microscopy in isolated spermatogenic cells, levels of both TNF-α and TNFR1 in spermatogonia, spermatocytes and spermatids were significantly higher in stressed mice than in control mice (Fig. 5). The results

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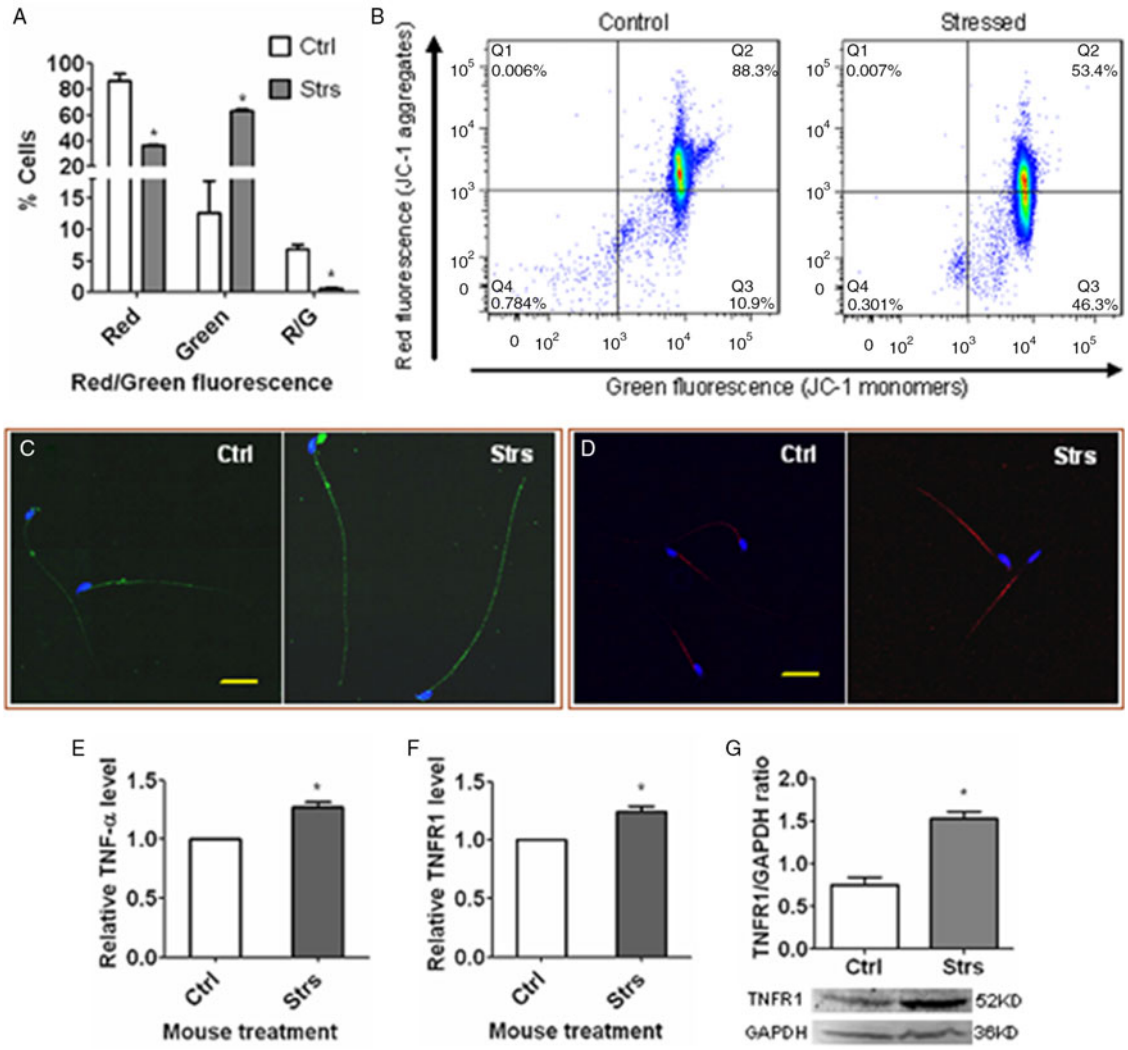

Figure 2. Effects of male restraint stress on MMP and expression of TNF-α and TNFR1 in spermatozoa. (*A*) Percentages of JC-1 red- and green-positive spermatozoa and ratios of red/green spermatozoa as assayed by flow cytometry following JC-1 staining. (*B*) JC-1 flow cytometry graphs of control and stressed spermatozoa, indicating that the control group contains more JC-1 red-positive but less JC-1 green-positive spermatozoa than does the stressed group. Each treatment was repeated three times with each replicate containing spermatozoa from three mice. (*C*, *D*) Sperm smears following TNF-α and TNFR1 immunostaining, respectively. While the Hoechst 33342 stained chromatin was coloured blue, the TNF-α and TNFR1 were coloured green and red, respectively. All the spermatozoa show TNF-α and TNFR1 staining on both heads and middle pieces. Bar is 30 μm and applies to all images in the same panel. (*E*, *F*) Relative level (fluorescence intensity) of TNF-α and TNFR1, respectively. Each treatment was repeated three times with each replicate containing about 40 spermatozoa from two mice. (*G*) Ratios of TNFR1/GAPDH as measured by western blotting. Each treatment was repeated three times with each replicate containing spermatozoa from three mice. *Significant difference ($P < 0.05$) from values in the control group.

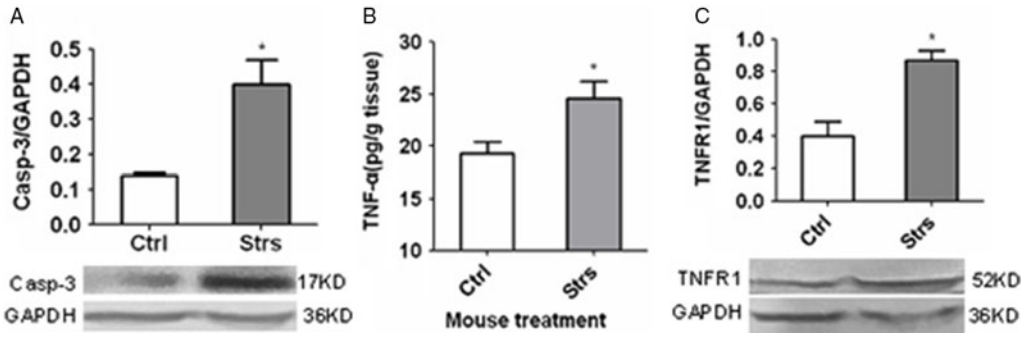

Figure 3. Expression levels of active caspase-3 (*A*), TNF-α (*B*) and TNFR1 (*C*) in caudae epididymides of control (Ctrl) and stressed (Strs) mice. While caspase-3 and TNFR1 were assayed by western blotting, TNF-α was measured by ELISA. Each treatment was repeated three times with each replicate containing caudae epididymides from four mice. *Significant difference ($P < 0.05$) from values in control mice.

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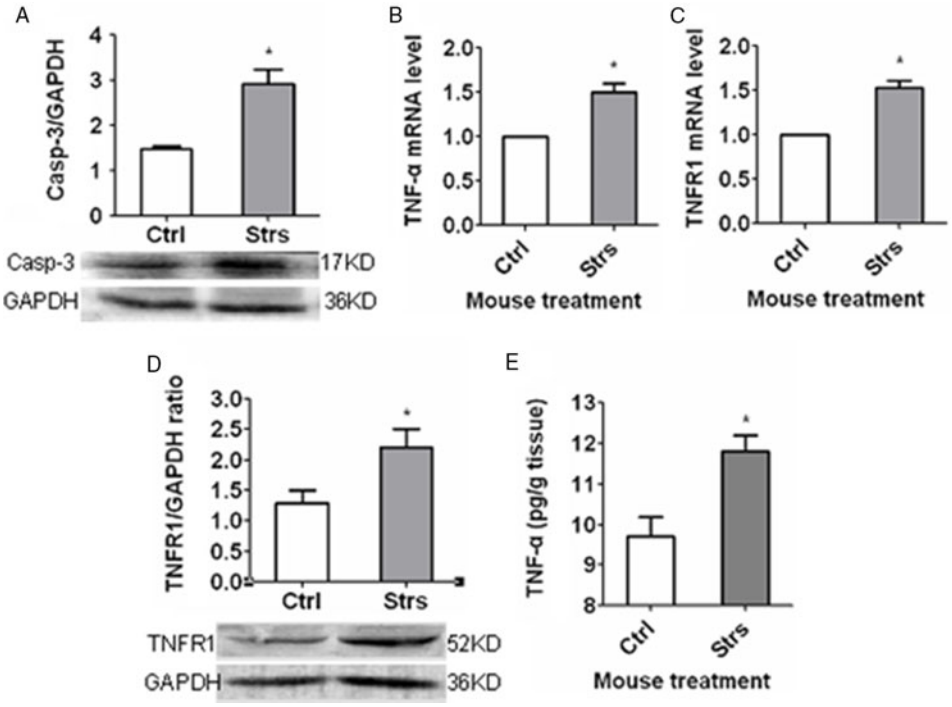

Figure 4. Expression levels of active caspase-3 protein (*A*) and TNF-α and TNFR1 mRNA (*B*, *C*) and protein (*D*, *E*) in seminiferous tubules of control (Ctrl) and restraint-stressed (Strs) mice. mRNA expression was measured by real-time PCR, protein expression of caspase-3 and TNFR1 was assayed by western blotting, and TNF-α protein was detected by ELISA. Each treatment was repeated three times with each replicate containing seminiferous tubules from three mice. *Significant difference ($P < 0.05$) from control values.

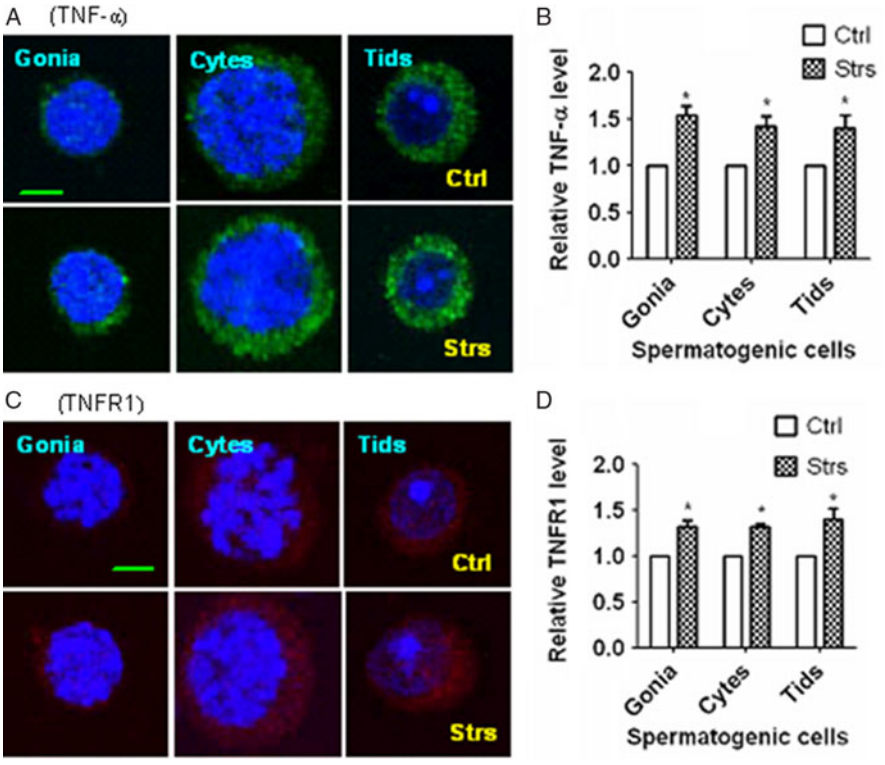

Figure 5. Effects of male restraint on expression of TNF-α and TNF receptor 1 in spermatogenic cells. (*A*, *C*) Micrographs of spermatogonia (Gonia), spermatocytes (Cytes) and spermatids (Tids) observed under a fluorescence microscope after immunostaining with TNF-α and TNFR1 antibodies, respectively. In both (*A*) and (*C*), while the upper rows show cells from control (Ctrl) mice, the lower rows show cells from stressed (Strs) animals. While the Hoechst 33342 stained chromatin was coloured blue, the TNF-α and TNFR1 were coloured green and red, respectively. Bars represent 5 μm and apply to all images in the same panel. (*B*, *D*) Relative levels of TNF-α and TNFR1, respectively, in different spermatogenic cells from control and stressed mice. Each treatment was repeated three times with each replicate including 40 cells from two mice. *Significant ($P < 0.05$) difference from values in control mice.

further confirmed that male restraint stress-induced apoptosis of spermatogenic cells through activating the TNF-α signalling, as our previous study demonstrated that the same male restraint treatment induced apoptosis in spermatogenic cells (Xiao *et al.*, 2019).

Restraint of male mice impaired the sperm quality 35 days later

To study whether the pro-apoptotic effect of male stress on spermatogenic cells can be translated into impaired quality of the spermatozoa derived from them, some mice restrained for

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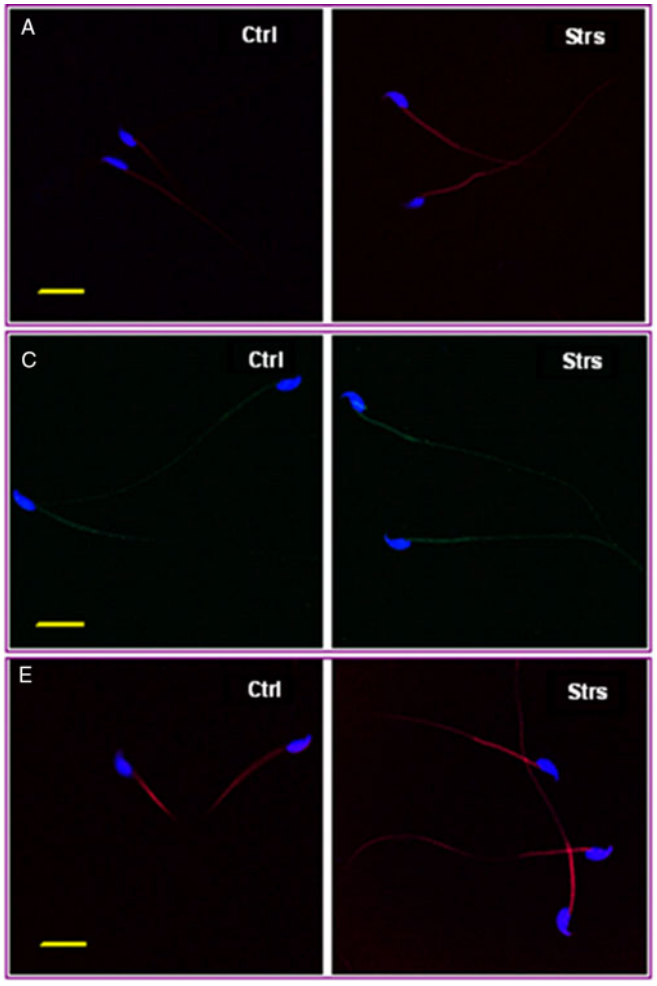

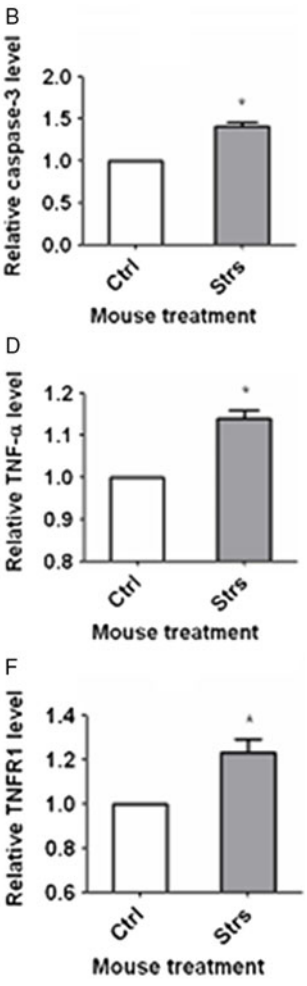

Figure 6. Levels of active caspase-3, TNF-α and TNFR1 in spermatozoa recovered from restraint-stressed (Strs) and unstressed control (Ctrl) mice 35 days after the end of a 48-h restraint. (*A*, *C*, *E*) Sperm smears following active caspase-3, TNF-α and TNFR1 immunostaining, respectively. While the Hoechst 33342 stained chromatin was coloured blue, the TNFR1/caspase-3 and TNF-α were coloured red and green, respectively. Bar represents 30 µm and applies to all images in the same panel. (*B*, *D*, *F*) Relative level (fluorescence intensity) of caspase-3, TNF-α and TNFR1, respectively. Each treatment was repeated three times with each replicate containing about 40 spermatozoa from two mice. *Significant difference ($P < 0.05$) from values in the control group.

48 h were raised for 35 days (to allow spermatogonia to develop to mature spermatozoa; Oakberg, 1956) before being sacrificed to examine sperm quality. The results showed that sperm motility was significantly ($P < 0.05$) lower in restrained mice (43 ± 2.5%) than in unstressed control mice (70.9 ± 3.7%). All spermatozoa showed caspase-3 (Fig. 6A), TNF-α (Fig. 6C) and TNFR1 (Fig. 6E) staining on both head and middle pieces. Furthermore, levels of active caspase-3 (Fig. 6B), TNF-α (Fig. 6D) and TNFR1 (Fig. 6F) were significantly higher in stressed mice than in control mice. The results verified that the pro-apoptotic effect of male stress on spermatogenic cells was translated into impaired quality of the spermatozoa derived from them through activation of the TNF-α system.

Experiments using the TNF-α gene knockout (TNF-α$^{-/-}$) mice

To further verify that male restraint stress induces apoptosis of spermatozoa and spermatogenic cells through activating the TNF-α signalling, wild-type or TNF-α$^{-/-}$ C57BL/6 J male mice were restrained for 48 h before fertilization potential of spermatozoa and the level of active caspase-3 in caudae epididymides or seminiferous tubules were examined by *in vitro* fertilization and western blotting, respectively. The results showed that, although restraint stress of males significantly decreased fertilization rates while increasing the level of active caspase-3 in caudae epididymides and seminiferous tubules in both wild-type and TNF-α$^{-/-}$ mice, the difference between stressed and control mice was significantly reduced in the TNF-α$^{-/-}$ mice compared with that in the wild-type mice (Fig. 7A-C). The results further substantiated the role of TNF-α signalling in male restraint stress-induced apoptosis of spermatozoa and spermatogenic cells.

Effects of in vitro *exposure to TNF-α on sperm motility and fertilizing potential*

Spermatozoa collected from caudae epididymides and vas deferens of unstressed mice were preserved for 24 h in M2 medium containing different concentrations of sTNF-α before examination for sperm motility or use for insemination. The results demonstrated that sperm motility declined significantly with increasing concentrations of sTNF-α and reached the lowest level at 100 ng/ml of TNF-α (Fig. 7D). Fertilization rates of the inseminated oocytes also decreased significantly after treatment of spermatozoa with 100 ng/ml TNF-α (Fig. 7E). Therefore, the results further confirmed that the apoptotic epididymides impaired sperm motility and fertilizing potential by secreting soluble TNF-α following male stress.

Discussion

The current results demonstrated that restraint of male mice significantly impaired sperm fertilizing potential while inducing oxidative stress and apoptosis. Therefore, male restraint decreased rates of *in vitro* fertilization in both wild-type and TNF-α$^{-/-}$ mice

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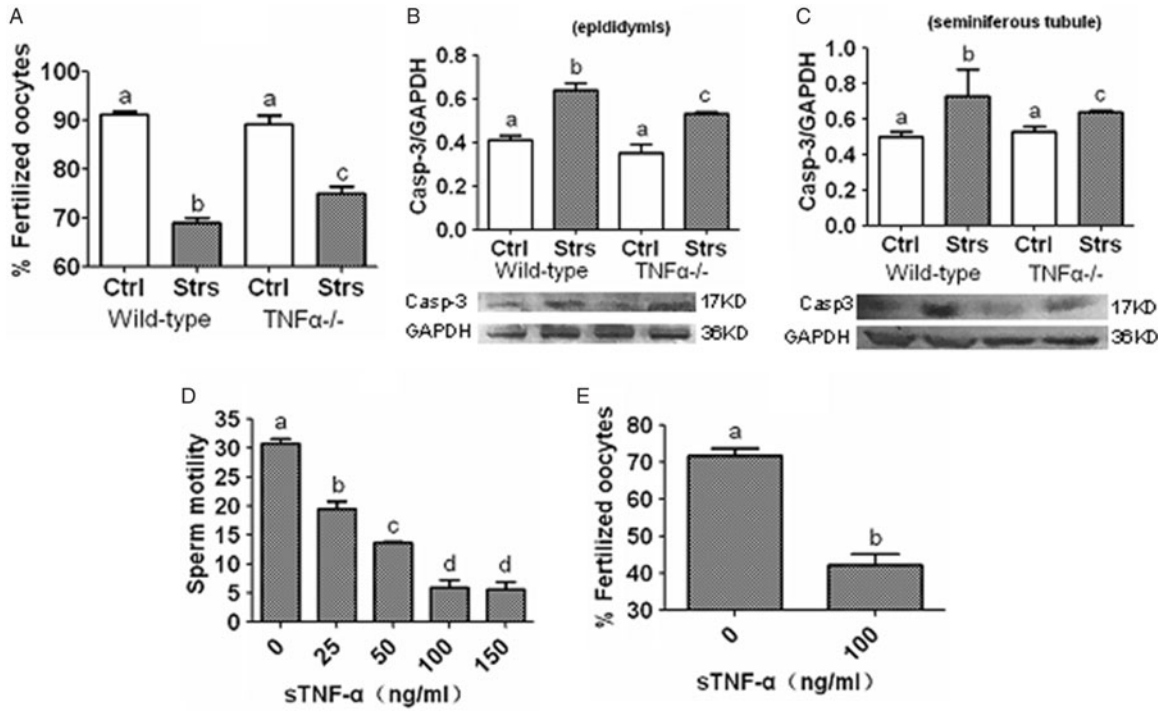

Figure 7. Experiments using TNF-$\alpha^{-/-}$ mice and *in vitro* exposure to TNF-α to verify role of the TNF-α signalling. (*A–C*) Fertilization potential of spermatozoa (*A*) and active caspase-3 level in caudae epididymides (*B*) and seminiferous tubules (*C*) in control (Ctrl) or stressed (Strs) wild-type or TNF-$\alpha^{-/-}$ C57BL/6 J mice. For *in vitro* fertilization experiments, each treatment was repeated three times with each replicate including about 60 oocytes inseminated with semen from two males. For western blotting, each treatment was repeated three times with each replicate containing caudae epididymides or seminiferous tubules from two mice. (*D*) Sperm motility after semen preservation with different concentrations of sTNF-α. Each treatment was repeated three times with each replicate including spermatozoa from four mice. (*E*) Percentages of fertilized oocytes after insemination with spermatozoa preserved without or with 100 ng/ml sTNF-α. Each treatment was repeated five or six times with each replicate containing about 30 oocytes inseminated with semen from three male mice. $^{a-d}$Values with a different letter above bars differ significantly ($P < 0.05$).

and reduced the MMP while increasing levels of MDA and active caspase-3 in spermatozoa recovered from caudae epididymides and vas deferens. MDA is well known as a marker for oxidative stress (Liu *et al.*, 2015), and its level has been measured in spermatozoa to reflect a state of oxidative stress (Moazamian *et al.*, 2015). For example, co-incubation of boar spermatozoa with landfill leachate, which caused a time- and dose-dependent decline in sperm motility and viability, increased both hydrogen peroxide and MDA levels (Adedara *et al.*, 2013).

Studies in various cells and tissues have indicated that oxidative stress can cause mitochondrial dysfunction and trigger apoptosis. In *Candida albicans*, for example, an increase in reactive oxygen species (ROS) induced oxidative stress and mitochondrial membrane depolarization, which causes the release of pro-apoptotic factors (Cho and Lee, 2011). Expression of the SHOX gene in osteosarcoma cells causes oxidative stress that, in turn, ruptures lysosomal membrane leading to releases of active cathepsin B to the cytosol and subsequent activation of the intrinsic apoptotic pathway with mitochondrial membrane permeabilization and caspase activation (Hristov *et al.*, 2014). Conversely, mitochondrial defects can cause ATP deficiency and ROS overproduction (Wu *et al.*, 2014). Spermatozoa are particularly susceptible to oxidative stress because they are exceptionally rich in vulnerable substrates like polyunsaturated fatty acids, proteins and DNA (Aitken *et al.*, 2012). Furthermore, the apoptotic cascade in senescent mature spermatozoa appears to be triggered by oxidative stress and lipid peroxidation, which leads to mitochondrial ROS generation (Aitken and Baker, 2013).

The present results suggested that restraint of male mice induced apoptosis in caudae epididymides, which facilitated apoptosis of maturing spermatozoa by releasing TNF-α. Therefore, we showed that the levels of active caspase-3, TNF-α and TNFR1 in the caudae epididymides were all significantly higher in stressed than in unstressed control mice. Furthermore, *in vitro* incubation with sTNF-α significantly impaired sperm motility and fertilizing potential. The mammalian epididymis is an essential organ for sperm transport, storage and maturation to acquire fertilization potential (Moore, 1998; Dacheux and Dacheux, 2013). However, although cytotoxic effects of apoptosis and oxidative stress in epididymis on epididymal spermatozoa have been observed after oral administration of nonylphenol (Lu *et al.*, 2014), how the apoptotic epididymis damages spermatozoa is unknown. Therefore, the present results have provided the first evidence that the stress-induced apoptosis of epididymis triggers sperm apoptosis by facilitating the release of TNF-α. It has been reported in rats that sleep restriction impairs postnatal epididymal development and sperm motility with increased oxidative stress (Siervo *et al.*, 2017). Glucocorticoids caused oxidative stress in rat epididymis through their effects on the antioxidant defence system (Dhanabalan *et al.*, 2010). Furthermore, restraint stress of rats elevated the levels of lipid peroxidation and hydrogen peroxide in the epididymis by suppressing the activities of antioxidant enzymes (Dhanabalan *et al.*, 2011).

This study showed that restraint stress of male mice triggered apoptosis in mature spermatozoa, epididymis, and spermatogenic cells by activating the TNF-α signalling. Therefore, the male

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restraint triggered apoptosis with increased expression of both TNF-α and TNFR1 in mature spermatozoa, epididymis and seminiferous tubules/spermatogenic cells. The restraint stress-induced adverse effects were significantly relieved in the TNF-α $^{-/-}$ mice. Furthermore, culture with sTNF-α significantly reduced sperm motility and fertilizing potential. The expression of TNF-α has been observed in spermatogenic cells (De *et al.*, 1993), mature spermatozoa (Payan-Carreira *et al.*, 2012), and in the epididymis (Payan-Carreira *et al.*, 2012; Oh *et al.*, 2016). Expression of the TNFR was detected in somatic cells of the testis including the Sertoli and Leydig cells (Lysiak, 2004) and in the whole sample of mammalian testes (Boekelheide *et al.*, 1998; Białas *et al.*, 2009), but its expression in spermatogenic cells remains to be established. For example, while Suescun *et al.* (2003) observed a significant increase in the number of TNFR1-positive germ cells in rats with autoimmune orchitis, De *et al.* (1993) found that RNA from mouse pachytene spermatocytes or round spermatids did not hybridize with human TNFR1 probe in northern blot analysis, although RNA from Sertoli cells did hybridize. Furthermore, TNFR expression in the epididymis has not been reported, to our knowledge. Therefore, the present results have shown for the first time that unfavourable conditions can trigger apoptosis in mature spermatozoa, epididymis and the spermatogenic cells by activating the TNF-α system.

In summary, by using a restraint-stressed mouse model, we have explored the mechanisms by which stressors trigger apoptosis in spermatozoa and spermatogenic cells, to reveal the mechanisms by which psychological stress impairs semen quality. The results demonstrated that male restraint stress triggered apoptosis in the maturing spermatozoa, epididymis, and spermatogenic cells via activating the TNF-α signalling. The stress-induced apoptosis of epididymis triggered sperm apoptosis by facilitating the release of TNF-α. The apoptotic spermatozoa might facilitate apoptosis of other spermatozoa by releasing TNF-α. Furthermore, the pro-apoptotic effect of male stress on spermatogenic cells can be translated into impaired quality of the future spermatozoa derived from them. The data are of great importance not only for animal breeding facilities but also may be relevant for the human reproduction, as it has been shown that the TNF-α system is active in the human semen (Estrada *et al.*, 1997; Lampiao and du Plessis, 2008).

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Conflicts of interest. The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical standards. Care and use of mice were conducted exactly in accordance with the guidelines and approved by the Animal Care and Use Committee of the Shandong Agricultural University P.R. China (Permit number: SDAUA-2001–0510).

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