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Ala-Gln improves varicocele-induced testicular injury by increasing HSP70 and antioxidant activity in male rats

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Abstract: This worked aimed to test the hypothesis that L-alanyl-L-glutamine (Ala-Gln) improves the varicocele-induced testicular injury, which causes male infertility. For this purpose, fifty adult male Wistar rats received the varicocele (VC) surgery at the left renal vein. Biomarkers were determined 2, 4, and 8 weeks after VC (n = 10/each detection). Four weeks after VC, rats received Ala-Gln (1.125 g/kg) treatment with and or saline for 1 week (n = 10/each group). Rats in the sham group were also detected for biomarkers at 2, 4, and 8 weeks (n = 10/each detection). VC caused testicular injury detected by hematoxylin–eosin (H&E) staining, immunohistochemistry, and TUNEL assay. HSP70 mRNA was detected quantitative RT-PCR, SOD, and CAT by nitroblue tetrazolium (NBT) method and 8-OHDG by ELISA. The results showed that varicocele induced injury in the testes. The weight of the left testes was lower than that of the right testes in VC-bearing rats ($p < 0.01$). The relative numbers of sustentacular and spermatogenic cells were decreased after VC ($p < 0.01$). In sham-4 wk, VC-4wk, VC-5wk and Ala-Gln groups, the apoptosis index was 5.10 ± 1.14, 13.22 ± 3.63, 33.62 ± 3.56 and 22.33 ± 2.61, relative level of HSP70 mRNA 1.00 ± 0.12, 0.53 ± 0.05, 0.51 ± 0.04 and 1.62 ± 0.15 fold, SOD 16.4 ± 0.23, 13.4 ± 0.17, 10.01 ± 1.06 and 19.53 ± 2.26 U/mg protein, CAT 2.16 ± 0.31, 1.07 ± 0.28, and 1.31 ± 0.26 and 3.46 ± 0.71 U/mg, 8-OHDG 5.23 ± 0.67, 6.81 ± 0.78, 7.16 ± 1.22 and 4.14 ± 0.73 pg/μg DNA, respectively ($p < 0.01$). Our results suggest that Ala-Gln prevented the VC-induced testicular injury. We have firstly reported that Ala-Gln protects against varicocele-induced testicular injuries by up-regulation of HSP70 and antioxidants, SOD and CAT, and down-regulation of oxidant 8-OHDG, resulting in reducing apoptosis in the testis.

Introduction

Varicocele (VC) is defined as the abnormal elongation, expansion, and distortion due to poor return flow of slender spermatic vein or venous valve damage (Agarwal *et al.*, 2016; Sadek *et al.*, 2011). Reported by the World Health Organization (WHO), the incidence of VC is about 25–40% in infertile men and may be the main cause of male infertility (Agarwal and Said, 2003; Sigman, 1998). VC enhances oxidative stress resulting in intensive DNA damage in germ cells and spermatozoa (Amin *et al.*, 2018; Khosravanian *et al.*, 2015; Salmani *et al.*, 2020). Heat-shock protein 70 (HSP70) located in the cytoplasm belongs to the HSP family as a protective protein to maintain normal cellular functions (De Maio, 1999; Hassanpour *et al.*, 2017). Due to the ability to bind with pro-apoptotic molecules, HSP70 is known as an anti-apoptotic molecule and protects the cellular DNA content from VCL-induced oxidative stress (Li *et al.*, 1997; Salmani *et al.*, 2020; Shamsi-Gamchi *et al.*, 2018). HSP70 is involved in the regulation of spermatogenesis in different stages with a role oxidative stress in male infertility. The abnormal expression of HSP70 may interfere the male fertility (Erata *et al.*, 2008; Feng *et al.*, 2001; Khosravanian *et al.*, 2015). Men with varicocele are usually with higher levels of seminal oxidants, including malondialdehyde (MDA), nitric oxide (NO), and 8-oxo-2'-deoxyguanosine (8-OHDG), but lower levels of seminal antioxidants, including superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) (Abd-Elmoaty *et al.*, 2010; Sakamoto *et al.*, 2008).

Glutamine amino acid (Gln) is the most abundant amino acid in the blood and the intracellular free amino acid pool of most tissues (Perriello *et al.*, 1995). Gln induces the expression of HSPs, including HSP70, to protect against endotoxin shock

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and lipopolysaccharides (LPS)-induced cardiomyocyte damage in rats (Gong and Jing, 2011; Jing *et al.*, 2007; Wischmeyer *et al.*, 2001). L-alanyl-L-glutamine (Ala-Gln) is a dipeptide with the highest stability, solubility, and heat-sterilization. When it intravenously infuses, Ala-Gln promptly hydrolyzed to glutamine and alanine (Albers *et al.*, 1989). In an experimental rat model, the acute Ala-Gln supplementation promotes significant increase of glutamine levels in plasma and tissues (Rogero *et al.*, 2006). In an animal model using Mongolian gerbils, preconditioning with L-Ala-Gln submitted to cerebral ischemia/reperfusion reduces the oxidative stress, nucleus degeneration, and red neuron of and death in the cerebral tissue (Pires *et al.*, 2011). Combining with fish oil containing the ω-3 polyunsaturated fatty acids (PUFAs), the dipeptide Ala-Gln has been used in patients with heart failure (HF) to improve both specific muscle functional parameters and fatigability and overall exercise tolerance (Wu *et al.*, 2015). In addition, the pre-administration of Ala-Gln just before lipopolysaccharide (LPS) in rats effectively protects the lung by enhancing HSP70 expression (Wang *et al.*, 2017). Therefore, Ala-Gln might be a stable HSP70 inducer.

In our study, we have investigated the hypothesis that Ala-Gln protects against the varicocele-induced testicular dysfunction. By administration with Ala-Gln in the VC-bearing rats, we have first found that, in contrast to the effects of varicocele, Ala-Gln increases HSP70 and seminal antioxidants and decreases seminal oxidants, resulting in the reduction of apoptosis in spermatogenic cells and the recovery of testicular injuries.

Methods

Animals and grouping

All experiments were approved by the Animal Experimental Ethics Committee of Shandong University, Jinan, China. Eighty male Wistar rats, at the age of 6.25 ± 0.75 months and weight of 203.20 ± 11.68 g, were provided by Shandong University animal center. Rats were randomly divided into sham (n = 30) and varicocele (VC) (n = 50) groups. All rats fasted for 4 h before the experiments.

Surgical procedures

Rats were anesthetized with an intraperitoneal (i.p.) injection of 4% chloral hydrate (1 mL/100 g body weight). The left renal vein of the rat in the VP group was carefully separated from the inside of the adrenal vein, the spermatic vein, and the outside the inferior vena cava. A smooth metal rod (0.8 mm diameter) was placed parallel to the left renal vein. The vein and metal rod were ligated with a 3/0 silk, which reduced the vein diameter to approximately half its original diameter. The metal rod was then pulled out. The contents were repositioned, and the skin was carefully sutured layer by layer (Fig. 1A, a). For rats in the sham group, we performed an abdominal median incision. Following exposure, the left renal vein was only separated without ligation. The incision was sutured with 3/0 silk (Fig. 1A, b).

Preservation of testis

To observe the effects of VC, the left and right testes of 10 rats in the VC or the sham group were taken 2, 4, and 8 weeks after surgeries (total 30 rats/each group) for measurement of VC biomarkers. To observe the effect of Ala-Gln on VC-induced testicular injury, 20 VC-bearing rats, 4 weeks after the varicocelectomy, were randomly divided into the Ala-Gln group (n = 10) with a daily i.p. of 1.125 g/kg body weight for one week and the control group (n = 10) with a daily i.p. of the same volume of saline for one week (also named VC-5wk group). The testes in both sides were taken, dissected out from the associated adipose tissue and fascia attached to their surface, and dried on a filter paper after washing with saline. The testes were weighted and then cut in half. One half was fixed in 4% paraformaldehyde for histological examination, and the other half immediately frozen and stored at −80°C for later analysis.

H&E staining, immunohistochemistry and TUNEL assay

After fixation in 4% paraformaldehyde, the half testis was embedded in paraffin and then cut to 5-μm sections. After routinely dewaxing and hydration, sections were stained with hematoxylin and eosin (H&E staining). After the peroxidase block, the section was incubated with the primary antibody, mouse anti-HSP70 monoclonal antibody (1:25) (Santa Cruz Biotech, USA), and then horseradish peroxidase-labeled secondary antibody. The results were observed with an Olympus BX50 light microscope (Olympus, Japan). The staining intensity was assigned a score of 0–3 (0-absent, 1-weak, 2-moderate, and 3-strong). The HSP70-positive staining was defined while score 2 and 3 staining was ≥10% of total cells. Five fields were randomly selected from each section and photographed under the same conditions. The average optical density values of the positive staining were analyzed using CellProfiler, a high-resolution color image reporting system (Matlab, USA). The apoptosis-detection kit (Promega, USA) was used to perform terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay following the manufacturer's instruction. The nuclei of the TUNEL positive cells stained yellow or dark brown. For calculation of the apoptosis index (AI), 10 fields were randomly selected from each section to count the TUNEL-positive cells.

Quantitative real time RT-PCR (RT-qPCR)

TRIzol reagents (Invitrogen, USA) was used to extract the total RNA from the rat testis tissue. First-strand cDNA was synthesized using a cDNA synthesis kit (Promega, USA) according to the manufacturer's protocol. RT-qPCR was performed with SYBR-Green mix kit (Applied Biosystems, USA), using primers: Hsp70 forward, 5'- ACC GTG CCC GCC TAC-3' and reverse, 5'- ACA GCG TCC TCT TGG CCC TC-3', and the internal control, GAPDH, forward, 5'-TGC CAC TCA GAA GAC TGT GG-3' and reverse, 5'-TTC AGC TCT GGG ATG ACC TT-3'. PCR was performed with 40 cycles of 95°C for 15 s, 60°C for 60 s, and 72°C for 30 s, using the ABI 7900 Real-Time PCR system (Applied Biosystems, USA).

Determination of testicular SOD, CAT and 8-OHDG

The activity of testicular superoxide dismutase (SOD) was assayed using the nitroblue tetrazolium (NBT) method (Sun *et al.*, 1988) and presented as U/mg protein of testicular tissue. The activity of catalase (CAT) was assayed (Aebi, 1984) and presented as U/mg protein of testicular tissue. One unit of CAT equates to

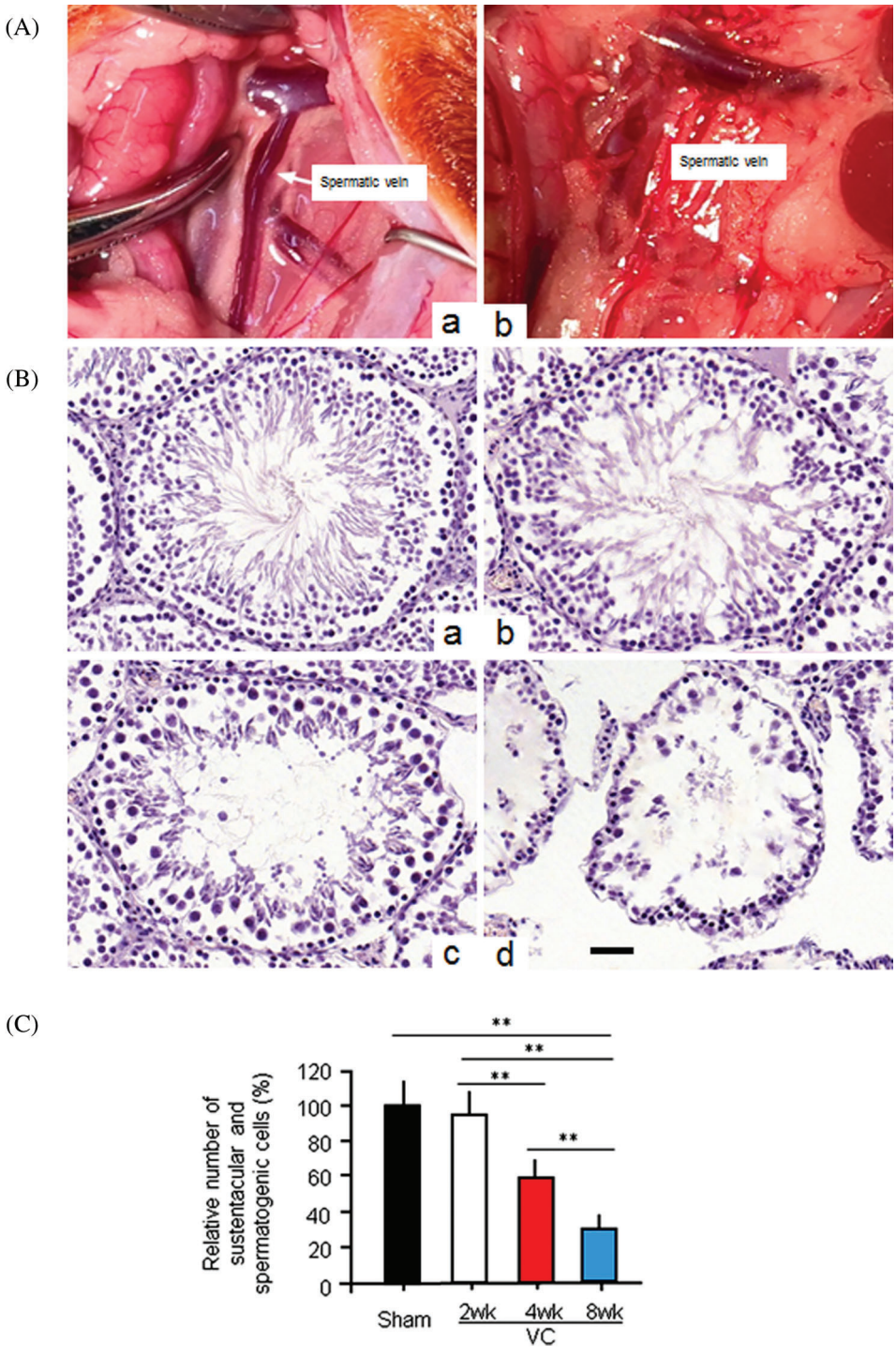

FIGURE 1. The experimental varicocele induces histopathological changes in rat testes.
(A) General observation of spermatic vein in rats of varicocele (VC) surgery (a) and shame (b) groups. The scale bar = 1 mm. (B) Testicular histopathology after VC. a. Sham-4wk: Sustentacular cells and spermatogenic cells were arranged neatly and orderly; b. VC-2wk: Sustentacular cells and spermatogenic cells were almost normal; c. VC-4wk: The number of seminiferous tubules decreased, the spermatogenic cells lost, the spermatogenic process blocked with interstitial edema; d. VC-8wk: Testicular seminiferous tubules severely atrophied, spermatogenic cells lost in large numbers, only sustentacular cells appeared. Scale bar = 100 μm. (C) Comparison of the relative number of sustentacular and spermatogenic cells (%) between sham, VC-2wk, VC-4wk, and VC-8wk groups (n = 10/each group). $^{**}p < 0.01$ (ANOVA and post-test).

the amount of CAT decomposing H_2O_2 at 1.0 mmol/min. The 8-Hydroxy-2 deoxyguanosine (8-OHDG) level was determined by using the 8-OHDG ELISA kit (Northwest, Canada). Testis DNA was extracted using DNasy Blood and Tissue Kit (Qiagen, USA). The extracted DNA was digested with nuclease P1, and then 8-OHDG was determined following the ELISA protocol. The level of 8-OHDG was presented as pg/μg DNA.

Statistical analysis

All data were presented as the mean ± standard deviation (SD). Statistical analysis was conducted using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). The means were compared using a one-way analysis of variance followed by the Student–Newman–Keuls test for the different groups. ANOVA and post-test were also used. $p < 0.05$ was considered to indicate a statistically significant difference. All experiments were performed at least three times.

Results

Varicocele induces injury in the testes of VC-model rats

Our primary aim was to test whether Ala-Gln could improve the varicocele (VC) induced testicular injury. Therefore, we successfully established the VC rat model 4 weeks after partial ligation of the left renal vein. The weight of the left testes was significantly lower than that of the right testes in

VC-bearing rats ($p < 0.01$) 4 and 8 weeks after the VC surgery (Tab. 1). The weight of the right testes of rats in the VC group was also significantly lower when compared to that in the sham group ($p < 0.05$). H&E staining showed the testicular histopathology in shame and VC groups: the cells were counted and normalized to the cell number in the sham group for the relative cell number (%). In the testis of the sham-4wk group, sustentacular and spermatogenic cells were arranged neatly and orderly (100.0 ± 15.3%) (Figs. 1B, 1a, 1C). Two weeks after VC, sustentacular and spermatogenic cells were almost normal (95.4 ± 14.2%) (Figs. 1B, 1b, 1C). Four weeks after VC, the number of seminiferous tubules was decreased, the spermatogenic cells lost (59.3 ± 7.8%), and the spermatogenic process blocked with the interstitial edema (Figs. 1B, 1c, 1C). At the same time, testicular seminiferous tubules were severely atrophied, a large number of spermatogenic cells lost, but sustentacular cells appeared in the vessel wall (32.4 ± 6.3%) (Figs. 1B, 1d, 1C). The bilateral testes obviously displayed the structural impairment, and the pathological changes in left side testis were more serious than that on the right side.

The experimental varicocele increases seminiferous tubule injury

Tab. 1 also showed that the injury rate of the seminiferous tubules in the left testis was 28.74 ± 8.85%, 52.38 ± 11.36%, and 87.64 ± 12.77% in VC-2wk, VC-4wk, and VC-8wk groups, and 3.29 ± 1.01%, 3.39 ± 1.09% and 3.41 ± 1.12% in Sham-2wk, Sham-4wk and Sham-8wk groups (n = 10/each group), respectively, with significant differences ($p < 0.01$). Two and four weeks after varicocele, the injury rate of the seminiferous tubules in the left testis was higher than that in the right testis ($p < 0.01$), but no significant difference when comparing the same feature 8 weeks after varicocele ($p > 0.05$) (Tab. 1). The data suggest that the experimental varicocele increases seminiferous tubule injury.

Varicocele decreases the number of HSP70-positive cells

Histological staining showed that the HSP70-positive score of spermatogenic cells was 3 (strong) in the sham-4wk group (n = 10), and 1 (weak) in the VC-4wk group (n = 10), respectively, with significant difference ($p < 0.01$). The average optical density value of the positive staining was 0.25 ± 0.07 in VC-4wk group (n = 10) and 0.42 ± 0.05 in Sham-4wk group (n = 10), respectively, with significant difference ($p < 0.01$) (Fig. 2A). The results suggest that HSP70 was widely expressed in rats of the Sham group, and the experimental varicocele reduces the HSP70 expression in spermatogenic cells.

Varicocele increases apoptosis but Ala-Gln decreases apoptosis in testicular spermatogenic cells

TUNEL was performed, and values of the apoptosis index (AI) were analyzed in rat testes. AI in rats in the sham-4 wk group, VC-4wk group (4 weeks after VC surgery), VC-5wk group (Treatment with saline for 1 week in VC-4wk group), and Ala-Gln group (treatment with Ala-Gln for 1 week in VC-4wk group) was 5.10 ± 1.14, 13.22 ± 3.63, 33.62 ± 3.56 and 22.33 ± 2.61, respectively (n = 10/each group) (ANOVA, $p < 0.01$) (Fig. 2B). The data suggest that the experimental varicocele increases, but Ala-Gln treatment decreases apoptosis in testicular spermatogenic cells.

VC reduces, but Ala-Gln enhances HSP70 transcription

HSP70 mRNA levels were measured by RT-qPCR in rats with different treatments (n = 10/each group). The relative level of HSP70 mRNA in rats in the sham-4 wk, VC-4wk, VC-5wk, and Ala-Gln groups (n = 10/each group) was 1.00 ± 0.12, 0.53 ± 0.05, 0.51 ± 0.04, and 1.62 ± 0.15 fold, respectively (ANOVA, $p < 0.01$), suggesting that the experimental varicocele decreases but Ala-Gln treatment increases the HSP70 expression at the transcriptional level (Fig. 3A).

VC reduces, but Ala-Gln enhances the activity of superoxide dismutase (SOD) and catalase (CAT)

The levels of SOD and CAT in the left testes of rats in the sham-4wk, VC-4wk, VC-5wk, and Ala-Gln groups (n = 10/group) were 16.4 ± 0.23 and 2.16 ± 0.31, 13.4 ± 0.17 and

TABLE 1

Weight of testis and bilateral seminiferous tubule injury in rats of VC and sham groups

Group	Post surgery (d)	Weight of testis (g)		*p*	Rate of seminiferous tubule injury (%)		*p*
		Left	Right		Left	Right	
VC-2wk	14	1.77 ± 0.06	1.75 ± 0.09	0.566	28.74 ± 8.85	17.37 ± 5.92	0.0034
Sham-2k	14	1.75 ± 0.03	1.76 ± 0.09	0.7427	3.29 ± 1.01	2.76 ± 1.03	0.2605
p		0.3583	0.8066		0.0001	0.0001	
VC-4wk	28	1.55 ± 0.08	1.71 ± 0.05	0.0001	52.38 ± 11.36	38.12 ± 9.46	0.007
Sham-4wk	28	1.78 ± 0.04	1.75 ± 0.03	0.0739	3.39 ± 1.09	2.81 ± 1.12	0.2559
p		0.0001	0.04		0.0001	0.0001	
VC-8wk	56	1.33 ± 0.07	1.59 ± 0.07	0.0001	87.64 ± 12.77	82.33 ± 10.58	0.3247
Sham-8wk	56	1.75 ± 0.05	1.77 ± 0.04	0.3364	3.41 ± 1.12	2.87 ± 1.08	0.2869
p		0.0001	0.0001		0.0001	0.0001	
P (VC-8wk: VC-4wk)		0.1557	0.222				

Note: n = 10/group.

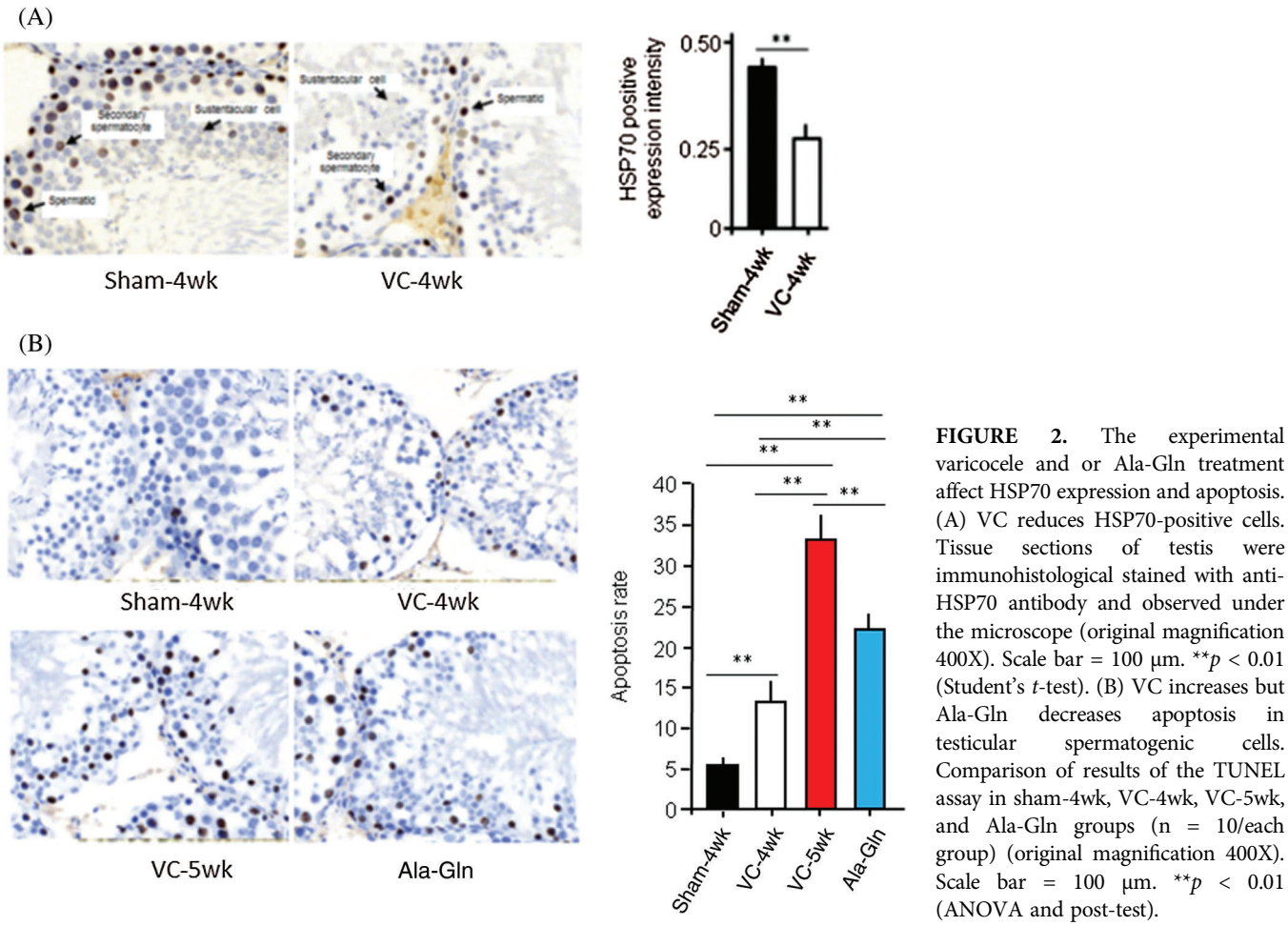

FIGURE 2. The experimental varicocele and or Ala-Gln treatment affect HSP70 expression and apoptosis. (A) VC reduces HSP70-positive cells. Tissue sections of testis were immunohistological stained with anti-HSP70 antibody and observed under the microscope (original magnification 400X). Scale bar = 100 μm. $^{**}p < 0.01$ (Student's *t*-test). (B) VC increases but Ala-Gln decreases apoptosis in testicular spermatogenic cells. Comparison of results of the TUNEL assay in sham-4wk, VC-4wk, VC-5wk, and Ala-Gln groups (n = 10/each group) (original magnification 400X). Scale bar = 100 μm. $^{**}p < 0.01$ (ANOVA and post-test).

1.07 ± 0.28, 10.01 ± 1.06 and 1.31 ± 0.26, 19.53 ± 2.26 and 3.46 ± 0.71 U/mg protein, respectively (ANOVA, $p < 0.01$) (Figs. 3B, C), suggesting that the Ala-Gln administration increases the activity of anti-oxidative stress enzymes.

VC increases, but Ala-Gln decreases 8-OHDG concentration in testes

The level of 8-OHDG in the left testes of rats in the sham-4wk, VC-4wk, VC-5wk and Ala-Gln group (n = 10/group) was 5.23 ± 0.67, 6.81 ± 0.78, 7.16 ± 1.22 and 4.14 ± 0.73 pg/μg DNA, respectively (ANOVA, $p < 0.01$) (Fig. 3D), suggesting that the Ala-Gln administration decreases the concentration of 8-OHDG in testes of rata after VC surgery.

Discussion

In the present study, we have tested the hypothesis that Ala-Gln enhances the expression of HSP70 and the concentration of antioxidants but reduces the concentration of oxidants and apoptosis in the varicocele (VC)-bearing rats. The VC surgery in rats causes a decrease in the weight of testes and the number of seminiferous tubules and spermatogenic cells, the injury of the bilateral seminiferous tubule, and the block of the spermatogenic process with interstitial edema. Varicocele reduces the HSP70 expression at the transcriptional level in spermatogenic cells and induces apoptosis in testicular spermatogenic cells. Importantly, we have administrated Ala-Gln in the VC-bearing rats, which increases the levels of HSP70 and antioxidants, SOD and CAT, as well as reduces oxidant 8-OHDG and apoptosis in spermatogenic cells. To our knowledge, this study examined, for the first time, the ability of Ala-Gln in the protection against testicular injuries caused by varicocele.

As a major cause of man infertility, varicocele impairs spermatogenesis accompanied by inducing a higher level of oxidative stress (Hendin *et al.*, 1999; Kao *et al.*, 2008). SOD and CAT provide an important defense against the toxicity of the superoxide radical (Chelikani *et al.*, 2004; Kheradmand *et al.*, 2009). The oxidative stress damages the sperm DNA, resulting in male infertility. 8-OHDG is a sensitive marker of the oxidative DNA damage caused by reactive oxygen species (ROS) in sperm and testicular tissue (Sakamoto *et al.*, 2008; Shen *et al.*, 1999; Tugcu *et al.*, 2010). Varicocele causes apoptosis in spermatogenic cells via the mitochondrial signal transduction pathway (Lee *et al.*, 2009; Ning *et al.*, 2017). Hsp70 is an apoptosis inhibitor and is constitutively expressed in spermatogenic cells during spermatogenesis (Dix *et al.*, 1996; Huang *et al.*, 2005). The reduction of HSP70 expression is also a biomarker of apoptosis in varicocele, resulting in male infertility (Purandhar *et al.*, 2014).

It is necessary to find a non-surgical method to improve varicocele-induced testicular injury (Johnson and Sandlow,

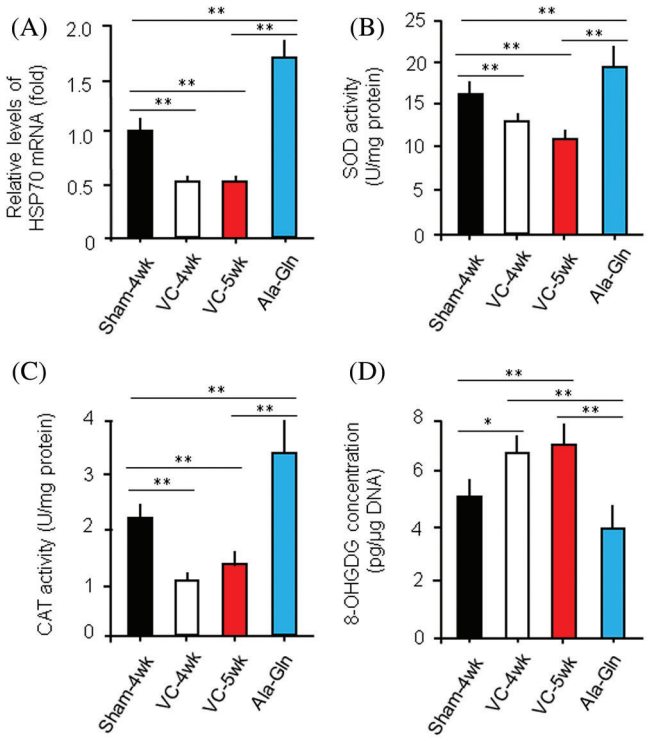

FIGURE 3. Ala-Gln improves the experimental varicocele (VC)-induced testis injury.
The relative HSP70 mRNA level (A), SOD activity (B), CAT activity (C), and 8-OHDG concentration (D) in sham-4wk, VC-4wk, VC-5wk, and Ala-Gln groups (n = 10) were compared. *$p < 0.05$; **$p < 0.01$ (ANOVA and post-test).

2017). Administration of Ala-Gln before torsion/detorsion of the spermatic cord decreases lipid peroxidation during ischemia and protects the testis from oxidative stress by upregulating glutathione (GSH) levels in during reperfusion (Leitao *et al.*, 2011). We have found the effects of Ala-Gln on the treatment of varicocele in a rat model, including the increase in the activity of anti-oxidants SOD and CAT, the decrease in the concentration of oxidant 8-OHDG, the enhancement of anti-apoptotic HSP70 expression, and the inhibition of apoptosis in spermatogenic cells. Our results suggest that Ala-Gln might be valuable for further study in the treatment of varicocele.

Conclusion

We have established a rat varicocele model and administrated Ala-Gln daily for 1 week after varicocele. We have found that Ala-Gln increases the activity of antioxidants SOD and CAT, decreases the concentration of oxidant 8-OHDG, enhances HSP70 expression, and inhibits apoptosis in spermatogenic cells. Therefore, Ala-Gln might be valuable for further study in varicocele treatment.

Availability of Data and Materials Statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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