GSH intervented spermatogenesis by oxygen free radicals- mitochondrial signaling pathway

Wei-Dong Zhang[1], Teng Fu[2], Zhan Zhang[1*], Li-Ting Jia[1], Lin-Lin Zhang[1], Hui-Zhen Zhang[1]

[1]The Third Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, China
[2]The New School, 66 West 12th Street, New York, NY 10011, USA
[*]Corresponding author. zhangzhan27@gmail.com

Abstract: **Objective:** Through establishing sperm oxygen radical damage model to study GSH interventing spermatogenesis by oxygen free radicals- mitochondrial pathway. **Methods:** Collecting normal semen by the packet, adding hypoxanthine, xanthine oxidase system to construct semen oxidative damage model, adding different concentrations of GSH simultaneously, after 2h, 1h and 12h incubation, the content of MDA and activity of T-SOD and GSH-Px were determined by enzymatic assays. The CASA system determined sperm motility parameters: sperm motility (%), fast forward movement, VCL, VSL, VAP, and ALH. The mRNA expression levels of apoptosis-related genes (Bcl-2, Bax, CytC, and Caspase-3) were measured by Real-time PCR. **Results:** 1. Compared with group a (control group), MDA content increased but T-SOD and GSH-PX activity and sperm motion parameters decreased in group b (plus oxidase reaction system) and group c (plus oxidase reaction system and a low concentration of GSH), The difference was significant statistically (all $P<0.05$). Compared with the group b, MDA levels were lower but T-SOD and GSH-PX activity and sperm motion parameters increased in group c and group d (plus oxidase reaction system and a high concentration of GSH), and the difference was significant statistically (all $P<0.05$). 2. Compared with group a, sperm Bcl-2 expression was reduced but Bax, CytC and Caspase-3 expression were increased in group b, and group c, and the difference was significant statistically ($P<0.01$, $P<0.05$, respectively). Compared with group b, sperm relative expression of Bax, CytC and Caspase-3 in group d were lower, the difference was significant statistically ($P<0.05$). **Conclusion:** ROS and GSH were responsible for sperm´s damage and protection respectively, in particular, reflecting the changes in motion parameters. GSH affected on ROS directly and blocked mitochondrial signaling pathway, and showed certain dose-effect relationship.
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1. Introduction

Molecular studies have demonstrated that the mitochondrial signaling pathway of apoptosis play a key part in the pathogenesis of male infertility[1]. Intriguingly, it has been reported that 25% to 40% of men diagnosed with semen disorders can be detected high levels of ROS (reactive oxygen species, also known as oxygen free radicals)[2]. The crosstalk between various apoptosis-inducing factors and ROS is an important component of mitochondria-dependent pathway of apoptosis. The pathogenic potential of stimulated ROS has also been involved in triggering of the mitochondrial apoptotic pathway and shown in some disease conditions (e.g. myocardial ischemia and traumatic brain injury) [3,4]. Therefore, we assume that ROS may start this pathway directly through abnormal expression of Bcl-2/Bax, the switch of this pathway, leading to the occurrence of infertility. If thus, by interventing ROS, it is possible to prevent the incidence of infertility through the mitochondrial pathway.

Since reduced glutathione (GSH) has a unique configuration, and therefore has a special antioxidant[5]. Although GSH was used in the clinical of Oligospermia and Asthenospermia[6], but what was the drug's targets and whether it worked through mitochondrial signaling pathway? Has not been reported.

Objective of this study was through establishing sperm oxygen radical damage model to study GSH interventing spermatogenesis by oxygen free radicals-mitochondrial pathway. Collecting normal semen by the packet, adding hypoxanthine, xanthine oxidase system to construct semen oxidative damage model, adding different concentrations of GSH simultaneously, after 2h, 1h and 12h incubation, the content of MDA and activity of T-SOD and GSH-Px were determined by enzymatic assays. Sperm motility parameters were determined by the CASA system including sperm motility(%), fast forward movement, VCL, VSL, VAP, and ALH. The mRNA expression levels of apoptosis-related genes (Bcl-2, Bax, CytC, and Caspase-3) were measured by Real-time PCR, so as to further explore the direct evidence of oxygen free radicals starting the mitochondrial pathway, and mechanism GSH interventing spermatogenesis through it.

2. Materials and Methods

2.1 Collection of ejaculate specimens

Adult males between the ages of 22 and 39 were recruited from the Department of Andrology in the Third Affiliated Hospital of Zhengzhou University (Henan, China) for study participation. A total of 30 participants were selected according to a previous diagnosis of normal sperm using the sperm count ($\geq 15\times10^6$ sperm/mL) and fast progressive motility ($\geq$32%) findings from standard semen analysis[7,8]. The infertility issue was attributed to a factor related to the female counterpart.

All donors provided written informed consent and the study was approved by the hospital's local ethics committee.

2.2 Isolation and grouping of seminal plasma and sperm

The liquefied semen samples from each participant were subjected to Percoll discontinuous gradient (40% and 80%) centrifugation (800 ×g, 20 min). Collected the seminal plasma (upper layer) 2ml to divide into four specimens (a, b, c, d) for MDA, T-SOD, GSH-Px assay. (Seminal groups were shown in Table 1). The accumulated sperm (bottom layer) were washed three times with phosphate-buffered saline (PBS; pH 7.4, 0.01 mol/L) and resuspended in fresh PBS 2ml to a final concentration of 60×10^6/mL, than, divided into four specimens (a, b, c, d). (Sperm suspension groups were shown in Table 2).

The seminal plasma of group a, group b, group c, and group d were incubated in a water bath box for 2h, for MDA, T-SOD and GSH-PX test. After the sperm suspension of group a, group b, group c, and group d were incubated in a water bath box for 1h, 5pl of them were used for sperm motion parameters test. The remainder were incubated 12h, which intended for Real-time PCR experiments. After centrifugated, washed, all prepared sperm samples were stored at -80ºC for extraction of total RNA.

2.3 Assay of MDA content and antioxidant enzymes' activity

The MDA test kit, the T-SOD test kit, and the GSH-Px test kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) were used to assess MDA content (based on the thiobarbituric method) and the activity of T-SOD (based on the xanthine oxidase colorimetric method) and GSH-Px (based on the enzymatic reaction method) , respectively.

2.4 The CASA system determined sperm motility parameters

It was the computer-assisted semen analysis (CASA) system that determined sperm motility parameters: sperm motility (%), fast forward movement, curve speed (VCL), linear velocity (VSL), average path velocity (VAP), and amplitude of lateral head displacement (ALH).

2.5 Quantitative assessment of apoptosis-related genes' expression

Total RNA from the sperm suspension was extracted by using the UltraPure RNA Kit from Cwbio Biotech Co. (Beijing, China) and quantified at 260 nm wavelength by UV spectrophotometry (Thermo, Wilmington, DE, USA). Reverse transcription was carried out using the First Strand cDNA Synthesis Kit from Dingguo (Beijing, China) and applied as template in quantitative Real-time PCR using the ABI Prism 9700 StepOneTM Real-Time PCR System (ABI, CA, USA). All primers were synthesized by the Sangon Biotech Co. (Shanghai, China); the primers used for gene-specific amplification were listed in Table 3. The PCR condition was as follows: one cycle of 95ºC for 10 min; followed by 40 cycles of 95ºC for 15 sec and 60ºC for 1 min. The $2^{-\triangle\triangle CT}$ method was used to calculate the transcripts' expression levels relative to GAPDH (normalization control).

2.6 Statistical analysis

Statistical analyses were performed with the SPSS 17.0 software program (SPSS, Chicago, IL, USA). Data were expressed as mean ± SD and inter-group differences were evaluated by Student's t-test. A value of P <0.05 was reported as statistically significant.

3. Results

3.1 Determination of the oxidation reaction of incubating seminal fluid in each group

Compared with group a, MDA content increased in group b, group c, and group d, and the difference was significant statistically (P<0.01, P<0.05, P<0.05, respectively). Compared with the group b, MDA levels were lower in group c and group d, and the difference was significant statistically (all P<0.05) (Figure 1).

3.2 Determination of the antioxidant enzymes of incubating seminal fluid in each group

Compared with group a, T-SOD and GSH-PX activity decreased in group b, and group c, and the difference was significant statistically (P<0.01, P<0.05, respectively); T-SOD and GSH-PX activity was reduced in group d, but there was no significant difference (P>0.05). Compared with group b, T-SOD and GSH-PX activity increased in group c, group d, the difference was significant statistically (P<0.05, P<0.01, respectively) (Figure 2,3).

3.3 Determination of sperm motility parameters

Compared with group a, sperm motion parameters were reduced in group b, and group c, and the difference was significant statistically (P<0.01); sperm motion parameters were reduced in group d, but there was no significant difference (P>0.05). Compared with group b, sperm motion parameters were increased in group c, and group d, and the difference was significant statistically (P<0.05) (Table.

4).

3.4 Determination of the relative expression of Bcl-2, Bax, CytC, Caspase-3 genes in each group by Real-time PCR

Compared with group a, sperm Bcl-2 expression was reduced in group b, and group c, and the difference was significant statistically (P<0.01, P<0.05, respectively); relative expression of sperm Bcl-2 was reduced in group d, but there was no significant difference (P>0.05).

Compared with group a, relative expression of sperm Bax, CytC and Caspase-3 were increased in group b, and group c, and the difference was significant statistically (P<0.01, P<0.05, respectively); relative expression levels of Bax, CytC and Caspase-3 in group d were increased, but there was no significant difference (P>0.05). Compared with group b, sperm relative expression of Bax, CytC and Caspase-3 in group d were lower, the difference was significant statistically (P <0.05). (Figure 4)

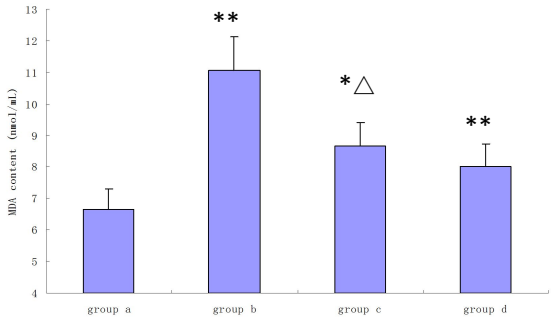

Figure 1. MDA content in seminal plasma from each group: Compared with group a, $^{*}P$<0.05, $^{**}P$<0.01; Compared with group b, $^{\triangle}P$<0.05.

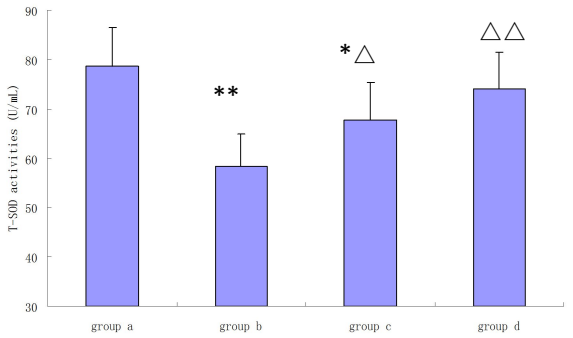

Figure 2. T-SOD activities in seminal plasma from each group: Compared with group a, $^{*}P$<0.05, $^{**}P$<0.01; Compared with group b, $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01.

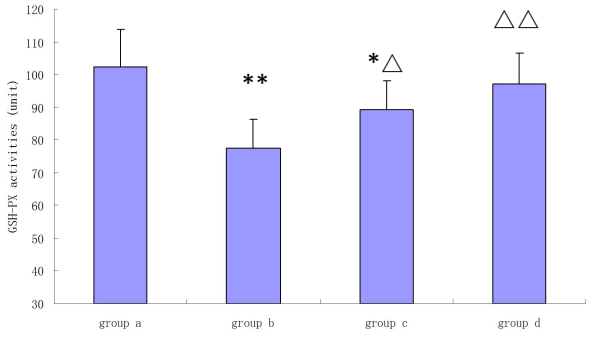

Figure 3. GSH-PX activities in seminal plasma from each group: Compared with group a, $^{*}P$<0.05, $^{**}P$<0.01; Compared with group b, $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01.

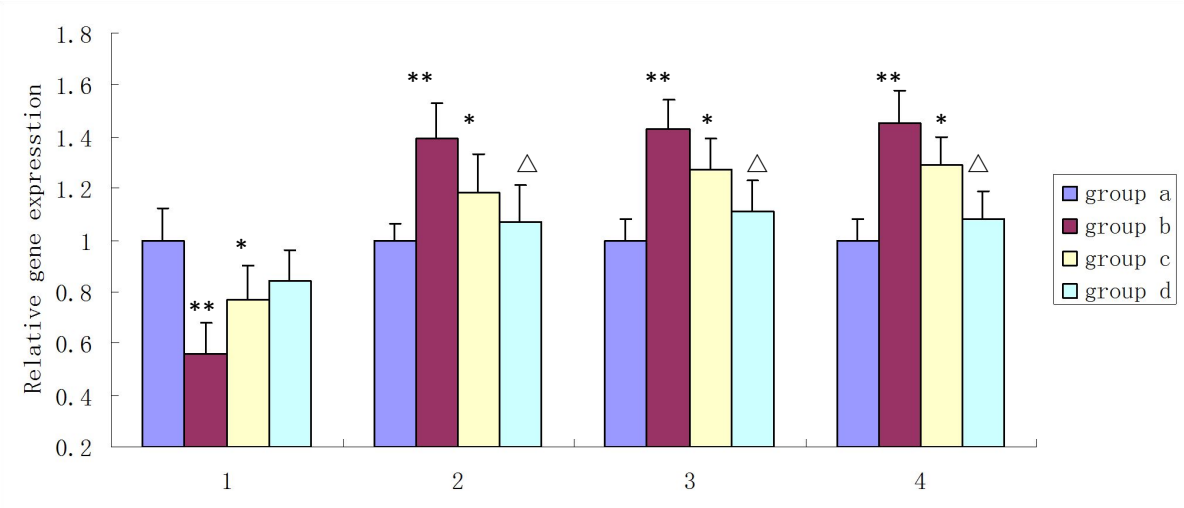

Figure 4. Relative gene expresstion of (1)Bcl-2, (2)Bax, (3)CytC and (4)Caspase-3: Compared with group a, $^{*}P$<0.05, $^{**}P$<0.01; Compared with group b, $^{\triangle}P$<0.05.

Table 1. Seminal groups situation

group	composition
a (control group)	seminal plasma
b	seminal + hypoxanthine (final concentration of 1mmol / L) + xanthine oxidase (final concentration 50mU/ml)
c	seminal + hypoxanthine (final concentration of 1mmol / L) + xanthine oxidase (final concentration of 50mU/ml) + GSH (final concentration of 0.75mmol / L)
d	seminal + hypoxanthine (final concentration of 1mmol / L) + xanthine oxidase (final concentration of 50mU/ml) + GSH (final concentration of 1.0mmol / L)

Table 2. Sperm suspension groups situation

group	composition
a (control group)	Sperm suspension
b	Sperm suspension+ hypoxanthine (final concentration of 1mmol / L) + xanthine oxidase (final concentration 50mU/ml)
c	Sperm suspension + hypoxanthine (final concentration of 1mmol / L) + xanthine oxidase (final concentration of 50mU/ml) + GSH (final concentration of 0.75mmol / L)
d	Sperm suspension + hypoxanthine (final concentration of 1mmol / L) + xanthine oxidase (final concentration of 50mU/ml) + GSH (final concentration of 1.0mmol / L)

Table 3. Gene-specific primers used in this study

Gene	GenBank ID	Primer sequence, 5′ → 3′		Fragment Length
		Forward	Reverse	
Bcl-2	NM_000633.2	GTGGATGACTGAGTACCTGAACC	AGACAGCCAGGAGAAATCAAAC	182bp
Bax	NM_004324.3	TTTTGCTTCAGGGTTTCAT	ACACTCGCTCAGCTTCTTG	178 bp
CytC	NM_006003.2	GCCTCAATGTCCCTGCTTCT	AGCACTCATGCTGGAAACGA	221 bp
Caspase-3	NM_004346.3	ATCACAGCAAAAGGAGCAGTTT	ACACCACTGTCTGTCTCAATGC	214 bp
GAPDH	NM_002046.3	TCGTGGAAGGACTCATGACC	AGGGATGATGTTCTGGAGAG	226 bp

Table 4. Sperm motility parameters from each group ($\bar{x} \pm s$)

group	N	sperm motility(%)	fast forward movement(%)	VCL	VSL	VAP	ALH
a	30	38.3±4.1	38.3±4.1	32.1±3.3	14.7±1.5	21.2±2.1	2.0±0.3
b	30	27.5±3.5[2)]	27.5±3.5[2)]	23.3±2.2[2)]	12.1±1.3[2)]	17.5±1.7[2)]	1.7±0.2[2)]
c	30	31.5±3.6[2), 3)]	31.5±3.6[2), 3)]	27.3±3.6[1), 3)]	13.0±1.4[2), 3)]	19.0±2.2[1)]	1.8±0.2[1), 3)]
d	30	35.4±3.1[4), 5)]	35.4±3.1[4), 5)]	30.3±3.0[4), 5)]	13.9±1.5[4), 5)]	20.2±2.1[4)]	1.9±0.2[4)]

Compared with group a, [1)]P<0.01, [2)]P<0.001; Compared with group b, [3)]P<0.05, [4)]P<0.01; Compared with group c, [5)]P<0.05.

4. Discussion

Mitochondrial Signal pathway is an important apoptosis pathway，in which, the Bcl-2 protein family plays a critical role. Among this protein family, Bcl-2 inhibits apoptosis, while others, like Bax, promotes it. Anti-apototic and pro-apototic proteins seem to be switched to apoptosis. After receiving apoptotic activation signal, the Bax oligomerizates and insertes into mitochondrial membrane, leading mitochondrial membrane permeability to change, and cause CytC to release. The CytC integrates with another important activating factor (Apaf-1) to recruit Caspase-9 precursor starting caspase cascade, activating caspase-3, which locates in the downstream of pathway. Therefore, Apoptosis has been induced finally[9,10].

In the male reproductive tract, excessive ROS can cause damage to sperm. Its mechanism of action is as follows: ①sperm membrane lipid peroxidation[11]; ② impacts on the ability of sperm motility[12]; ③ impacts on sperm DNA[13]. In addition to the direct damage to sperm, whether can increased ROS start mitochondrial signaling pathway of apoptosis?

Because reduced glutathione (GSH) has a unique configuration, it has a special anti-oxidation[14,15,16]. After Lenzi A, etc. conducted antioxidant therapy to male infertility with GSH for 4 weeks, sperm motility had been improved significantly[17]. But what is the molecular mechanism of drug action, especially, whether is it by any signaling pathways ? Unclear.

In this study, adding hypoxanthine, xanthine oxidase reaction system to construct semen oxidative damage model, adding different concentrations of GSH simultaneously, after incubation, MDA content in each group was assayed by TBA method (thiobarbituric method). The results of this study showed that hypoxanthine, xanthine reaction system produced high levels of MDA concentration in semen, accompanied by reduced activities of antioxidant enzymes included T-SOD and GSH-PX, which confirmed directly injury of semen by ROS. After incubation by GSH, MDA levels reduced, and high concentrations of GSH were stronger than low concentrations of GSH. It showed that the protection of GSH for semen presented a certain dose-effect relationship. As for increased activities of T-SOD and GSH-PX after GSH incubatied, and high concentrations of GSH were stronger than low concentrations of GSH, even closed to the control group, which described a synergy between GSH and antioxidant enzymes.

This study showed that the sperm motion parameters (including total sperm vitality, fast forward movement, VCL, VSL, VAP, ALH) decreased after the role of hypoxanthine, xanthine reaction system. After incubation by GSH, the sperm motion parameters increased, and high concentrations of GSH were stronger than low concentrations of GSH, even closed the control group in total sperm vitality. It confirmed the impact of excessive ROS on sperm motility, especially on total sperm vitality, fast forward movement, and VSL. Conversely, The GSH confrontated and removed ROS in semen, and presented a certain dose-effect relationship, which consistented with the research of Coyan K[18].

This study also showed that the sperm Bcl-2 relative expression decreased, but Bax relative expression increased after the role of hypoxanthine, xanthine reaction system. After incubation by GSH, the sperm relative expression of Bcl-2 and Bax were opposited with the former, and high concentrations of GSH were stronger than low concentrations of GSH. After effected by high concentrations of GSH, Bcl-2 and Bax sperm relative expression levels were closed to the control group. It showed excessive ROS in semen activated mitochondrial signaling pathways, but GSH affected on ROS directly and blocked this pathway. Subsequent changes of CytC, Caspase-3 was the reflection of ROS and different concentrations of GSH effected on mitochondrial signaling pathway.

In short, with deeper study of GSH and ROS effected on male infertility, it is possible that provide a new therapeutic approach for the treatment of male infertility in the future.

Corresponding Author:
Zhan Zhang
The Third Affiliated Hospital of Zhengzhou University Zhengzhou University
Zhengzhou, Henan 450052, China
E-mail: zhangzhan27@gmail.com

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