



# Association of Zinc deficiency, oxidative stress and increased double-stranded DNA breaks in globozoospermic infertile patients and its implication for the assisted reproductive technique

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**Background:** Sperm DNA fragmentation and its adverse impact on outcomes of assisted reproductive techniques (ART) in globozoospermic infertile patients has been previously reported. However, the association of Zinc element with DNA damage and intracytoplasmic sperm injection (ICSI) outcome in globozoospermic infertile patients remains unclear.

**Methods:** Using flame atomic absorption spectrophotometer and superoxide dismutase (SOD) assay, the levels of Cu, Fe, Mn, Zn and SOD activities in seminal plasma from both globozoospermic infertile patients and fertile volunteers were tested respectively. Using sperm chromatin dispersion (SCD) test and Comet assay, the DNA damages in their semen samples from the two groups was detected. In addition, using Aniline Blue staining, their sperm nucleus maturations were also examined.

**Results:** The levels of seminal Zinc and SOD activities were lower in the globozoospermic infertile patients and the double-stranded break DFI (DSB-DFI) were significantly higher than that in the fertile controls. Antioxidative insufficiency of SOD with a low Zn level might be responsible for oxidative stress, which may lead to DNA damage in globozoospermic spermatozoa. Zn deficiency might also have influence on the chromatin stabilization of globozoospermic spermatozoa during spermiogenesis, causing its more vulnerable to oxidative attack.

**Conclusions:** Serious DSBs in globozoospermia and antioxidative insufficiency due to Zinc element deficiency in spermatozoa might be responsible for the failure of ICSI in globozoospermia.

**Keywords:** Globozoospermia; Zinc; oxidative stress; sperm DNA damage; intracytoplasmic sperm injection (ICSI)

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## Introduction

Globozoospermia, primarily characterized by the presence of round-headed spermatozoa with no acrosome, is a severe form of teratozoospermia in male infertility. As a result of deficiency in acrosome, globozoospermic sperm cannot bind and penetrate zona pellucida, resulting in failure of fertilizing an oocyte and then cause sterility (1,2). Failure on *in vitro* fertilizations were also thought to be related to increased DNA damage in globozoospermic spermatozoa (3,4). Although successful pregnancies have been achieved with globozoospermic spermatozoa using intracytoplasmic sperm injection (ICSI) along with artificial oocyte activation (AOA) (5-7), uncertainty of clinical outcome remains for most cases. Several studies have demonstrated sperm DNA fragmentation and its adverse impact on outcomes of assisted reproductive techniques (ART) in individuals affected by globozoospermia (4,8,9). Therefore, it is imperative to further investigate and monitor the sperm DNA damage levels in these patients.

Sperm DNA damage could be affected by several factors and reactive oxygen species (ROS) production remains one of the most important factors to determine the DNA damage levels in fertilizing sperm (10,11). Sperm are particularly sensitive to ROS due to high proportion of polyunsaturated fatty acids in their membranes, limited ability to repair DNA and intrinsic deficiencies in intracellular antioxidant enzymes (12,13). Fortunately, rich antioxidants in natural seminal plasma can scavenge excess ROS to protect spermatozoa from oxidative injuries, especially after leaving the testis (14). Among these antioxidants, Zinc plays significant roles in the antioxidant networks (15). Zinc ion not only has antioxidative effect for sperm survival and function (16,17) but also can regulate the sperm nuclear chromatin structures (18,19). Moreover, extracellular Zinc ions incorporated into seminal fluid during ejaculation were found to regulate sperm motility (20), sperm morphology (17), membrane stabilization (21) and antioxidant activity for lipid peroxidation (22). In fact, Zinc ion is involved in the whole aspects of sperm structure and function from spermatogenesis, maturation, capacitation, fertilization to embryo development (23). Given the antioxidative roles of Zinc, its deficiency in seminal plasma can lead to oxidative damage induced by ROS. Indeed, Zinc deficiency was reported to result in arrest of spermatogenesis at the round

and elongated spermatid stages (24). Reduced level of Zinc ion in seminal plasma was also found in azoospermic, oligozoospermic and asthenozoospermic individuals (25). Apart from Zinc ion, other metal ions such as Fe, Mn and Cu were also reported about their functions as prosthetic groups of SOD activity (26). For example, it was reported that Fe overload likely cause an increase in sperm DNA damage (27) and the level of Cu and Mn elements displayed correlation with DNA fragmentation (28). However, whether the level of Zinc ion is associated with sperm DNA damage as well as IVF outcome in globozoospermic infertile patients remain unclear.

Therefore, in this study we investigated the association of Zinc ion dysregulation, oxidative stress, and increased DNA damage and its clinical applications for the globozoospermia patients. Using semen samples from fertile volunteers and globozoospermic individuals who went routine infertile ART, we measured the level of Zn, Fe, Mn and Cu ions in globozoospermic semen using the flame atomic absorption spectrophotometer. We then tested their SOD activities using the superoxide dismutase (SOD) assay. Their DNA fragmentation level and type of the DNA damage were further investigated through sperm chromatin dispersion (SCD) test, Comet assay, and Aniline Blue staining. We hope this study would help to understand the relationship between serious DSBs and antioxidative insufficiency due to Zinc element deficiency in spermatozoa and provide useful information on future IVF treatment for globozoospermic infertile patients. We present the following article in accordance with the MDAR reporting checklist (available at: <http://dx.doi.org/10.21037/tau-20-1116>).

## Methods

### *Subjects and sample collection*

Semen samples were collected from healthy fertile volunteers (n=12) and globozoospermic patients (n=5) who underwent the routine infertile ART outcomes evaluation. The average age of the globozoospermic patients was about 29.2±4.6 years. All samples were obtained by masturbation after 2–5 days of sexual abstinence. The seminal samples were collected and evaluated according to WHO guidelines (29). The study was conducted in accordance with the Declaration of Helsinki

(as revised in 2013). The study was approved by the Ethical Review Board of West China Second University Hospital, Sichuan University (K2018089). Informed consent was obtained from each subject otherwise the specimens would have been discarded.

### *Morphologic observation on sperm*

#### **Papanicolaou staining**

Papanicolaou staining were proceeded according to the instruction of the Papanicolaou staining kit (PUHUA, Chengdu, China). Ten  $\mu\text{L}$  of semen was smeared onto a glass slide and placed horizontally and air dried. Fixed sufficiently in 95% alcohol, the coated slides were dipped orderly into the alcohol of 80%, 50% and distilled water. After immersion in the Hematoxylin solution and infiltration with acidic ethanol, the slides were submerged in Orange G dye following the dehydration in 50%, 80% and 95% alcohol. When redundant Orange G dyes was removed by continue dissolution in 95% alcohol, the slides were stained in EA-50 staining solution. Finally, the stained slides underwent dehydration in 95% and absolute alcohol in turn. At least 200 spermatozoa were scored per sample during morphometric analysis.

#### **Electron microscope observation**

Firstly, semen samples were rinsed in Sperm Washing Medium. Secondly, the semen samples were fixed with 3% glutaraldehyde, post-fixed by 1%  $\text{OsO}_4$  fixative solution, dehydrated in increasing gradient acetone, and then embedded in Epon 812. Before ultrathin sperm-sectioning, a half-thin section was made to ensure that sperm was located under a light microscope. The ultrathin sections were stained with lead citrate-uranyl acetate and then photographed using a transmission electron microscopy (TEM) (TECNAI G2 F20, Philips) under an accelerating voltage of 80 kV.

### *Determination of metal elements and SOD activity in seminal plasma*

#### **Determination of metal elements**

0.5 mL of thawed seminal plasma was mixed with 0.5 mL of 30%  $\text{H}_2\text{O}_2$ . The mixtures were shaken for 30 min, added up to 5 mL with 5%  $\text{HNO}_3$  solution, and mixed thoroughly. Then, the level of Mn, Cu, Zn and Fe elements were determined by using flame atomic absorption spectrophotometer (PerkinElmer PE900T; Waltham,

Massachusetts, USA) according to routine protocol. Certified standards were used as references.

#### **SOD assay**

SOD activity was measured in all samples from fertile volunteers and globozoospermic infertile patients. The seminal plasma was isolated from semen by centrifugation at 2000 rpm for 15 min. SOD activity in the seminal plasma was measured by inhibiting nitrite (NIT) reduction due to the superoxide anion which was generated by the combination of xanthine and xanthine oxidase using a SOD Assay Kit (No. A001-3, Jiancheng bioengineering institute, Nanjing, China). Each of samples were conducted three times as technical replicates. In this reaction system, SOD active unit (U) referred to the enzymatic amount that caused a 50% inhibition for the rate of NIT reduction, and the SOD activity was defined as U/mL seminal plasma.

### *Detection of DNA fragmentation in semen samples*

#### **SCD test**

An improved SCD test were implemented following the instructions of the SCD kit supplier (PUHUA, China). Briefly, semen samples were diluted to 10 million/mL in PBS, and 60  $\mu\text{L}$  was mixed fully with 120  $\mu\text{L}$  of 1% LMP agarose (type VII; Sigma, St Louis, USA). Twenty  $\mu\text{L}$  of the semen-agarose mixtures were pipetted onto pre-coated slides and covered with coverslips in the dark until solidification at 4 °C. After removal of the coverslip and incubation in HCl solution, the slides were submerged in lysing solution and then washed in distilled water. After dehydrated in 50%, 70% and 95% ethanol, the slides were stained with Wright's solution and washed briefly in tap water and then allowed to dry. Stained sperm cells were visualized using a bright-field microscopy (Olympus, CX31RBSF; Tokyo, Japan) and their image information was collected. Digital images were analyzed using a routine designed with Visilog 7.0 software (Noesis, France). The frequency of sperm cells with fragmented nuclear DNA, i.e., the DNA fragmentation index (DFI), was calculated by counting at least 200 spermatozoa per slide.

#### **Comet assay**

Comet assay test was performed according to a previous protocol (30). Same as in the SCD test, after the gel solidified, coverslips were removed and the slides were submerged in lysing solution 1 for 60 min, followed by lysing solution 2 for 60 min, then washed in TBE buffer.

For the neutral Comet assay, slides were subjected to electrophoresis in freshly-made 1×TBE electrophoresis buffer (pH8.5) at 20 V (1 V/cm) for 12.5 min; For the alkaline Comet assay, slides were incubated in an alkaline denaturing solution for 2.5 min, and then electrophoresed in 0.03M NaOH at 20 V (1 V/cm) for 4 min. Both neutral and alkaline slides were rinsed once in the neutralizing solution, thereafter, washed briefly in TBE buffer. Slides finally underwent dehydration in gradient ethanol and air dried. Nucleoid DNA in Comets was stained with SYBR Green I (Gibco, S7585; Grand Island, NY, USA) and visualized fluorescent images were obtained using fluorescence microscope (Nikon 80I, Japan), and stored as TIFF files format with Spot imaging software (Version4.6, Diagnostic instruments Inc., USA). Comet images were analyzed using the CASP software and the thresholds including Head center threshold, Comet threshold, Tail threshold and Head threshold were set as 0.8, 0.05, 0.04 and 0.05, respectively. At least 400 spermatozoa comets from different fields of view on several slides were counted for each sample. The parameter of the tail moment was chosen as the standard of assessment DNA damage and the value of 0.1 in the tail moment was set as the threshold of sperm with DNA damage in our study. Such threshold values were set by analyzing the mean, median and range of the tail moment values for each assay, and consistent with real ART outcome in the globozoospermic infertile patients and fertile volunteers.

#### ***Aniline Blue staining of nuclear maturity of sperm***

Aniline Blue staining was carried out according to the instructions on Aniline Blue Staining Kit (PUHUA, Chengdu, China). After sperm was washed with 0.9% NaCl, 5 µL of the washed samples were spread onto slides and allowed to dry in the air. The smears were fixed in Fast Green solution for 5 min and subsequently stained with Aniline Blue mixture with acetic acid. After redundant Aniline Blue was removed, the slides were submerged into acid ethanol followed by redyeing with Eosin solution. At least 200 sperm per slide were evaluated and the percentage of sperm with dark blue/blue-purple head was calculated as percentage of immature sperm in nucleus. Stained spermatozoa were evaluated using light microscopy with oil immersion.

#### ***Statistical analysis***

Statistical analyses were performed using the Statistics

Package for the Social Sciences software (SPSS, version 26; IBM Crop, Armonk, NY, USA). Ion content, SOD activity and DFI values in SCD and Comet assay as well as sperm maturation were respectively compared between infertile and normal groups using a non-parametric Mann-Whitney U-test. Unless proposed separately all values were given as mean ± standard deviation. Values of P<0.05 were considered statistically significant and P<0.01 as extremely significant. The relationships between SOD activity and Zn level in seminal plasma were examined by Pearson correlation analysis.

## **Results**

### ***Clinic characteristics and routine infertile ART outcome evaluations of globozoospermic infertile patients***

The clinical data of patients were presented in *Table 1*. Among 5 patients, only Patient No. 2 had one good embryo transferred and achieved clinical pregnancy. Patient No. 3 was Type I globozoospermic patient and had one embryo transferred but failed clinical pregnancy. All other patients didn't receive IVF/ICSI treatment due to personal economic reasons. Their sperm concentration and motility parameters were variable, indicating spermatogenesis and sperm motility were both affected. All the controls were fertile and presented normal sperm parameters according to WHO criteria (29).

### ***Morphological observation on sperm***

Morphologic analysis of semen samples was carried out using Papanicolaou staining to verify the type of globozoospermic sperm. Based on WHO criteria, the stain results showed round-headed and irregular features in sperm of the infertile patients under light microscopy (*Figure 1*). Further results from electron microscopic clearly displayed the major characteristics of typical globozoospermia, such as round sperm head, the lack of an acrosome and residual cytoplasmic droplets surrounding the nucleus or the midpiece. Also, the chromatin rarefactions distribution was found inside of the nucleus (*Figure 2*).

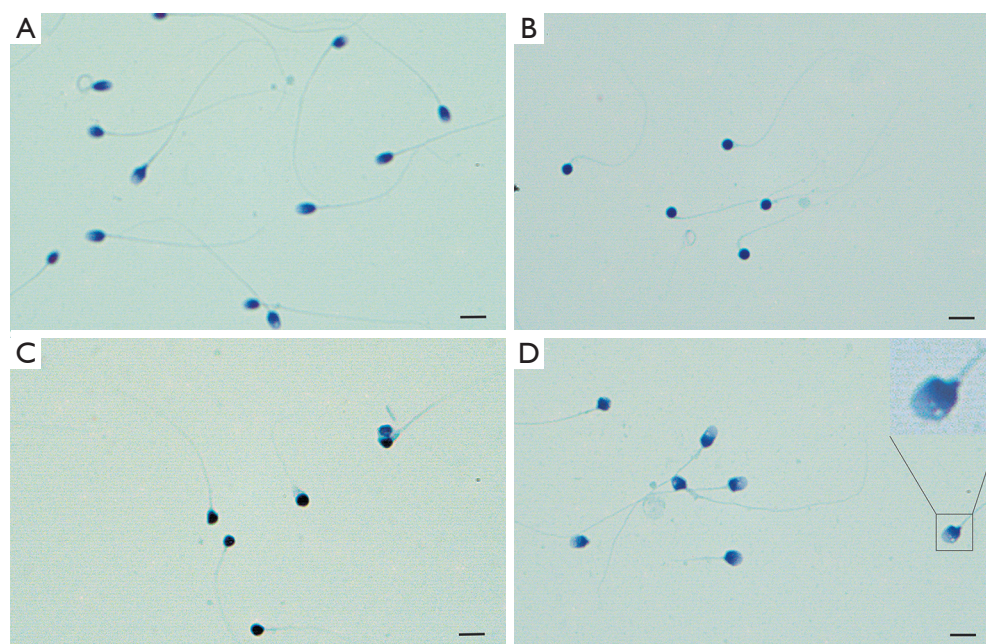
### ***Determination of metal elements and SOD activity***

The levels of Cu, Fe, Mn and Zn ion in seminal plasma from both the fertile volunteers (the control group) and the globozoospermic infertile patients (the globozoospermic group) were tested using flame atomic absorption

**Table 1** The related information on clinical outcomes of the globozoospermic patients involved in the study

Patients	Globozoospermia type	Consanguinity	Fertility in family members	Concentration of sperm (mL <sup>-1</sup> )	ICSI result
1	I	No	1 infertile sister, 1 fertile sister	14.7 million	No ICSI
2	II	No	2 brothers and 1 sister, all fertile	2.3 million	1× ICSI: 83.33% fertilization, pregnancy from two frozen embryos transfer, one healthy girl was born
3	I	Yes (the patient's grandmother and grandma are sisters)	2 brothers and 1 sister, all fertile	60.8 million	3× ICSI: 13.33% fertilization, no success
4	I	No	1 infertile sister	1.1 million	No ICSI
5	I	Yes (unknown degree)	1 brother and 1 sister, all fertile	22.7 million	No ICSI

ICSI, intracytoplasmic sperm injection.

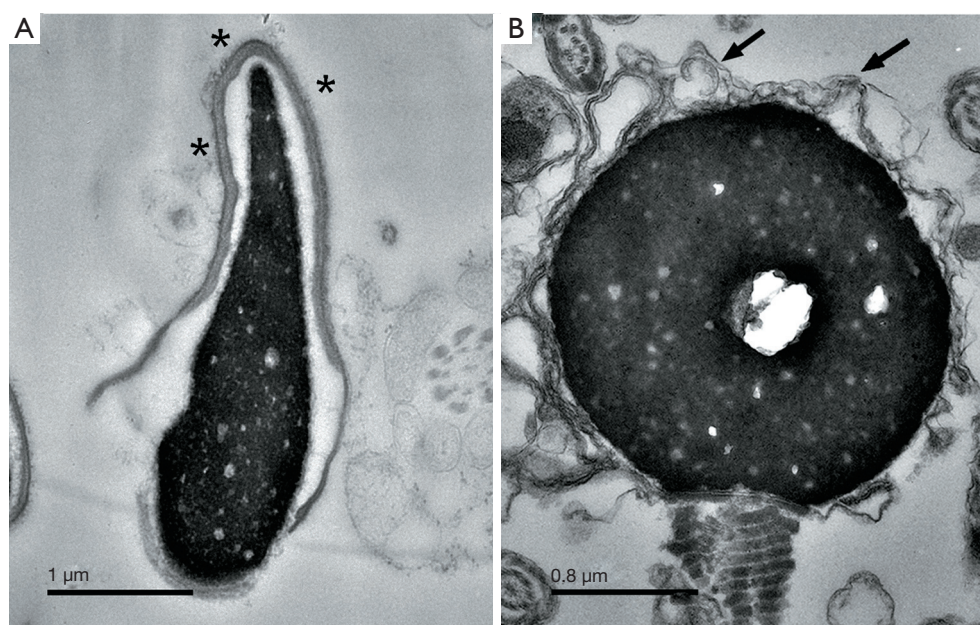


**Figure 1** Papanicolaou stain showing morphologic features of globozoospermia (1,000×). (A) Normal sperm head. (B) 100% round head of type I globozoospermia. (C) Irregular (round, tapered and pyriform) head in type II globozoospermia. (D) Vacuolation in head of globozoospermia (scale bar, 5 µm).

spectrophotometer. Our results showed that the level of Zn in seminal plasma from the globozoospermic group was significantly lower than that from the control group ( $106.667 \pm 36.147$  vs.  $158.274 \pm 31.398$  µg/mL,  $P < 0.05$ ). The level of Cu, Fe, Mn in semen between the globozoospermia

and the fertile groups were also tested. There were no significant differences shown (Table 2).

To understand the ability to counteract ROS for the sperm, the SOD activity in seminal plasma was tested in these two groups with different fertility. A lower activity in



**Figure 2** Electron microscopy of globozoospermic sperm. (A) Normal sperm head with acrosome (asterisks). Its elongated shape with a normal length to transversal diameter ratio. (B) A rounded acrosomeless head of globozoospermic sperm. The presence of chromatin rarefaction in the middle of the nucleus and residual cytoplasmic droplets surrounded the round nucleus (black arrows). Panel magnification is indicated by length of the bars.

the globozoospermic group ( $319.515 \pm 38.219$ ) was observed than that in the control group ( $358.148 \pm 23.935$ ) ( $P < 0.05$ ) (Table 2). Statistical analyses displayed that the SOD activity was positively correlated with Zn content in all semen samples ( $r = 0.485$ ,  $P = 0.049$ ) (Figure 3). Therefore, the above results suggested that in the globozoospermic group, the lower Zn level was related to the lower SOD activity.

### DNA fragmentation analysis

The SCD test was used to analyze the DNA fragmentation produced in the sperm. The nuclear chromatin was very well preserved with a high density whereby the halos can be accurately stained and assessed by bright-field microscopy (Figure 4). The mean DFI revealed by SCD test was significantly higher in the globozoospermic group compared with that in the control group ( $58.30\% \pm 4.63\%$  vs.  $23.44\% \pm 4.46\%$ ) ( $P < 0.01$ ) (Table S1). This result indicated there was significant amount of DNA fragmentation produced in the globozoospermic spermatozoa according to the WHO criteria (DFI value  $> 30\%$ ).

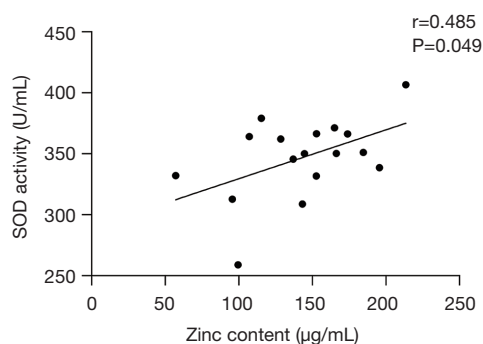
Subsequently, the Comet assay was carried out since the assay can not only identify the total level of sperm

DNA fragmentation but also distinguish different types of DNA breaks of single-stranded DNA break (SSB) and double-stranded DNA break (DSB). This allowed a more precise and extensive analysis of the DNA damage. To assess the level of DNA fragmentation in sperm, a threshold for quantitative parameters such as tail length, olive tail moment is normally established according to previous studies (31,32), or the mean of DNA breaks in ART outcome is usually calculated as threshold values of Comet assay results (33,34). Our results showed that a significant higher double-stranded break DFI (DSB-DFI) in the globozoospermia than that in the control group ( $38.36 \pm 11.88$  vs.  $14.09 \pm 1.95$ ,  $P < 0.01$ ). As shown in Table 3, the DSB-DFI ( $38.36 \pm 11.88$ ) was also higher than SSB-DFI ( $18.59 \pm 6.32$ ) in the globozoospermic group. Moreover, the levels of the section with low SDF within DSB were also dramatically higher in globozoospermic group than in the control group ( $23.36 \pm 10.24$  vs.  $7.91 \pm 1.94$ ) ( $P < 0.01$ ). This meant that the incidence of sperm with short comet tail was relatively high in the globozoospermic sample. There was no difference in total SSB-DFI between the globozoospermic group and the control group whereas the proportion of high levels of SDF within total SSB-DFI

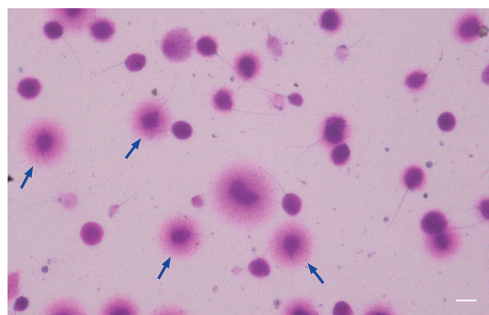
**Table 2** Four elements content and SOD activity in seminal plasma from globozoospermic patients and fertile controls

Groups	n	Element content ( $\mu\text{g/mL}$ )				SOD activity (U/mL)
		Mn	Cu	Fe	Zn	
Globozoospermia (n=5)	1	0.784	0.523	0.275	128.576	362.156
	2	0.110	0.290	1.530	99.450	258.930
	3	0.300	0.135	0.975	57.090	332.079
	4	0.610	0.190	1.790	95.580	312.675
	5	0.350	0.360	2.150	152.640	331.737
	$\bar{x}\pm\text{SD}$		0.431 $\pm$ 0.266	0.300 $\pm$ 0.152	1.344 $\pm$ 0.735	106.667 $\pm$ 36.147*
Fertile control (n=12)	1	0.440	0.210	1.190	144.720	350.014
	2	0.290	0.280	0.460	165.030	371.186
	3	0.250	0.320	1.780	115.350	379.129
	4	0.438	0.350	1.850	143.250	308.704
	5	0.500	0.480	1.760	107.130	364.1061
	6	0.270	0.210	0.220	136.980	345.505
	7	0.410	0.190	0.800	152.820	366.425
	8	0.380	0.420	1.800	166.290	350.188
	9	0.038	0.550	1.638	195.563	338.553
	10	0.660	0.170	2.080	173.970	366.252
	11	0.030	0.230	0.940	213.480	406.663
	12	0.180	0.430	1.930	184.710	351.056
$\bar{x}\pm\text{SD}$		0.324 $\pm$ 0.186	0.320 $\pm$ 0.126	1.371 $\pm$ 0.627	158.274 $\pm$ 31.398	358.148 $\pm$ 23.935

\*, significant differences, with respect to fertile controls ( $P<0.05$ ). SOD, superoxide dismutase.



**Figure 3** Correlation analysis between SOD activity and Zinc concentration in seminal plasma. SOD, superoxide dismutase.



**Figure 4** Representative Sperm Chromatin Dispersion (SCD) test on one globozoospermic patient (400 $\times$ ). Sperm cells with halo (blue arrows) showed normal sperm chromatin dispersion. The frequency of sperm cells without halo was reflective of a degree of nuclear damage (scale bar, 10  $\mu\text{m}$ ).

**Table 3** DSB-DFI and SSB-DFI calculated from semen samples from infertile patients and fertile control subjects

Groups	Samples	SSB (%)		DSB (%)		SSB-DFI (%)	DSB-DFI (%)
		Low	High	Low	High		
Globozoospermia (n=5)	1	4.44	4.00	23.64	10.47	12.00	51.55
	2	9.09	6.49	38.10	0.95	18.18	41.90
	3	6.06	4.76	17.90	5.68	12.98	27.07
	4	8.99	9.52	26.56	11.72	23.28	46.48
	5	12.00	8.00	10.62	10.18	26.50	24.78
Fertile control (n=12)	1	12.77	0.85	7.01	3.82	16.17	11.46
	2	10.84	2.96	4.36	0.55	14.78	17.13
	3	9.16	5.46	8.96	1.24	16.34	14.93
	4	12.75	2.75	9.98	2.99	17.50	16.96
	5	7.48	2.24	7.73	2.49	13.22	12.97
	6	6.50	2.00	6.00	2.00	11.50	10.75
	7	8.46	2.49	5.25	3.25	13.93	13.00
	8	8.47	2.66	8.25	4.00	17.68	14.75
	9	2.90	3.38	9.93	2.73	15.70	15.38
	10	6.73	1.50	8.75	3.00	10.72	14.50
	11	6.95	4.22	8.25	2.25	15.14	14.00
	12	8.25	3.25	10.50	0.75	13.75	13.22
Globozoospermia		8.12±2.94	6.55±2.27**	23.36±10.24**	7.80±4.46*	18.59±6.32	38.36±11.88**
Fertile controls		8.44±2.77	2.81±1.21	7.91±1.94	2.42±1.12	14.70±2.19	14.09±1.95

\*, P<0.05, \*\*, P<0.01, respectively, globozoospermia compared with fertile control groups. DFI, frequency of the sperm DNA fragmentation (SDF); SSB-DFI, frequency of single-stranded breaks; DSB-DFI, frequency of double-stranded breaks. The percentage of high and low levels of SDF was respectively counted within SSB and DSB for each sample according to the threshold of tail moment in comet results. The tail moment values of 0.1–0.5 and >1.5 respectively were set as low and high SSB while 0.1–10 and >25 values were set for low and high DSB, respectively.

between the two groups was significantly different (P<0.01) (Table 3), indicating the serious extent rather than universality of single strand fragment in globozoospermic infertile patients.

### Nuclear maturation of sperm

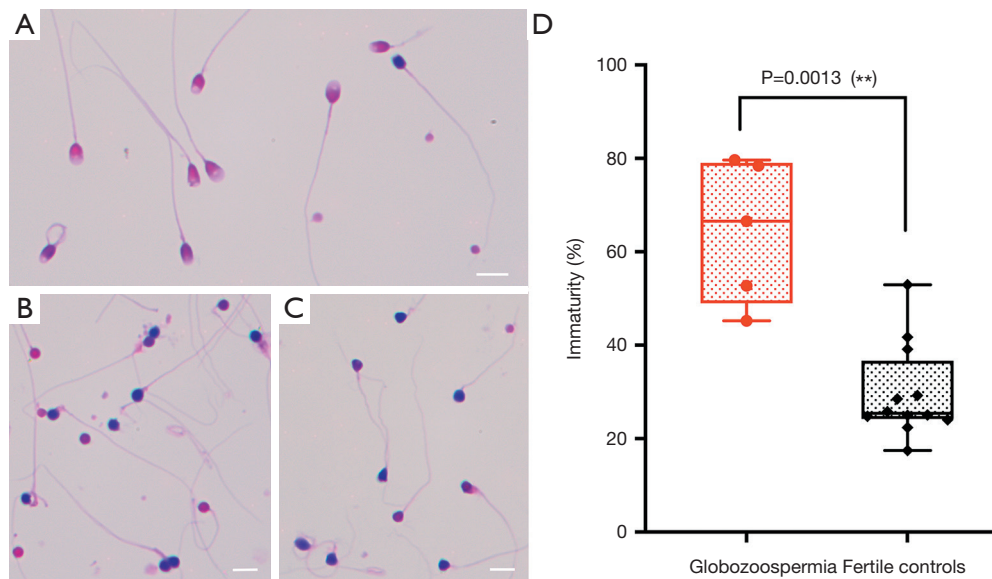
During spermiogenesis, the replacement of histones by protamines allows spermatozoa to acquire a highly packed mature structure. Aniline blue selectively binding to lysine-rich histones while Eosin specifically combining with rich arginine and cystine residues of protamines in spermatozoa showed the presence of histones for blue and protamines for red in the sperm nucleus respectively (Figure 5). Thus, the indirect approach was used to evaluate mature status

of sperm by calculating the ratio of the sperm with extra histones in the sperm nucleus for sperm samples. Our results showed the percentage of reacted spermatozoa to Aniline Blue in the globozoospermic group (64.51%±15.31%) was significant higher (up to 2-fold) than that in the control group (29.67%±9.97%) (P<0.01) (Figure 5D). This meant that histones which should have been successfully replaced by protamines in sperm nucleus were excessive present, in other words, there was more abnormal immature sperm in globozoospermic infertile patients.

### Discussion

Understanding the DNA damage in patients with





**Figure 5** Aniline blue staining (1,000 $\times$ ). Sperm with normal protamine (red stained) and immature sperm with low protamine or high histone (while dark blue/blue-purple stained). (A) Normal sperm. (B) Sperm from globozoospermia I. (C) Sperm from globozoospermia II. (D) Box-plot of nuclear immaturity between globozoospermia and fertile control groups, \*\*,  $P < 0.01$  (scale bar, 5  $\mu\text{m}$ ).

teratozoospermia and providing intervention to improve the quality of sperm is crucial to the success of conception in ART (35-39). In this study, we used both SCD test and Comet assay to exam globozoospermia samples and observed obvious DNA fragmentation and severe DSBs. A significant increase of DSB in sperm might explain the failure of ICSI in these patients compared with the clinical evaluation data of patients on ART (Table 1). We also found that the level of seminal Zinc and SOD activities were lower in the globozoospermic infertile patients and their DSB-DFI was significantly higher than that in the fertile controls. Anti-oxidative insufficiency of SOD with low Zn level therefore might be responsible for oxidative stress, which may lead to higher DNA damage in globozoospermic spermatozoa.

To our knowledge, this is the first study which systemically investigate the ion concentration, oxidative stress marker and DFI index in globozoospermia samples. A correlation of low Zn level with higher DFI index observed in this study indicated that a low Zn level may be responsible for the high oxidative stress and the higher DNA damage in sperm. However, this needs to be further confirmed with a larger cohort of samples as this study only included 5 samples due to its rare availability. Using simple Comet assay, this study enabled the identification of DNA fragmentation and clear characterisation in the sperm

from teratozoospermic infertile patients. Identifying the association of Zn, SOD activities and DNA fragmentation in spermatozoa patients showed potentially how the reduced SOD activities under zinc deficiency could disturb the balance and be responsible for oxidative stress in globozoospermia. Understanding this important correlation of zinc and globozoospermia could provide a useful treatment strategy for teratozoospermic infertile patients in their ART treatment.

#### *DNA fragmentations in spermatozoa patients and its clinical implications*

Men with teratozoospermia would normally need ART to achieve conception, but the outcome is often very poor at the present and many patients are even confronted with a higher risk of selecting spermatozoa with damaged DNA for ART (35,36). Overwhelming evidences suggest that spermatozoa with DNA fragmentation is associated with higher miscarriage rates, lower good-quality embryo rate, lower clinical pregnancy rate and lower live birth rate after ART (37-39). In this study, using Comet assay we were able to characterise the DNA fragmentation and compare the parameters related to DSB/SSB in spermatozoa patients to determine how the different types of DSB could have impact on the IVF/ICSI outcome.

There are normally two types of DSB, extensive DSB and localized DSB. The extensive DSB has very long comet tails separated from the sperm core and is very small part of total DSB, while the localized DSB is the most common DSB and largely localized and attached to the matrix attachment regions (MAR) (40). One previous study reported that a large number of sperm with extensive DSB (long tails) and those with SSB were present in a normal seminal sample (40). On the contrary, our data showed the higher incidence of sperm with DSB (short Comet tail) as well as relatively lower SSB-DFI in the globozoospermic samples than the normal controls. Therefore, it seemed obvious that localized DSB in the MAR was the main form of DSB in our globozoospermic sperms. The report has also claimed that localized DSB in the MAR of human sperm may lead to the risks of recurrent miscarriage and implantation failure as well as slow embryo kinetics in ICSI cycles (40). In our study, there were also significant differences in the proportion of high fragmentation levels within total SSB-DFI between the globozoospermic patients and the fertile controls, suggesting the presence of high level SSB and poor ability to repair SSB for globozoospermia. The impaired function of nuclear matrix due to its damaged integrity caused by the localized DSB have been reported to be the possible reason for the failure outcomes of ART (41,42). The localized DSB has also been demonstrated to attach to the sperm nuclear matrix probably through the TOP2B or similar protein (43,44). It is now clear that topoisomerase II mediates DSBs in somatic cells (45). In spermatozoa, supraphysiological ROS levels disturb sperm TOPII, which is involved in the nuclear remodeling via breaking and reconnecting DNA during spermiogenesis, causing large amounts of DNA nicks in elongating spermatids, and finally result in sperm DNA fragmentation (46). Our results showed the higher incidence of sperm with DSB (short Comet tail) as well as relatively lower SSB-DFI could therefore be used to predict the outcome of IVF/ICSI in globozoospermic spermatozoa, and possibly in other types of teratozoospermia samples. However, this needs to be further validated in larger cohort of samples.

#### ***The role of Zn in spermatozoa patients and its clinical implications***

Ion measurement on semen samples in this study showed that Zn deficiency is one of the important characteristics in globozoospermic spermatozoa samples. In addition to

be a cofactor for various proteins and electron transport, Zn was reported to be crucial in the stabilization of sperm chromatin (18,47). When the condensation of the nucleus starts, Zinc is incorporated into the sperm nucleus and participates in the process of protamination by the formation of S-Zn-S bonds with protamine, which maintains the stabilization of chromatin during spermiogenesis (18). We observed abnormal sperm chromatin in form of impaired chromatin condensations by TEM in the globozoospermic samples (*Figure 2B*). At the same time, the Aniline Blue staining also displayed protamination level deficiency in sperm nucleus of the infertile patients (*Figure 5*). This is entirely possible that in our patients with globozoospermia, the impaired chromatin compaction induced DNA breaks and free radicals by reducing protamination and disulphide bond formation owing to deficient Zn (48). Alternatively, in the absence of Zinc, globozoospermic spermatozoa carrying poor chromatin compaction is more vulnerable to oxidative attack (49). In fact, the Zn level in the sperm of Patient No. 3 is the lowest and he was a type I globozoospermic patient. This indicated that a low Zn level might be responsible for his unsuccessful ICSI outcome. Moreover, reduced Zinc transport proteins might be another possible cause for Zn dysfunction because they regulate Zinc uptake into the cytoplasm or from the lumen of intracellular compartments (50,51). A novel member of RING finger proteins, the RNF151, was found to possess a TRAF-type Zinc finger domain and C3HC4-type RING finger domain, and this Zinc finger protein probably plays a role in acrosome formation by interaction with dysbindin (52). All the above suggested that Zinc might function in acrosomal biogenesis indirectly.

Reportedly, oral Zinc supplementation can restore the level of the semen parameters including semen volume, sperm count, progressive sperm motility, normal sperm form in asthenozoospermic subjects. At the same time, the Zinc supplementation also returns to normal value for HMW-Zn, one of three types of Zinc binding proteins, which include high (HMW), intermediate (IMW), and low molecular weight ligand (LMW) proteins in human seminal plasma (53). Clinical results indicated that zinc sulfate treatment for infertile patients with low Zinc level in semen may be an effective therapy for infertile individuals especially after varicocelelectomy (54). Our results in this study, together with previous studies, certainly provided more evidences and inspiration to treat globozoospermia by zinc supplementation in the future.

### *Association of Zn, SOD activities and DNA fragmentation in spermatozoa patients and its clinical implications*

In this study, we investigated the association of reduced SOD activity and the Zn level in the sperm samples of spermatozoa patients. SODs initiate the conversion of superoxide anion and scavenge superoxide free radical whereby spermatozoa maintain membrane fluidity and internal environments, thereby ensuring the normal morphologic structures and function for sperm (55,56). It has been found that SOD activity was negatively associated with sperm DNA fragmentation (57). Given the crucial importance of SOD in defense strategy, the level of Zn, Cu, Mn and Fe elements in semen and SOD activity in seminal plasma were examined in the globozoospermic and normal samples in this study. Previous studies found a higher level of Fe content presented in semen from teratozoospermia than that from the spermatozoa patients, and therefore caused morphological anomalies of spermatozoa due to Fe-induced lipid peroxidation (58-60). Unlike these results, our results did not establish the difference between globozoospermia and normal samples in the level of Fe, together with Cu and Mn elements. However, we observed a strong positive correlation between SOD activity and the level of Zn in semen, which confirmed previous reports on the zinc-dependent enzymatic activity of the sperm (58,61).

Testes contain cytosolic (CuZnSOD), mitochondrial (MnSOD) and secretory extracellular SOD (Ex-SOD) forms of SOD (26). Among them, 75% activities attribute to CuZnSOD, the main SOD isoform in spermatozoa SOD. In this study, the level of Zn content and SOD activities were both found significantly lower in globozoospermic semen. Thus, we assumed that this compromised SOD activities could be attributed to Zinc deficiency in globozoospermia. It could be the case that the expression of SOD gene actually increased probably as a response to oxidative stress, but their activities still remained at a low level due to the lack of Zinc as a prosthetic group. It has been reported that for proper function of spermatozoa, the adequate balance is vital between superoxide radical generation and dismutation (62). Therefore, it is well accepted that reduced SOD activities under Zinc deficiency would necessarily disturb the balance and be responsible for oxidative stress in globozoospermia. Our results further confirmed this. Understanding this important correlation could provide useful treatment strategies for teratozoospermia patients in their ART treatment.

In conclusion, our study found that serious DSBs were

presented in globozoospermia and occurred mainly in MAR of sperm nucleus. Antioxidative insufficiency of SOD under the lack of Zn might be responsible for oxidative stress, which led to DNA damage in globozoospermic spermatozoa. Zn deficiency might also have influence on the chromatin stabilization of globozoospermic spermatozoa during spermiogenesis, resulting in its more vulnerable to oxidative attack. All the above suggested that it is critical to determine a detailed profile of the Zn level, oxidative stress marker, SOD and DSB value for globozoospermic infertile patients so that more comprehensive outcome prediction of ICSI can be planned for these patients. To help to achieve a high successful rate of ICSI, we hypothesize that Zinc therapy for patients suffering from globozoospermia can be beneficial in improving ART outcomes.

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### **Footnote**

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*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are

appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethical Review Board of West China Second University Hospital, Sichuan University (K2018089). Informed consent was obtained from each subject otherwise the specimens would have been discarded.

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