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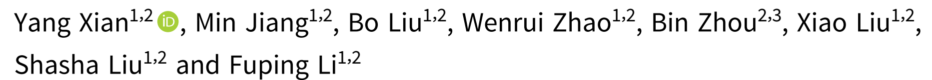
A cryoprotectant supplemented with pentoxifylline can improve the effect of freezing on the motility of human testicular sperm



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Summary

This study examined the effect of a cryoprotectant with and without pentoxifylline supplementation on the motility and viability of human testicular sperm, both before and after freezing. Testicular samples were obtained from 68 patients with azoospermia who came to the Andrology Service of West China Second University Hospital, Sichuan University, for testicular biopsies from December 2019 to April 2020. All patients were assigned randomly to two groups: experimental, whose testicular sperm were added to the cryoprotectant with pentoxifylline, and the control, whose testicular sperm were added to the cryoprotectant without pentoxifylline. Both groups used the same freezing and thawing methods. Testicular sperm motility in the experimental group was significantly higher than that of the control group, both before and after cryopreservation. The recovery rate of sperm motility in the experimental group was significantly higher than that of the control group. The percentage of samples with motile testicular sperm in the experimental group was significantly higher than that of the control group after thawing. Sperm viability was unchanged between the experimental and control groups, both before and after freezing. Overall, a pentoxifylline-supplemented cryoprotectant can significantly improve the motility of testicular sperm before and after cryopreservation.

Introduction

With the development of social industrialization and increasingly serious environmental pollution, there is a growing number of infertile couples, among which male factor-related infertility accounts for 40–50% of the cases (Jackson *et al.*, 2010). Azoospermia constitutes 10% of male factor infertility (AbdelHafez *et al.*, 2009). Since 1992 (Palermo *et al.*, 1992), the intracytoplasmic sperm injection (ICSI) technique has been a breakthrough in the treatment of patients with severe male factor infertility, and allowed patients to obtain access to *in vitro* fertilization procedures. In 1993, ICSI was performed successfully using testicular spermatozoa (Gil-Salom *et al.*, 1996). This laid the foundations for the clinical application of testicular sperm. Subsequently, Kamal *et al.* (2010) found that the ICSI outcomes of patients with ejaculated sperm were similar to those of obstructive azoospermia patients using testicular and epididymal spermatozoa.

Following exclusion of hereditary diseases, a testicular diagnostic biopsy is usually performed for azoospermia patients prior to ICSI to determine the presence of testicular sperm. To avoid multiple testicular biopsies, it is important to freeze testicular sperm when they are found in the diagnostic testicular biopsy.

The theory of epididymal sperm maturation was first proposed in 1994 (Bedford, 1994). This theory implies that sperm produced by the testis are immature, and lack motility and sperm-oocyte binding ability (Bedford, 1994; Orgebin-Crist, 1998). Sperm motility is enhanced during epididymal maturation. Therefore, sperm retrieved from the epididymis show relatively higher developmental maturity and exhibit greater motility. In contrast, testicular sperm are often characterized by the absence of motility, especially after a cryopreservation procedure (Navas *et al.*, 2017; Morin *et al.*, 2020). Furthermore, it is difficult to distinguish immotile viable spermatozoa from immotile non-viable spermatozoa in the ICSI procedure (Rubino *et al.*, 2016). This makes the identification of motile sperm from testicular sperm extraction samples extremely important.

Pentoxifylline (PF), a phosphodiesterase inhibitor, has been used to stimulate the motility of epididymal and testicular sperm (Angelopoulos *et al.*, 1999; Terriou *et al.*, 2000). Compared with the use of PF in fresh cultures of testicular biopsies before ICSI, the use of a PF-supplemented cryoprotectant in testicular diagnostic biopsies for testicular sperm cryopreservation appears to

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be more effective, and can decrease damage by minimizing repeated testicular punctures in patients.

This study was designed to investigate whether a PF-supplemented cryoprotectant could improve the quality of human testicular sperm following freezing. The outcome of our study was assessed in terms of sperm motility and viability before and after cryopreservation.

Materials and methods

Study design

This prospective study included 68 patients with azoospermia who came to the Andrology Service of West China Second University Hospital, Sichuan University, for diagnostic testicular biopsies from December 2019 to April 2020. Azoospermia was confirmed in at least two semen samples after centrifugation at 3000 *g* for 15 min and observing the entire pellet according to the World Health Organization Laboratory Manual for the Examination and Processing of Human Semen (5th edition, 2010). Testicular sperm were found during diagnostic testicular fine needle sperm aspiration in all participants.

The 68 patients were assigned randomly to two groups: experimental and control. In the experimental group, a PF-supplemented cryoprotectant was used for testicular spermatozoa cryopreservation. In the control group, a cryoprotectant without PF was used for testicular spermatozoa cryopreservation.

This project was approved by the Ethics Committee of West China Second University Hospital, Sichuan University. All participants, who voluntarily joined this study, provided informed consent.

Processing and evaluation of testicular samples

Testicular samples were obtained by testicular fine needle sperm aspiration. After surgery, testicular tissue was placed into a 1.8 ml Eppendorf tube with Tyrode's solution (Sigma-Aldrich, St. Louis, MO, USA), then transferred to the laboratory. The tissue was finely shredded with mechanical ophthalmic scissors in a 1.8 ml Eppendorf tube containing 300 μl G-IVF Plus (Vitrolife, Göteborg, Sweden) to create a testicular suspension.

Experimental group suspensions were diluted 1:3 (v:v) with the PF-supplemented cryoprotectant; the final concentration of PF was 2.5 mmol/l. Control group suspensions were diluted similarly with cryoprotectant without PF. All testicular suspensions were placed at 25°C for 5 min. Subsequently, the motility and viability of the testicular sperm were examined.

The motility of testicular sperm [(number of motile sperm/total number of sperm) × 100%] was calculated using a CX30 microscope at ×200 magnification (Olympus, Tokyo, Japan). Next 10 μl of the testicular suspension were delivered onto a clean glass slide, covered with a 22 mm × 22 mm coverslip providing a chamber of depth 20.7 μm, then evaluated in, in total, 10 fields. All immotile and motile sperm were counted (motility included all kinds of sperm movement; i.e. swimming in small circles, the flagellar force hardly displacing the head, or when only a flagellar beat could be observed).

Viability assay

The eosin technique was used to determine sperm viability. As recommended by the World Health Organization, after mixing, a 5 μl aliquot of the testicular suspension was combined with 5 μl of eosin solution on a microscope slide, mixed with a pipette tip by swirling the sample on the slide, then covered with a 22 mm × 22 mm coverslip and left for 30 s. Light microscopy (×400 magnification) was used to calculate the numbers of stained (dead) and unstained (live) cells with the aid of a laboratory counter.

Freezing and thawing of the testicular suspensions

Fresh testicular suspensions with cryoprotectant (modified Tyrode's solution with 30% glycerol; the experimental group added 2.4 mmol/l PF) were incubated for 5 min at 25°C. Then, 200 μl of the mixture were loaded into 300 μl CBS High Security Semen Straws (Cryo Bio Systems, Normandy, France). The straws were heat-sealed at both ends, then placed 8 cm above the liquid nitrogen surface for 10 min. Subsequently, the straws were transferred to liquid nitrogen for storage. All samples were kept in liquid nitrogen for at least 24 h before thawing.

The straws were thawed in a 37°C water bath for 5 min, both ends of the straws were removed, and the testicular suspensions decanted into 1.8 ml Eppendorf tubes. Each tube was placed in a 37°C CO_2 incubator for 15 min. Subsequently, testicular sperm motility and viability were examined.

Statistical analyses

Statistical analyses were performed using SPSS v.16.0 (IBM, Chicago, IL, USA). Data are expressed as means ± standard deviations, or numbers and percentages. Differences between the two groups were assessed using the *t*-test or χ^2 test, as appropriate. A P-value < 0.05 was considered statistically significant.

Results

Patient characteristics

Table 1 shows the clinical characteristics of the 68 participants in this study. The average age of the experimental group was 32.2 ± 5.0 years (range 23–45 years). The average age of the control group was 32.2 ± 5.2 years (range 23–42 years). Most of the study participants exhibited primary infertility (91.2%). The mean durations of infertility in the experimental and control groups were 3.02 ± 2.41 and 2.61 ± 2.32 years, respectively. The average left testicular volumes of the experimental and control groups were 14.4 ± 6.7 and 15.0 ± 5.5 ml, respectively. The average right testicular volumes of the experimental and control groups were 16.1 ± 4.9 and 16.0 ± 4.6 ml, respectively. There were no significant differences on these basic characteristics between the experimental and control groups.

The percentage of motile sperm of testicular samples before and after cryopreservation

The before freezing results were obtained using testicular suspensions with (the experimental group) or without (the control group) PF-supplemented cryoprotectant that were placed for 5-min at 25°C. Among the 34 samples in the experimental group, all exhibited motile sperm (100%), while 32 of the 34 samples in the control group had motile sperm (94.1%). There was no significant difference between these two groups ($P = 0.473$) (Fig. 1).

After the freeze–thaw process, all 34 samples in the experimental group contained motile sperm (100%). In contrast, only 15 samples of the 34 samples in the control group had motile sperm (44.1%). The percentage of motile sperm of testicular samples in the experimental group was significantly higher than that of the control group ($P = 0.000$) (Fig. 1).

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Table 1. Basic characteristic of study population

Characteristics		Experimental group ($n = 34$)	Control group ($n = 34$)	P-value
Age	≥35 years	10	12	
	<35 years	24	22	
	Mean ± SD	32.17 ± 4.98	32.17 ± 5.16	1.000
Infertility type	Primary	31 (91.17%)	31 (91.17%)	
	Secondary	3 (8.83%)	3 (8.83%)	
Infertility duration (years)	≥3 years	16	13	
	<3 years	18	21	
	Mean ± SD	3.02 ± 2.41	2.61 ± 2.32	0.475
Testicular volume (ml)	Left	14.38 ± 6.68	15.02 ± 5.47	0.664
	Right	16.08 ± 4.87	16.00 ± 4.57	0.939

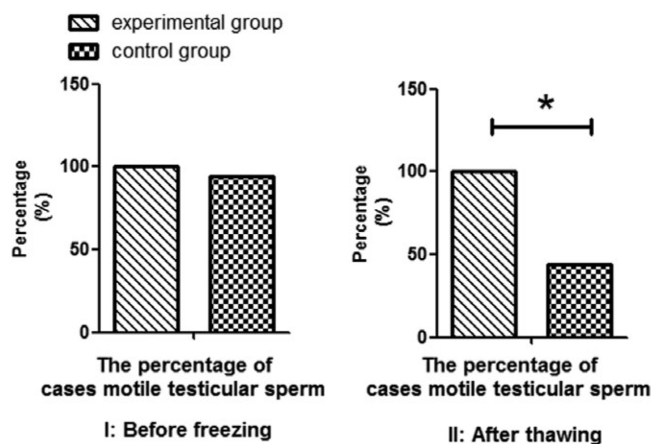

Figure 1. Percentage of cases of motile testicular sperm before and after cryopreservation in experimental group and control group.

Testicular sperm motility and viability before and after cryopreservation

The effects of the PF-supplemented cryoprotectant on testicular sperm motility and viability before freezing are shown in Fig. 2. The motility of testicular sperm in the experimental group was significantly higher than that of the control group (15.1 ± 6.6 % vs. 4.01 ± 3.02 %, respectively) ($P = 0.000$). There was no significant difference in sperm viability between the experimental (69.2 ± 11.4 %) and control (72.0 ± 8.1 %) groups ($P = 0.237$).

After thawing, the motility of testicular sperm in the experimental group was significantly higher than that of the control group (10.3 ± 5.5 % vs. 0.75 ± 0.98 %, respectively) ($P = 0.000$) (Fig. 2). There was no significant difference in viability between the experimental (41.1 ± 10.8%) and the control (41.0 ± 13.1%) groups ($P = 0.984$).

The motility recovery rate of frozen testicular sperm after thawing in the experimental group was significantly higher than that of the control group (70.1 ± 25.4 % vs. 38.2 ± 36.1%, respectively) ($P = 0.038$).

Discussion

Since the 1990s, assisted reproductive technology (ART) has made a great progress using ICSI, especially for those infertile couples with male factor infertility. ICSI can achieve high fertilization and pregnancy rates, not only with ejaculate spermatozoa, but also with epididymal or testicular sperm (Devroey *et al.*, 1994, 1996; De Croo *et al.*, 2000; Corona *et al.*, 2019). The use of frozen testicular (Gil-Salom *et al.*, 1996) and epididymal (Nagy *et al.*, 1995a) sperm in ICSI has become an effective and standard approach to treat obstructive and non-obstructive azoospermia patients as the technology became more sophisticated. However, in a non-selected population with non-obstructive azoospermia, the probability of finding sperm is only approximately 50% (Tournaye *et al.*, 1997).

The techniques used to obtain testicular sperm, such as extraction and aspiration, are invasive procedures that require local anaesthesia and a percutaneous needle puncture (Al-Malki *et al.*, 2017). This may cause adverse events such as hematoma, inflammation, fibrosis, and even permanent androgen deficiency (Schlegel and Su, 1997; Schill *et al.*, 2003). Therefore, cryopreservation of testicular sperm is necessary when patients undergo a diagnostic biopsy. The cryoprotection of testicular sperm is effective for ART; there were no differences in pregnancy rates using epididymal or testicular spermatozoa, and both fresh and frozen spermatozoa gave similar results (Silber *et al.*, 1995). A meta-analysis found that there were no differences in the fertilization outcome, cleavage rate, good embryo rate, clinical pregnancy rate, and live birth rate when comparing fresh versus frozen–thawed testicular sperm (Yu *et al.*, 2018).

Freezing causes mechanical and osmotic damage to sperm due to the temperature decrease; the addition of cryoprotectant agents can minimized this damage (Palomar Rios *et al.*, 2018). Cryodamage affects sperm viability by damaging the membrane integrity (Zhu and Liu, 2000; Hossain *et al.*, 2010). Because of the part mitochondrial damage caused by freezing, a decreased motility of human sperm can be observed in thawed samples (Satirapod *et al.*, 2012). Moreover, the DNA fragmentation may increase due to the increased production of reactive oxygen species (ROS) during cryopreservation (Thomson *et al.*, 2009; Kopeika *et al.*, 2015).

Compared with freezing ejaculated sperm, the cryopreservation of testicular sperm appears to be more difficult due to the low concentration and the lack of motility of testicular sperm, which because of the sperm motility is obtained during the maturation of the epididymis (Morin *et al.*, 2020). Furthermore, testicular suspensions contain a large number of erythrocytes, germinal cells and cellular debris. This makes the identification of motile testicular sperm much easier than for immotile testicular sperm (Kovacic

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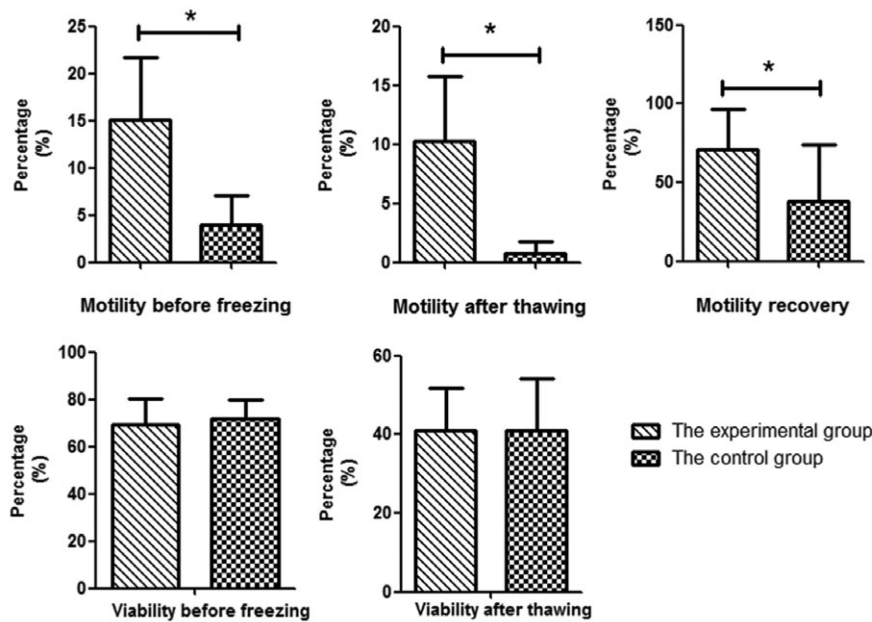

Figure 2. Motility and viability of testicular sperm before and after cryopreservation and the motility recovery rate of testicular sperm. *: $P < 0.05$.

et al., 2006). Therefore, improving the low motility of testicular sperm, especially following freezing, would make their clinical application easier and more effective.

Previous research found that the random injection of immotile spermatozoa could lead to disappointing outcomes of ICSI, with intact cells remaining in the ooplasm 20 h after injection (Liu *et al.*, 1995). Moreover, for testicular sperm, Terriou *et al.* (2000) found that the fertilization rate (FR) of ICSI using PF-treated testicular sperm was three-fold greater than with ICSI using non-PF-treated testicular sperm. Kovacic *et al.* (2006) found that, using PF with immotile testicular sperm, they could elicit movement of these sperm, allowing easier identification of vital sperm. This also shortened the procedure [30 min (minimum 10, maximum 90) vs. 120 min (minimum 60, maximum 240)], improved the fertilization rates (66% vs. 50.9%), and increased the number of embryos (4.7±3.3 vs. 2.7±2.1), which allowed the selection of better embryos for transfer and freezing. Overall, it appears that the most important factor for the successful outcome of ICSI is associated with using ejaculated motile spermatozoon for oocyte microinjection (Nagy *et al.*, 1995b).

Pentoxifylline is a cyclic adenosine monophosphate (cAMP) phosphodiesterase enzyme inhibitor. Inhibiting this enzyme prevents the normal functions of cAMP in human sperm respiration, motility, and regulation of the acrosome reaction by increasing the intracellular cAMP concentration (Tournaye *et al.*, 1994; Sharma *et al.*, 1996). Adding PF to a sperm sample before the ICSI procedure can drive the flagellar movement of live sperm cells through the inhibition of cyclic 3′,5′-nucleotidase phosphodiesterase and the resulting increased concentration of intracellular cyclic nucleotides (Nassar *et al.*, 1999). Although safety concerns have been raised regarding the use of PF in ICSI, its use seems to be universally accepted based on extensive and in-depth research on its mechanism. In 2003, the french Agence de la biomédecine included PF in the official list of compounds allowed for ART procedures that use phosphodiesterase inhibitors for selecting live spermatozoa before ICSI. It was reported that PF can improve sperm movement without any adverse effects on sperm chromatin/DNA integrity in vitrification (Nabi *et al.*, 2017). Furthermore, Navas *et al.* (2017) found that the use of PF in ICSI did not appear to increase adverse obstetric and neonatal outcomes.

Although most previous studies added PF to the sperm sample before the ICSI procedure (Griveau *et al.*, 2006), we used PF as a cryoprotectant supplement to improve the motility of testicular sperm before and after cryopreservation, and before the ART procedure. As shown in Figure 1, before freezing, all experimental group (PF-supplemented cryoprotectant) samples had motile sperm in the testicular suspensions, and 94% had motile sperm in the control group (cryoprotectant without PF). After thawing, the percentage of samples with motile testicular sperm in the experimental group was significantly higher than that of the control group (100% vs. 44.1%). Furthermore, PF-supplemented cryoprotectant significantly improved the motility of testicular sperm before and after cryopreservation (Figure 2).

Previous research implied that PF enhanced post-thaw motility of cryopreserved human spermatozoa when added after thawing, but there were no differences in motion characteristics with sperm samples treated by adding 3 mmol/l PF as a supplement to the cryoprotectant (Stanic *et al.*, 2002). The difference compared with our results can be explained from the following two aspects: (1) the specimen sources were different, we chose immotile testicular sperm where the motion characteristics were easier and more obviously improved, while the previous study utilized ejaculated sperm with normal motility; and (2) the concentrations of PF added into cryoprotectant were different (2.4 mmol/l vs. 3 mmol/l), which may affect the ability of PF to improve sperm motility.

Immotile but viable sperm were detected by the eosin test that focuses on spermatozoon membrane integrity. There was no significant difference in viability between the experimental and control groups, both before and after cryopreservation. This indicates that PF improves the motility of immotile but viable testicular spermatozoa. We believe that the cryopreservation of testicular sperm with a PF-supplemented cryoprotectant has the same effect as adding PF to a sperm sample before the ICSI procedure, and has the advantage of avoiding patients undergoing repeated punctures.

In summary, the results of this study demonstrated that the motility and motility recovery rates of testicular sperm were higher

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in the cryoprotectant supplemented with PF group than the cryoprotectant without PF group. Therefore, PF-supplemented cryoprotectant can improve the motility of testicular sperm before and after cryopreservation.

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Conflicts of interest. The authors declare that they have no competing interest.

Ethical standards. This project was approved by the Ethics Committee of West China Second University Hospital, Sichuan University.

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