

# Effect of peroxiredoxin 6 on total and progressive motility of human spermatozoa after cryopreservation

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**Abstract:** Sperm cryopreservation is useful in assisted reproductive technology and male fertility preservation. However, freezing and thawing significantly reduces the total and progressive motility of human spermatozoa. In the present study, we explored the effects of peroxiredoxin 6 (PRDX6) on total and progressive motility of human spermatozoa after cryopreservation. Semen samples of 20 males with normal parameters were collected and frozen in media supplemented with different concentrations of PRDX6 (0 mM,  $10^{-5}$  mM,  $10^{-7}$  mM, and  $10^{-9}$  mM, respectively). Post-thaw total and progressive motility of sperms were measured. The results showed that in comparison with 0 mM, the concentrations of  $10^{-5}$  mM,  $10^{-7}$  mM, and  $10^{-9}$  mM of PRDX6 all significantly improved total motility and progressive motility of sperms ( $p < 0.05$ ). The  $10^{-7}$  mM of PRDX6 showed the best performance. In conclusion, the supplementation of PRDX6 helps to maintain the total and progressive motility of human spermatozoa.

## Introduction

Human sperm cryopreservation is a simple and practical approach that has been widely used in the preservation of male reproductive capacity. It serves as a potential method for some cancer patients who need to preserve their fertility before chemotherapy or radiotherapy (Dohle, 2010). In addition, donor semen stored in human sperm bank is clinically widely used in artificial insemination and *in vitro* fertilization (Daudin *et al.*, 2015; Nangia *et al.*, 2013). However, cryopreservation has been shown to negatively affect sperm motility/viability, membrane stability, and the fertilization capacity of oocytes (Ferrari *et al.*, 2016; Nijs *et al.*, 2009; O'Connell *et al.*, 2002; Ozkavukcu *et al.*, 2008).

Peroxiredoxins (PRDXs) have been widely used as antioxidant enzymes (Fisher, 2019; Perkins *et al.*, 2015; Rhee,

2016). Peroxiredoxin 6 (PRDX6), as a unique member of the PRDXs family, has three enzymatic activities of peroxidase, phospholipase A2 (PLA2), and acyltransferase (Feinstein, 2019; Fisher *et al.*, 2016). It can translocate to damaged mitochondria, especially those with antioxidant and anti-apoptosis features (Fisher, 2019). It is reported that the PRDX6 level and total spermatozoa counts were lower in the seminal plasma of infertile men than those in healthy donors (Gong *et al.*, 2012). Therefore, PRDX6 is a major factor that protects sperm motility, viability, and DNA integrity (O'Flaherty, 2018).

To date, there are few studies about the effects of PRDX6 on sperm quality. The protective effects of PRDX6 on human spermatozoa after thawing have not been reported. This study aims to evaluate the protective effect of PRDX6 on human sperm quality after cryopreservation.

## Materials and Methods

### Subjects

A total of 20 healthy males from the Reproductive Medical Center in Peking University International Hospital were

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enrolled in this study. Semen samples were collected after sexual abstinence for 2–7 days. This study was approved by the Ethics Committees of Peking University International Hospital. All subjects signed informed consent.

#### *Cryopreservation and thawing of human spermatozoa*

The samples were subdivided into four groups and cryopreserved in SpermFreeze Medium (Irvine Scientific, USA). Briefly, after semen liquefaction, all samples were diluted 1:1 with SpermFreeze medium supplemented with 0 mM,  $10^{-5}$  mM,  $10^{-7}$  mM, and  $10^{-9}$  mM of PRDX6 (Sigma-Aldrich, USA). The samples were placed 5–10 cm above the surface of liquid nitrogen for 30 min and then kept in liquid nitrogen. After cryopreservation for two weeks, semen samples were taken out, immediately thawed at room temperature for 1 min and then incubated in a water bath at  $35 \pm 2^\circ\text{C}$  for another 30 s. Finally, total motility, progressive motility (PR), and morphology of sperms were assessed within 30 min after thawing.

#### *Measurement of sperm motility*

The sperm motility was analyzed by a computer-assisted sperm analysis program (CASA, WLJY-9000, China), using pre-cryopreservation and post-thaw samples. Briefly, 10  $\mu\text{L}$  of each sample was mixed with the dye and dropped onto the slide. No less than 200 spermatozoa in each sample were counted. The percentage of stained and unstained spermatozoa was calculated on each slide under a phase-contrast microscope (Sun *et al.*, 2018). Sperm motility parameters, including total motility, PR, nonprogressive motility (NP), and immobility (IM), were recorded. Each experiment was repeated at least three times.

#### *Statistical Analyses*

SPSS 19.0 software (IBM Corp.) was used for statistical analysis. Data were expressed as mean  $\pm$  SD and analyzed using Student's paired *t*-test. A value of  $p < 0.05$  was considered statistically significant.

## Results

#### *Characteristics of the study population*

The characteristics of 20 males, as well as their fresh semen samples, were presented in Tab. 1. All characteristics of semen (volume, concentration, PR, NP, total motility, normal morphology) were normalized according to the 5th

edition of WHO Laboratory Manual for the Examination and Processing of Human Semen (Shu *et al.*, 2013).

#### *Effects of PRDX6 on sperm total motility, PR, and NP after cryopreservation*

To explore the effects of PRDX6, we treated the sperm samples with different concentrations of PRDX6 (0 mM,  $10^{-5}$  mM,  $10^{-7}$  mM, and  $10^{-9}$  mM) and tested the sperm activity after cryopreservation. We showed that activities of PR, NP, and total motility, were significantly reduced after cryopreservation compared to the fresh semen ( $p < 0.05$ ) (Tab. 2). Total motility and PR of PRDX6-treated samples were significantly different compared to those of pre-cryopreservation and post-thawing without PRDX6 treatment ( $p < 0.05$ ) (Tab. 2). These results suggest that PRDX6 improves total motility and PR of sperm.

#### *Determination of the optimum concentration of PRDX6 for protecting sperm total motility and PR after cryopreservation*

We further investigated the optimum concentration of PRDX6 for protecting sperm total motility and PR after cryopreservation. We found that the difference in sperm total motility at  $10^{-7}$  mM concentration was significant compared to the other two dosages ( $p < 0.05$ ) (Fig. 1). In contrast, there were no statistical differences among 0 mM,  $10^{-5}$  mM, and  $10^{-9}$  mM PRDX6 treatments (Fig. 2). Overall, these results indicate that  $10^{-7}$  mM is the optimum concentration of PRDX6 for protecting sperm total motility and PR after cryopreservation.

## Discussion

In the past few decades, semen cryopreservation is usually used in male fertility preservation and assisted reproductive technology. However, sperm motility gets impaired during the freezing-thawing process. Moreover, the freezing-thawing also influences early embryo development and decreases clinical pregnancy rates in assisted reproductive technology (Ferrari *et al.*, 2016; Nijs *et al.*, 2009; O'Connell *et al.*, 2002; Ozkavukcu *et al.*, 2008).

Sperm motility of post-thaw semen is decreased compared to fresh semen (Biagi *et al.*, 2019; Mehaisen *et al.*, 2020; Pariz *et al.*, 2019). The cryopreserved spermatozoa have also been found to adversely affect assisted reproductive outcomes (Depalo *et al.*, 2016; Wang *et al.*, 2009). This study found that PRDX6 had a very good protective effect on human spermatozoa. Our findings show, for the first time, that PRDX6 can act as a cryoprotectant to maintain high total motility and PR of human spermatozoa.

Many studies have shown that the reactive oxygen species (ROS) generation may be the main disadvantage of sperm cryopreservation, which, however, can be relieved by antioxidants (Banihani *et al.*, 2014; Deng *et al.*, 2017). PRDX6 is the only antioxidant present in seminal plasma with wide subcellular distributions (plasma membrane, mitochondria, cytosol, nucleus, and endoplasmic reticulum) (Gong *et al.*, 2012; Rhee *et al.*, 2005; Wood *et al.*, 2003). Therefore, it is an essential component of tissues and cells. In subfertile Prdx6 $^{-/-}$  male mice, sperm motility was lower than that in the C57BL/6J wild-type controls due to the inhibition of energy-generating enzymes and the thiol

TABLE 1

#### Characteristics of the subjects and the semen samples before cryopreservation

Characteristics	
Male age (y)	31.62 $\pm$ 4.62
Volume (ml)	3.15 $\pm$ 1.32
Concentration (mM)	76.86 $\pm$ 10.45
Progressive motility, PR (%)	53.46 $\pm$ 15.65
Non-progressive motility, NP (%)	14.71 $\pm$ 7.41
Total motility (%)	68.17 $\pm$ 17.98
Normal morphology (%)	4.67 $\pm$ 1.06

TABLE 2

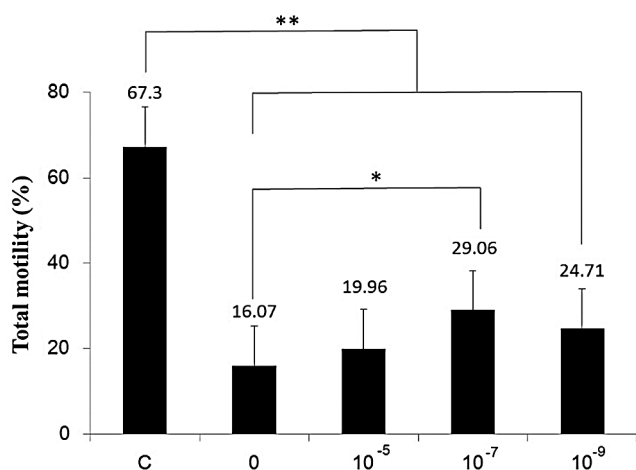
Effect of different PRDX6 concentrations on sperm total motility, PR, and NP after cryopreservation

Groups (PRDX6 concentration)	Total motility	Progressive motility (PR)	Non-progressive motility (NP)
Pre-cryopreservation			
Control	67.30 ± 13.55	54.96 ± 10.44	12.35 ± 6.14
Post-thawing			
0 mM	16.07 ± 10.44	13.58 ± 9.90	2.60 ± 1.73
10 <sup>-5</sup> mM	19.96 ± 13.95	16.67 ± 13.16	3.29 ± 1.87
10 <sup>-7</sup> mM	29.06 ± 18.96*#	23.87 ± 18.38*#	3.9 ± 1.89
10 <sup>-9</sup> mM	24.71 ± 14.02	21.70 ± 12.90	3.01 ± 1.40

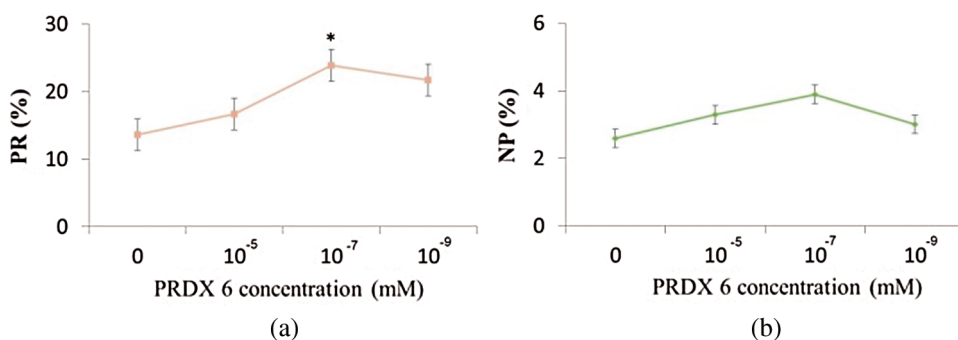
The average values for series of experiments are given and showed as means ± SD.

\*There is a significant difference between the data of post-thawing and pre-cryopreservation ( $p < 0.05$ ).

#there is a significant difference between the data of post-thawing samples with PRDX6 treatment and post-thawing samples without PRDX6 treatment ( $p < 0.05$ ).



**FIGURE 1.** Effect of different PRDX6 concentrations on sperm total motility. In the control (fresh) group, the spermatozoa were counted directly without PRDX6 supplementation. The final concentration of PRDX6 were 0, 0.001, 0.01, 0.1, and 1 mM, respectively. Data are the mean ± SD from three separate experiments. \* $p < 0.01$  compared to other groups; \*\* $p < 0.05$ , compared to 0 mM group.



**FIGURE 2.** The optimum concentration of PRDX6 on sperm PR and NP after cryopreservation. (A): progressive motility (PR); (B): non-progressive motility (NP). In the 0 mM-group, the spermatozoa were frozen in medium without PRDX6 supplementation. The other groups of PRDX6 were 0.001, 0.01, 0.1, and 1 mM, respectively. Data are the mean ± SD from three separate experiments. \* $p < 0.05$  compared to 0 mM group.

oxidation of tubulin (Moawad *et al.*, 2017). This is also consistent with our observations that PRDX6 exerted its protective effect on total motility and PR of freeze-thawing sperm through its antioxidant properties.

Spermatozoa are sensitive to high levels of ROS that cause lipid peroxidation in sperm (Rao *et al.*, 1989). Many studies showed that both peroxidase and iPLA2 activities of PRDX6 are essential to control ROS levels in human spermatozoa (O'Flaherty, 2014; O'Flaherty and Souza, 2011; Zini *et al.*, 1993). A few other studies revealed the protective

role of PRDX6 against ROS in a dose-dependent manner (Fisher, 2019; Moawad *et al.*, 2017; O'Flaherty and Souza, 2011). We observed a similar protective role of PRDX6 in this study, suggesting that PRDX6 might act as a ROS scavenger and a human sperm cryoprotectant. In addition, we found that the optimum concentration of PRDX6 was 10<sup>-7</sup> mM.

In conclusion, supplementation of PRDX6 to human spermatozoa freezing medium could improve total sperm motility and PR. Further studies regarding the effects of

PRDX6 on improving the integrity of the plasma membrane and mitochondrial membrane will be conducted to better understanding the mechanisms of PRDX6.

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**Availability of Data and Materials:** The datasets used or analyzed during the current study are available from the corresponding authors 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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