ORIGINAL ARTICLE

JOURNAL OF
Obstetrics and Gynecological
INVESTIGATIONS

J Obstet Gynecol Investig 2018; 1: e6–e12
DOI: https://doi.org/10.5114/jogi.2018.73423

Submitted: 20 July 2017
Accepted: 28 January 2018

Cryopreservation of human sperm in the presence of Zn^{2+} increases the motility rate

Arie Berkovitz[1], Deborah Allouche-Fitoussi[2], Diana Izhakov[2], Haim Breitbart[2]

[1]Meir Hospital, Kfar-Saba, Israel
[2]Bar-Ilan University, Ramat Gan, Israel

Abstract

Introduction: The aim of the study was to study the effect of Zn^{2+} addition to human sperm prior to and after cryopreservation on post-thaw sperm motility.

Material and methods: Human semen was liquefied, and the semen was loaded on a gradient and centrifuged for 10 min at 3000 g at room temperature. The lower layer containing the sperm was collected, resuspended in Ham's F-10 medium then centrifuged again, and the sperm were allowed to "swim up" after the last wash at 37°C. The motile cells were collected, resuspended in capacitation medium and supplemented with 0.1% human serum albumin. Samples with or without $ZnCl_2$ were kept at room temperature for 10 min, after which they were mixed with equal volumes of cryoprotective medium and aliquots were transferred to screw-top cryovials and stored in liquid nitrogen for several days. The samples were removed from liquid nitrogen, subjected to rapid thawing and washed in HamF-10 medium. Sperm motility was analyzed using computer-assisted sperm analysis (CASA).

Results: Freezing of human sperm in the presence of Zn^{2+} led to a significant increase in the number of motile sperm as well as preserving motility for a longer period of time. The percentage of sperm presenting progressive motility was also increased by Zn^{2+} depending on the fluid volume used for freezing. The presence of Zn^{2+} in the freezing medium also protects sperm motility after second freezing.

Conclusions: In order to achieve good motility after freezing, human sperm should be frozen in 0.1 ml of medium containing 50 μM Zn^{2+}.

Key words: sperm, cryopreservation, motility, Zn^{2+}.

Corresponding author: Prof. Haim Breitbart, Bar-Ilan University, The Mina and Everard Goodman Faculty of Life Sciences, Max & Anna Webb St, 5290002 Ramat Gan, Israel, phone: 972-3-5318201, e-mail: Haim.Breitbart@biu.ac.il

To cite this article: Berkovitz A, Allouche-Fitoussi D, Izhakov D, Breitbart H. Cryopreservation of human sperm in the presence of Zn^{2+} increases the motility rate. J Obstet Gynecol Investig 2018; 1: e6–e12. DOI: https://doi.org/10.5114/jogi.2018.73423

Introduction

The practice of human sperm freezing using liquid nitrogen was first introduced in the 1960s [1] and is currently routinely used in the assisted reproductive laboratory. Recovery of motile sperm after thawing is essential for further fertility treatment. However, risks following thawing including thermal shock, formation of intracellular ice crystals and osmotic shock can cause cell membrane damage and cell death that might lead to a reduced percentage of motile sperm [2, 3]. Indeed, the most common detrimental effects of the freeze-thaw sperm process include reduced motility, membrane damage, ultrastructural changes [4], DNA damage [5] and loss of mitochondrial function [6]. Various substances have been used for enhancing human sperm resistance to the stress of cryopreservation-thawing. Of these progesterone and/or acetyl carnitine were found not to be adequately effective in preventing human sperm stress after thawing [7]. Brugnon *et al.* reported that density gradient centrifugation prior to cryopreservation and hypotaurine supplementation improve post-thaw quality of sperm. However, total motility and progressive motility were in the low range of 18% and 11%, respectively [8]. A different method for sperm freezing by vitrification (without the use of conventional cryoprotectants) which requires 1% human serum albumin and 0.25 M of sucrose was compared to the conventional cryoprotectant freezing protocol. Compared to the initial sperm motility prior to freezing (74%), no statistically significant differences between the two methods were observed after thawing, in total motility and rapid progressive motility (20% and 11%, respectively) [9].

The aim of the present study was to assess the improvement in sperm cryopreservation conditions by adding Zn^{2+} to the freezing medium. Cryopreservation causes oxidative stress to the spermatozoa [2, 3] while it is known that Zn^{2+} acts as an antioxidant [10]. In human sperm, Zn^{2+} has been shown to improve membrane integrity and reduce DNA damage [11, 12]. Moreover, the addition of Zn^{2+} to human semen before freezing prevents DNA damage and preserves sperm function [13].

It is known that protein kinase A(PKA) mediates sperm motility [14]. In a recent study we demonstrated that Zn^{2+} activates PKA in sperm, which is mediated by the G-protein-coupled receptor GPR39 [15]. Zn^{2+} activates adenylyl-cyclase in sperm [15], and the increase in cAMP leads to actin polymerization [16, 17], a process necessary for the regulation of sperm motility [18]. The increase in flagellar beat required for the development of hyper-activated motility is also mediated by cAMP [19, 20]. Zn^{2+} is required for normal physiological function and development [21, 22]. The testes contain a high concentration of Zn^{2+} [23], which is essential in both the earlier and late stages of spermatogenesis [24, 25]. It has been suggested that extracellular Zn^{2+} activates a signal transduction pathway by several mechanisms, including the activation of ZnR, a member of the G-protein-coupled receptor (GPCR) family [26].

A high concentration of Zn^{2+} is needed for the earlier and the late stages of spermatogenesis in the testes [24, 25]. However, after penetration into the female reproductive tract, sperm are exposed to 1.0–1.5 μM Zn^{2+} [27], which is about 1000 times lower compared to its concentration in the seminal plasma. Thus, under physiological conditions in the female reproductive tract Zn^{2+} may enhance sperm capacitation and fertilization ability. Sperm motility is known to be extremely reduced after the freeze-thaw process, thus further highlighting the importance in improving the cryopreservation protocol, in order to maintain a higher motility rate after freezing. The aim of the present study was to verify the enhancement of sperm motility by Zn^{2+} after thawing.

Material and methods

Sperm preparation

After obtaining informed consent, the left over spermatozoa after IVF or ICSI procedures were transferred for freezing evaluation to the andrology laboratory of Bar-Ilan University. It should be emphasized that since our research was performed with left over spermatozoa, the length of time between ejaculation and freezing was approximately 4 to 6 h, in all the samples.

Human semen was liquefied by incubation for 30 min at room temperature; afterwards, the semen was loaded on a gradient (PureCeption Lower and Upper Phase Gradient 80% and 40%) and centrifuged for 10 min at 3000 g at room temperature. The lower layer containing the sperm was collected and washed in Ham's F-10 medium. The motile cells were collected and resuspended in Ham's F-10 medium containing 21 mM HEPES, 4 mM sodium bicarbonate, 0.6% human serum albumin, and 3.6 ml sodium lactate (60% syrup). The lower layer containing the sperm was collected and washed twice in Ham's F-10 medium, then centrifuged again, and the sperm were allowed to "swim up" after the last wash at 37°C. The motile cells were collected without the pellet and resuspended in capacitation medium and supplemented with 0.1% human serum albumin (HSA, Sigma-Aldrich, USA, Cat. No. A1653).

This procedure allowed us to obtain motile sperm without leukocyte contamination.

Statement about IRB: An informed consent form was signed to allow further evaluation of the remaining spermatozoa. No further statement of Institutional Review Board is required.

Cryopreservation and thawing

The liquefied ejaculates were divided into two aliquots; $ZnCl_2$ was added to one aliquot and the other aliquot served as a control. The samples were kept at room temperature for 10 min, after which they were mixed with equal volumes of cryoprotective medium (sperm freezing medium kit, Quinn's Advantage, SAGE). The aliquots were transferred to screw-top cryovials and stored in liquid nitrogen for several days, as previously described. The samples were removed from liquid nitrogen, subjected to rapid thawing and washed in HamF-10 medium.

Sperm motility determinations

Sperm cells (1×10^7 cells/ml) were incubated in capacitation medium HamF-10 for 1, 2, 3 or 4 h. Samples (5 μl)

were taken out at the indicated time and placed in a pre-warmed standard counter four-chamber slide (20 l m-depth) (Leja, Nieuw-Vennet, Netherlands) at 37°C, and analyzed using a CASA (computer-aided sperm analysis) device with IVOS software (version 12, Hamilton-Thorne Biosciences). Up to 10 sequences, each 30 s long, were acquired for each sample. At least 700 cells were analyzed in each sample, according to parameters identifying human sperm motility.

Results

Freezing of human sperm in the presence of 50 µM Zn^{2+} revealed a 26%-184% increase in the number of motile sperm in 10 out of 13 semen samples (Table 1). In 3 semen samples a decrease in total motility was observed after freezing with Zn^{2+}. Freezing in the presence of Zn^{2+} also showed a 130% increase in sperm presenting progressive motility (Figure 1). Similar results were obtained when motility determination was performed at zero time or after 1 h of incubation (Figure 1). These data clearly indicate that the presence of Zn^{2+} in the freezing medium significantly improved sperm motility after freezing-thawing. Incubation of frozen sperm after thawing revealed a gradual reduction in the number of motile cells, reaching zero motility after 4 h of incubation; however, this reduction did not occur when sperm were frozen in medium containing Zn^{2+} (Figure 2).

Table 1. Effect of Zn^{2+} in freezing medium on sperm motility: Human sperm were frozen in freezing medium with or without 50 µM $ZnCl_2$. After thawing 1×10^7 cells/ml were incubated in the capacitation medium Ham's F-10 for 1 h. Samples were taken for total motility measurement analyzed by CASA using the IVOS device as described in Material and methods

Sample no.	Semen volume [ml]	Concentration in millions	Total motility cont. (%)	Total motility Zn^{2+} (%)	Enhancement by Zn^{2+} (%)
1	0.5	45	7.40	4.70	None
2	0.5	150	8.00	18.00	125
3	2.0	7	5.00	10.00	100
4	0.5	10	2.30	5.00	117
5	0.5	35	1.70	1.07	None
6	0.5	60	1.30	2.10	61
7	0.5	15	1.03	1.30	26
8	0.5	5	12.60	17.30	37
9	0.5	10	18.30	11.00	None
10	3.2	55	9.00	16.00	77
11	3.0	28	2.20	3.10	40
12	2.5	17	9.20	17.00	85
13	1.5	15	10.10	17.20	70
14	0.6	25	7.50	21.33	184

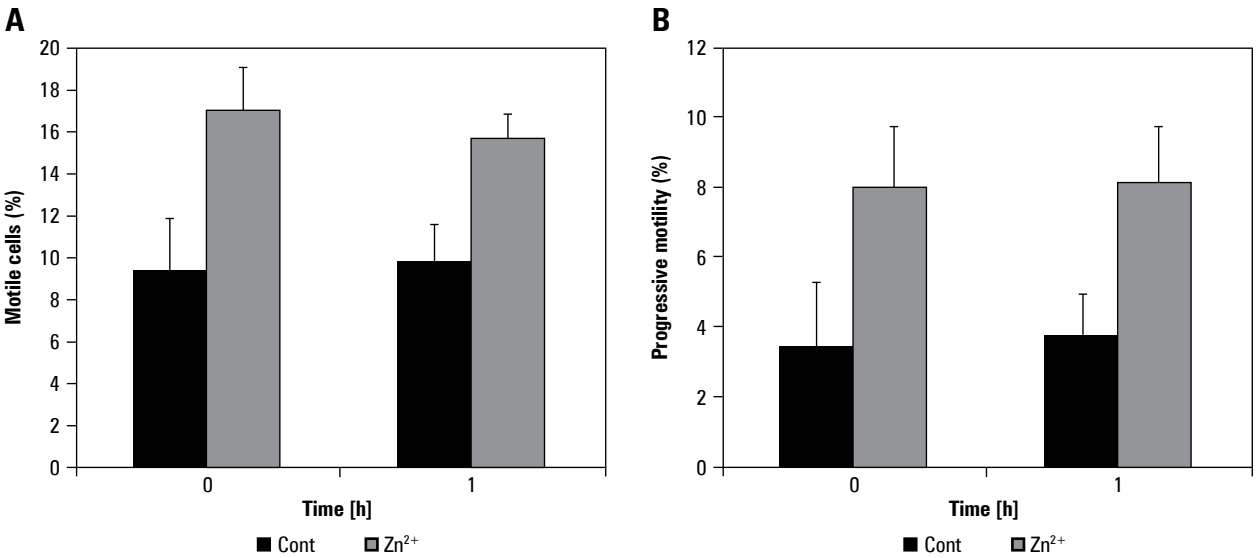

Figure 1. Human spermatozoa were frozen in freezing medium, with or without Zn^{2+} (50 µM). After thawing 1×10^7 cells/ml were incubated in the capacitation medium Ham's F-10 for 1 h. Samples of cells were taken out at the indicated time and (**A**) total motility and (**B**) progressive motility were analyzed by CASA using the IVOS device, as described in Material and methods

Thus, we conclude that the presence of Zn^{2+} in the freezing medium enhances the percentage of motile sperm and preserves motility for a longer period of time.

The percentage of cells presenting progressive motility was also significantly increased depending on the fluid volume used for freezing (Figure 3). Freezing in 100 µl resulted in a two-fold improvement compared to freezing in 50 µl when both total and progressive motility were determined (Figure 3). The presence of Zn^{2+} in the freezing medium revealed about a 2-fold increase in the number of motile cells and cells presenting progressive motility when 50 µl of freezing medium was used, whereas in 100 µl there was about a 3-fold increase in total and progressive motility (Figure 3). We can conclude that the sample volume used for freezing the cells is critical for achieving a higher proportion of motile cells, especially in medium containing Zn^{2+}.

The presence of Zn^{2+} also protects sperm motility after a second freezing. When frozen cells were thawed and then refrozen in the presence of Zn^{2+}, there was a 48% and 93% increase in total and progressive motility, respectively (Figure 4). After the second freezing and thawing 2% and 1% of the cells were motile or showed progressive motility, respectively, compared to slightly higher rates of 3% and 2% (total and progressive motility, respectively) when the second freezing was performed with Zn^{2+}. These data clearly indicate that Zn^{2+} protects the cells against loss of motility even after a second freezing.

We also tested the effect of glucose and pentoxifylline on sperm motility after freezing with Zn^{2+}. Glucose increases intracellular ATP levels by operating the glycolysis pathway. Pentoxifylline, an inhibitor of the enzyme phosphodiesterase which hydrolyzes cAMP, will cause an increase in intracellular levels of cAMP. It is well known that both ATP and cAMP are necessary molecules for functional sperm motility. The data in Figure 5 show that glucose plus pentoxifylline caused 93% stimulation of progressive motility after the second freezing and thawing, whereas when cells were frozen with Zn^{2+} there was a 154% increase in progressive motility. No significant differences were observed by adding glucose or pentoxifylline when cells were frozen in the presence or absence of Zn^{2+} in the freezing medium. Addition of Zn^{2+} plus glucose caused 43% and 71% increases in progressive motility when cells were frozen in the absence or presence of Zn^{2+}, respectively (Figure 5). Thus, we conclude that the best conditions to achieve good motility are to freeze the sperm in 100 µl of medium containing 50 µM Zn^{2+} and to add glucose and pentoxifylline to the cell suspension after thawing.

Discussion

The data described here clearly indicate that the presence of Zn^{2+} in the freezing medium significantly improved sperm motility after freezing-thawing. It was previously shown that the presence of 50 µM Zn^{2+} in human sperm cryopreservation increases sperm total motility by 12.6% and progressive motility by 8.4% [13], while under the conditions

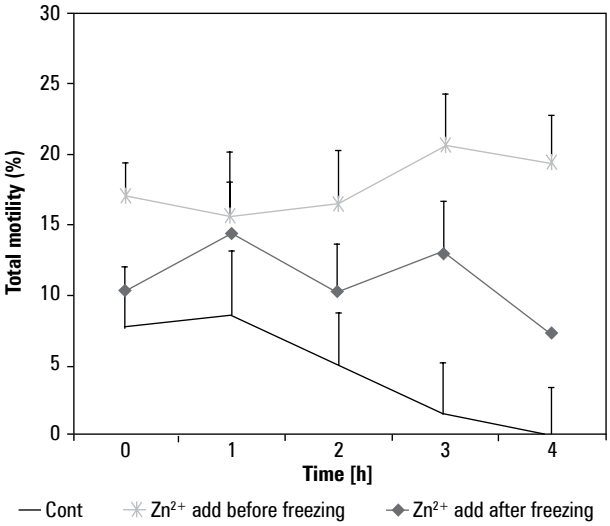

Figure 2. Human spermatozoa were frozen in freezing medium, with or without Zn^{2+} (50 µM). After thawing, Zn^{2+} (50 µM) was added to 1×10^7 cells/ml before incubation in the capacitation medium Ham's F-10 for 1, 2, 3 or 4 h. Samples of cells were taken out at the indicated time and total motility was analyzed by CASA using the IVOS device, as described in Material and methods

of our study a considerably larger increase of motility was demonstrated (Table 1).

The presence of Zn^{2+} in the freezing medium increases the percentage of motile sperm and preserves motility for a longer period of time (Figure 2). There are contradictory reports regarding the effect of Zn^{2+} on sperm motility. Several studies have suggested that Zn^{2+} inhibits motility [28, 29], whereas other reports show that Zn^{2+} enhances sperm motility [30, 31]. The higher percentage of motile sperm after freezing in the presence of Zn^{2+} observed in the present study can be attributed to the stabilizing effect of Zn^{2+} on microfilaments in the outer dense fibers [32]. We found that Zn^{2+} stimulates production of actin filaments in human sperm (data not shown), a process found to enhance human sperm motility [18]. In addition, Zn^{2+} may stabilize the sperm plasma membrane during the freeze-thaw process [33, 34], thus protecting the sperm from motility damage.

The data also clearly indicate that Zn^{2+} protects the cells against loss of motility even after a second freezing (Figure 4).

One of the main problems in semen cryopreservation concerns DNA damage [5] and loss of mitochondrial function [6]. It was previously shown that semen samples cryopreserved in the presence of Zn^{2+} had a higher percentage of sperm with intact DNA and better mitochondrial function compared to freezing without Zn^{2+} [13]. These data provide further support to our suggestion to add Zn^{2+} to the cryopreserved medium.

We also tested the effect of glucose and pentoxifylline on sperm motility after freezing with Zn^{2+}. The data in Figure 5 show that glucose plus pentoxifylline caused 93% stimulation of progressive motility after the second freezing and thawing, whereas when cells were frozen with Zn^{2+} there was a 154% increase in progressive motility. Thus, we conclude

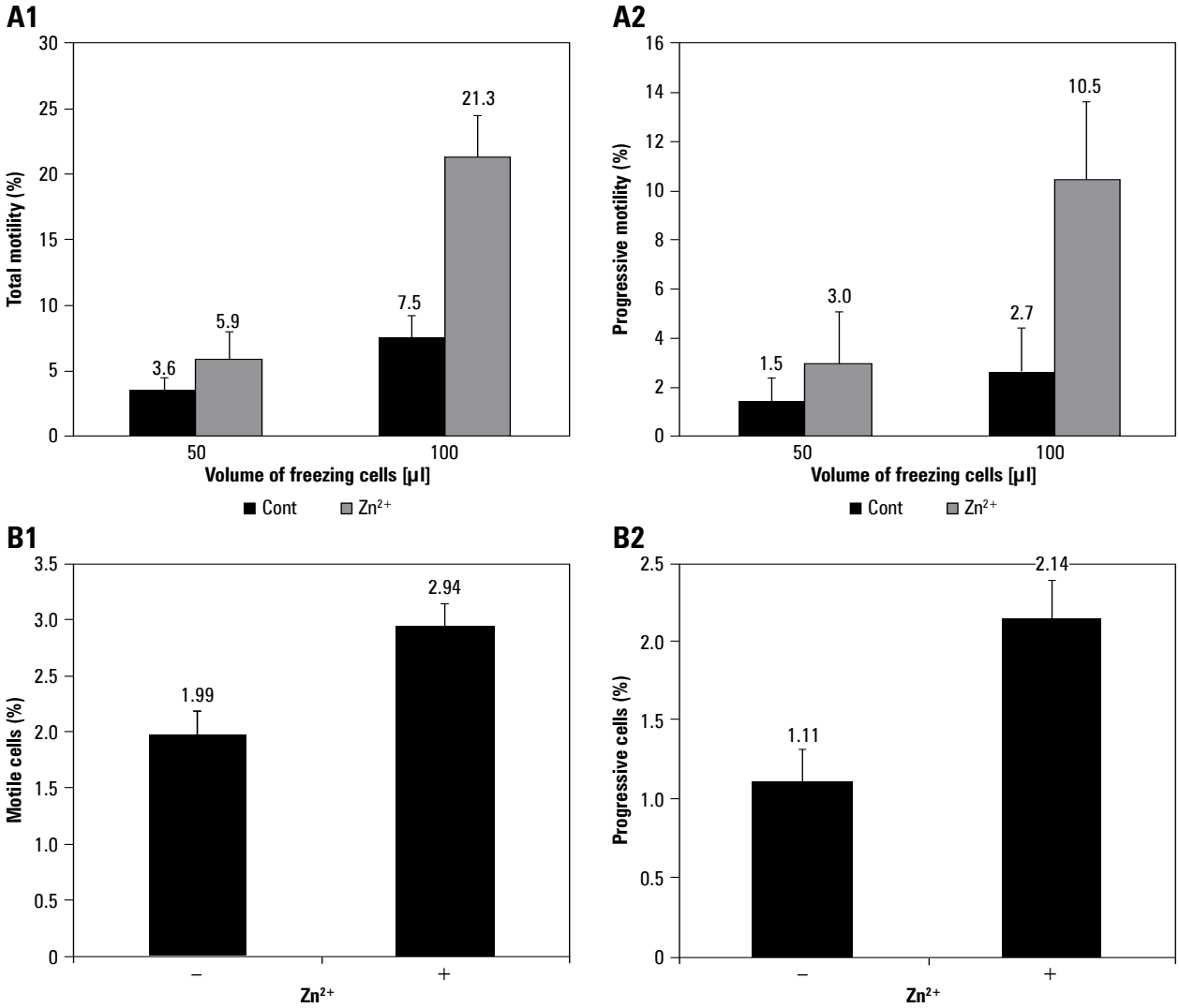

Figure 3. **A** – 50 and 100 µl of human spermatozoa were frozen in freezing medium, with or without Zn^{2+} (50 µM). After thawing 1×10^7 cells/ml were incubated in the capacitation medium Ham's F-10 for a few minutes. (1) Total and (2) progressive motility was analyzed by CASA using the IVOS device, as described in Material and methods. **B** – 100 µl of human sperm were frozen for the second time in freezing medium, with or without Zn^{2+} (50 µM). After thawing 1×10^7 cells/ml were incubated in the capacitation medium Ham's F-10 for a few minutes. (1) Total and (2) progressive motility was analyzed by CASA using the IVOS device, as described in Material and methods

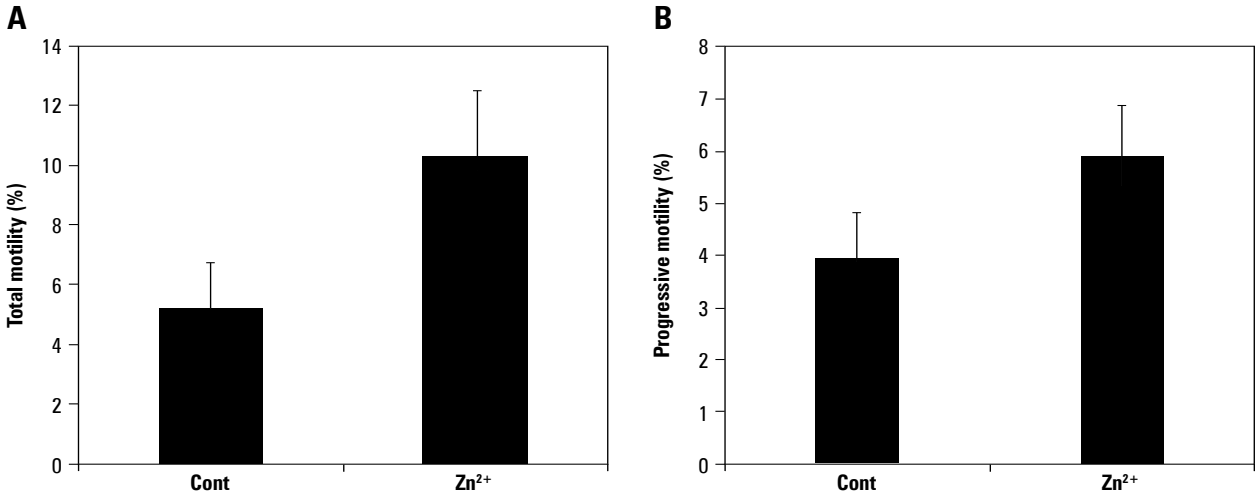

Figure 4. Human spermatozoa were frozen in freezing medium, with or without Zn^{2+} (50 µM). After thawing 1×10^7 cells/ml were incubated in the capacitation medium Ham's F-10 for a few minutes. Samples of cells were taken out and (**A**) total and (**B**) progressive motility was analyzed by CASA using the IVOS device, as described in Material and methods

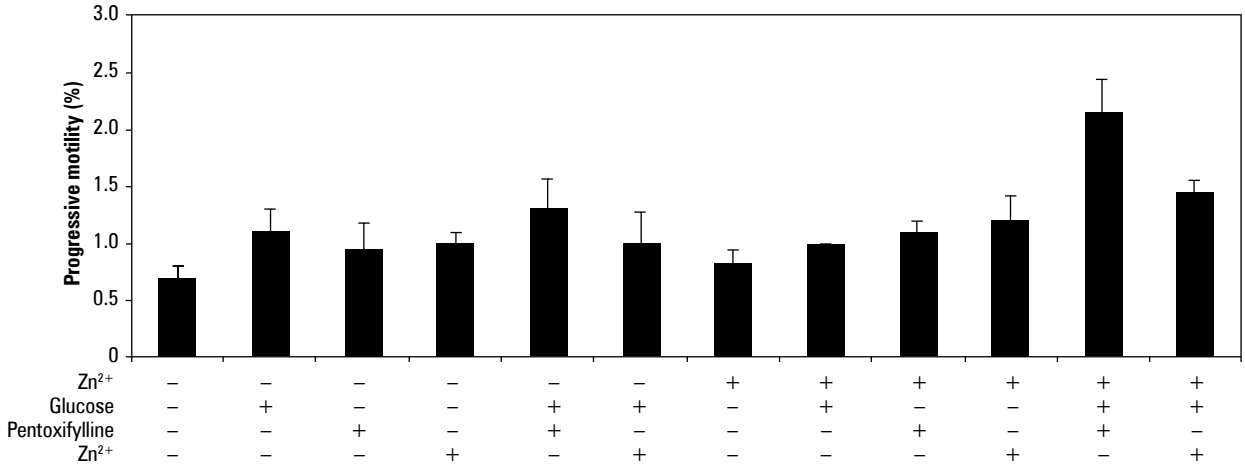

Figure 5. Human spermatozoa were frozen a second time in freezing medium, with or without Zn^{2+} (50 µM). After thawing, Zn^{2+} (50 µM), glucose (5 µM), and pentoxifylline (3.6 mM0 were added to 1×10^7 cells/ml before incubation in the capacitation medium Ham's F-10 for a few minutes. Samples of cells were taken out and the progressive motility was analyzed by CASA using the IVOS device, as described in Material and methods

that the best conditions to achieve good motility are to freeze the sperm in 100 µl of medium containing 50 µM Zn^{2+} and to add glucose and pentoxifylline to the cell suspension after thawing.

Several reports have suggested that Zn^{2+} inhibits sperm capacitation and the acrosome reaction, two processes essential for successful fertilization [29, 35, 36]. In our recent study we showed that Zn^{2+} in micromolar concentration stimulates bovine sperm capacitation and the acrosome reaction [15]. In human sperm we also found that Zn^{2+} stimulates sperm capacitation including stimulation of protein tyrosine phosphorylation and hyper-activated motility (submitted for publication). It was also demonstrated elsewhere that survival, the rate of capacitation and the acrosome reaction are significantly enhanced in human semen samples cryopreserved in the presence of Zn.

Conclusions

Our data clearly demonstrated that freezing sperm in the presence of Zn^{2+} significantly enhanced sperm total and progressive motility.

Conflict of interest

The authors declare no conflict of interest.

References

1. Sherman JK. Synopsis of the use of frozen human semen since 1964: state of the art of human semen banking. Fertil Steril 1973; 24: 397-412.
2. Donnelly ET, Steele EK, McClure N, Lewis SE. Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation. Hum Reprod 2001; 16: 1191-9.
3. Donnelly ET, McClure N, Lewis SE. Cryopreservation of human semen and prepared sperm: effects on motility parameters and DNA integrity. Fertil Steril 2001; 76: 892-900.
4. Critser JK, Huse-Benda AR, Aaker DV, Arneson BW, Ball GD. Cryopreservation of human spermatozoa. III. The effect of cryoprotectants on motility. Fertil Steril 1988; 50: 314-20.
5. Zribi N, Feki Chakroun N, El Euch H, Gargouri J, Bahloul A, Ammar Keskes L. Effects of cryopreservation on human sperm deoxyribonucleic acid integrity. Fertil Steril 2010; 93: 159-66.
6. O'Connell M, McClure N, Lewis SE. The effects of cryopreservation on sperm morphology, motility and mitochondrial function. Hum Reprod 2002; 17: 704-9.
7. Duru NK, Morshedi M, Schuffner A, Oehninger S. Semen treatment with progesterone and/or acetyl-L-carnitine does not improve sperm motility or membrane damage after cryopreservation-thawing. Fertil Steril 2000; 74: 715-20.
8. Brugnon F, Ouchchane L, Pons-Rejraji H, Artonne C, Farigoule M, Janny L. Density gradient centrifugation prior to cryopreservation and hypotaurine supplementation improve post-thaw quality of sperm from infertile men with oligoasthenoteratozoospermia. Hum Reprod 2013; 28: 2045-57.
9. Slabbert M, du Plessis SS, Huyser C. Large volume cryoprotectant-free vitrification: an alternative to conventional cryopreservation for human spermatozoa. Andrologia 2015; 47: 594-9.
10. Powell SR. The antioxidant properties of zinc. J Nutr 2000; 130 (5S Suppl): 1447S-54S.
11. Omu AE, Al-Harmi J, Vedi HL, Mlechkova L, Sayed AF, Al-Ragum NS. Magnesium sulphate therapy in women with pre-eclampsia and eclampsia in Kuwait. Med Princ Pract 2008; 17: 227-32.
12. Barratt CL, Aitken RJ, Bjorndahl L, et al. Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications: a position report. Hum Reprod 2010; 25: 824-38.
13. Kotdawala AP, Kumar S, Salian SR, et al. Addition of zinc to human ejaculate prior to cryopreservation prevents freeze-thaw-induced DNA damage and preserves sperm function. J Assist Reprod Genet 2012; 29: 1447-53.
14. Mizrahi R, Breitbart H. Mitochondrial PKA mediates sperm motility. Biochim Biophys Acta 2014; 1840: 3404-12.
15. Michailov Y, Ickowicz D, Breitbart H. Zn^{2+}-stimulation of sperm capacitation and of the acrosome reaction is mediated by EGFR activation. Dev Biol 2014; 396: 246-55.

16. Cohen G, Rubinstein S, Gur Y, Breitbart H. Crosstalk between protein kinase A and C regulates phospholipase D and F-actin formation during sperm capacitation. Dev Biol 2004; 267: 230-41.
17. Etkovitz N, Rubinstein S, Daniel L, Breitbart H. Role of PI3-kinase and PI4-kinase in actin polymerization during bovine sperm capacitation. Biol Reprod 2007; 77: 263-73.
18. Itach SB, Finklestein M, Etkovitz N, Breitbart H. Hyper-activated motility in sperm capacitation is mediated by phospholipase D-dependent actin polymerization. Dev Biol 2012; 362: 154-61.
19. Shahar S, Hillman P, Lubart R, Ickowicz D, Breitbart H. Activation of sperm EGFR by light irradiation is mediated by reactive oxygen species. Photochem Photobiol 2014; 90: 1077-83.
20. Shahar S, Wiser A, Ickowicz D, Lubart R, Shulman A, Breitbart H. Light-mediated activation reveals a key role for protein kinase A and sarcoma protein kinase in the development of sperm hyper-activated motility. Hum Reprod 2011; 26: 2274-82.
21. MacDonald RS. The role of zinc in growth and cell proliferation. J Nutr 2000; 130 (5S Suppl): 1500S-8S.
22. Vallee BL, Falchuk KH. The biochemical basis of zinc physiology. Physiol Rev 1993; 73: 79-118.
23. Bedwal RS, Bahuguna A. Zinc, copper and selenium in reproduction. Experientia 1994; 50: 626-40.
24. Sorensen MB, Stoltenberg M, Henriksen K, Ernst E, Danscher G, Parvinen M. Histochemical tracing of zinc ions in the rat testis. Mol Hum Reprod 1998; 4: 423-8.
25. Yamaguchi S, Miura C, Kikuchi K, et al. Zinc is an essential trace element for spermatogenesis. Proc Natl Acad Sci USA 2009; 106: 10859-64.
26. Hershfinkel M, Moran A, Grossman N, Sekler I. A zinc-sensing receptor triggers the release of intracellular Ca2+ and regulates ion transport. Proc Natl Acad Sci USA 2001; 98: 11749-54.
27. Menezo Y, Pluntz L, Chouteau J, et al. Zinc concentrations in serum and follicular fluid during ovarian stimulation and expression of Zn2+ transporters in human oocytes and cumulus cells. Reprod Biomed Online 2011; 22: 647-52.
28. Danscher G, Hammen R, Fjerdingstad E, H. R. Zinc content of human ejaculate and the motility of sperm cells. Int J Androl [Internet] Blackwell Publishing Ltd 1978; 1: 576-81.
29. Riffo M, Leiva S, Astudillo J. Effect of zinc on human sperm motility and the acrosome reaction. Int J Androl 1992; 15: 229-37.
30. Stankovic H, Mikac-Devic D. Zinc and copper in human semen. Clin Chim Acta 1976; 70: 123-6.
31. Caldamone AA, Freytag MK, Cockett AT. Seminal zinc and male infertility. Urology 1979; 13: 280-1.
32. Calvin HI, Hwang FH, Wohlrab H. Localization of zinc in a dense fiber-connecting piece fraction of rat sperm tails analogous chemically to hair keratin. Biol Reprod 1975; 13: 228-39.
33. Bettger WJ, O'Dell BL. A critical physiological role of zinc in the structure and function of biomembranes. Life Sci 1981; 28: 1425-38.
34. Kendall NR, McMullen S, Green A, Rodway RG. The effect of a zinc, cobalt and selenium soluble glass bolus on trace element status and semen quality of ram lambs. Anim Reprod Sci 2000; 62: 277-83.
35. Andrews JC, Nolan JP, Hammerstedt RH, Bavister BD. Role of zinc during hamster sperm capacitation. Biol Reprod 1994; 51: 1238-47.
36. Liu DY, Sie BS, Liu ML, Agresta F, Baker HW. Relationship between seminal plasma zinc concentration and spermatozoa-zona pellucida binding and the ZP-induced acrosome reaction in subfertile men. Asian J Androl 2009; 11: 499-507.