

Effects of different Zinc levels in the sperm culture medium on sperm recovery and quality of sperms in the swim up procedure for sperm processing

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Abstract

A controlled *in vitro* study was carried out to observe the effect of different Zinc (Zn) levels on sperm recovery rate, chromosome integrity, cell membrane integrity and motility in the swim up procedure. Semen samples were obtained from males who underwent seminal fluid analysis at the Infertility Laboratory, Department of Obstetrics and Gynaecology, Faculty of Medicine, Ragama. Twenty normozoospermic samples were randomly selected for the study and each sample was processed with supplemented Earl's Balanced Salt Solution (EBSS) containing different concentrations of Zn [0.5ml of supplemented EBSS with 25 μ l of solution containing 0.6 μ mol (group 1) and 1.2 μ mol (group 2) of Zn respectively]. One aliquot processed with 25 μ l of physiological saline with added EBSS served as the control. Pre and post wash sperm counts and motility were recorded immediately after processing. Post wash sperms from the three groups were observed for chromosome integrity, cell membrane integrity, and motility. Motility changes after four hours of incubation were also observed.

The mean sperm concentration showed an increase in group 1 compared to the control sample [21.87 \pm 21.61 (SD) millions/ml

compared to 18.34 \pm 19.73 millions/ml, $P < 0.05$] whereas a reduction was observed in group 2 [16.25 \pm 17.73 (SD) millions/ml compared to 18.34 \pm 19.73 millions/ml, $P > 0.05$]. The mean differences in sperm concentration compared to the control showed statistically significant differences in both groups where an increase was observed in group 1 [3.52 \pm 4.96 (SD) millions/ml] and a reduction in group 2 (- 2.08 \pm 6.59 millions/ml).

The mean differences in sperm recovery rate showed significant differences in group 1 [8.97 \pm 14.04 (SD) millions/ml] and group 2 (-4.85 \pm 17.92 millions/ml) compared to the control. It was an increase in group 1 and a reduction in the sperm recovery rate in group 2.

A significant reduction in mean sperm motility was observed in group 2 [67.33% \pm 18.52 (SD) vs. 91.00% \pm 9.60, $P < 0.05$] after four hours of incubation. Though a reduction was observed in group 1 it was not statistically significant (83.33% \pm 8.72 vs. 93.60% \pm 5.01, $P > 0.05$). The motility reduction was significantly greater in group 2 compared to group 1 (26.01% \pm 20.24 vs. 10.97% \pm 8.35, $P < 0.05$). Chromosome integrity and cell membrane integrity of sperms were not affected by different Zn levels.

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In conclusion, low levels of Zn in the sperm processing medium (EBSS) have a beneficial effect on sperm recovery in the swim up procedure.

Key Words: Zn added EBSS, Harvesting rate, Chromosome integrity, Cell membrane integrity, motility.

Introduction

Management of male factor subfertility involves assisted reproductive technologies (ART) (1). Obtaining a good yield of high quality spermatozoa is a vital step in these techniques. Commonly used sperm processing techniques include swim up, density gradients centrifugation, glass wool filtration, migration sedimentation and centri-swim procedure (2, 3, 4). In practice they have their own advantages and shortcomings, and there is no clear evidence to suggest that one method is superior to another when pregnancy rates are considered (4, 5).

Whatever the technique used, culture media with minor differences in composition are used in washing the sperm. All culture media generally contain essential salts, energy substrates, a nitrogen source and a buffer. Several adjuvants including caffeine, pentoxifylline, follicular fluid, progesterone, platelet activating factor, cytokines, kallikrein, bicarbonate, metal chelators and some vitamins have been used to enhance sperm function and to minimize inter-cellular stress by providing a protective environment (6,7,8,9). Some have also incorporated antioxidants to sperm culture media but their beneficial effect on sperm function is not clearly established. Whether antioxidants improve the sperm function and subsequent fertilization capacity is an important area for further research (10).

Zinc (Zn) is present in high concentrations in seminal fluid and plays a multifaceted role in sperm functions (11). It helps to stabilize the cell membrane and nuclear chromatin of spermatozoa and is reported to be the primary factor responsible for antibacterial activity (12). Zn influences sperm motility and plays a regulatory role in the process of capacitation and acrosome reaction (13, 14). The antioxidant capacity of Zn has been reported by many authors (15). EBSS is one of the recommended media in the WHO manual for sperm processing (16). However, Zn is not a component of this medium, though other cations such as magnesium and calcium are included. The aim of the present study was to investigate whether the incorporation of Zn in the EBSS culture medium improves sperm recovery rate in the swim up procedure.

Material and Methods

The study was carried out as a laboratory based controlled study at the University Infertility Laboratory at Ragama. Ethical clearance was obtained from the Ethics Committee, Faculty of Medicine, Ragama.

Twenty males attending the infertility clinic with normozoospermia [based on the selection criteria in WHO manual 1999 (16)] over a period of three months from January 2006 were included. Semen samples were collected into sterile containers (Bibby Sterilin Ltd., England) and analysed within 30 minutes of collection. Immediately after the analysis, each sample was processed according to the swim-up sperm preparation method using the supplemented EBSS with added Zn.

Preparation of Zn solutions: Zn solutions of different concentrations were prepared using analytical reagent grade $ZnSO_4$ (BDH, Poole, England) and normal saline (0.9% w/v);

13.8 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ was dissolved in 1 ml of physiological saline; 25 μl of this solution contains 1.2 μmol of Zn.

Sperm processing: For a given seminal fluid sample three conical centrifuge tubes containing 0.5 ml of human serum albumin added EBSS (Sigma Chemical Co., St. Louis, USA) were prepared. To one tube 25 μl of solution containing 0.6 μmol of Zn was added (group 1); 25 μl of solution containing 1.2 μmol of Zn was added to another tube (group 2). A third tube with 25 μl of physiological saline solution without Zn served as the control. Each tube was gently under layered with 0.5 ml of a well mixed semen sample. Tubes were inclined at an angle of 45° and placed in an incubator (Sanyo MCO-17AIC-U, Loughborough, England) for one hour at 37°C in 5% CO_2 environment. Subsequently the uppermost 0.4 ml was diluted with 2.8 ml of EBSS medium and centrifuged (Fisher Scientific Model 225, Litho, U.S.A.) at 500 g for 5 minutes. The sperm pellet formed was re-suspended in 0.5 ml of EBSS. The re-suspended sperms were assessed for motility, chromosome integrity and cell membrane integrity. Samples were incubated at 37°C in 5% CO_2 for further four hours and reassessed for motility. Groups 1 and 2 were compared against the control group with regard to the above parameters. Percentage of sperm harvested from the raw sample (recovery rate) was calculated using the following equation.

Percentage of sperm harvested from the raw sample = $(\text{Total motile sperm population in washed sample} / \text{Total motile sperm population in raw semen sample}) \times 100$.

Assessment of motility and count: Sperm motility and count were assessed using a Makler counting chamber (Sefi Medical, Israel). Samples were observed under a

phase contrast microscope (Magnus Mxt, New Delhi, India) at a magnification of 400. **Evaluation of chromosome integrity:** The method followed was originally described by Tajeda et. al. in 1984 (17). Sperm smears were prepared on clean glass slides and air dried. They were fixed in Carnoy's solution (3 part methanol and 1 part glacial acetic acid) overnight at room temperature. Slides were removed from the fixer and air dried for three minutes, stained with acridine orange (AO) (BDH, Poole, England) for five minutes. The slides were then gently rinsed with deionized water and mounted with cover glass before drying. They were read under a fluorescence microscope (Olympus CH 40, Tokyo, Japan) using 490nm excitation and 530nm barrier filters ($\times 40$). At least 200 spermatozoa were counted. Green heads were counted as normal and yellow to red sperm heads as abnormal.

Assessing cell membrane integrity by the hypo-osmotic swelling test (HOS Test): Washed sperm samples (0.1 ml) were mixed with 1 ml swelling solution (0.735 sodium citrate dihydrate and 1.351 g fructose in 100 ml of distilled water) and incubated at 37°C for 30 minutes. A drop of the mixture was placed on a clean glass slide and covered with a cover glass (22 mm \times 22 mm). The number of swollen sperms (sperms with curved or bent tails) in each slide was counted using a phase contrast microscope at a magnification of 400.

Statistical Analysis

Analysis was done using SPSS 10.0 for windows. The results are presented as mean \pm standard deviation (SD) except sample population means which are expressed as mean \pm standard error of the mean (SEM). Comparison between means of Zn treated groups and controls was done using the Student's t-test and ANOVA.

Results

The sample population had a mean seminal fluid volume of 3.04 ± 0.26 ml, a mean concentration of 68.30 ± 6.9 millions/ml and a mean sperm motility of $61.60 \pm 3.8\%$.

The mean post wash sperm concentration, percentage of motile sperms in the processed samples and the sperm recovery rates are shown in Table 1. The mean \pm SD sperm concentration showed an increase in the group 1 compared to the control sample (21.87 ± 21.61 millions/ml compared to 18.34 ± 19.73 millions/ml, $P < 0.05$) whereas a reduction was observed in group 2 (16.25 ± 17.73 millions/ml compared to 18.34 ± 19.73 millions/ml, $P > 0.05$). The mean differences in sperm concentration compared to the control showed statistically significant differences in both groups where an increase was observed in group 1 (3.52 ± 4.96 millions/ml) and a reduction in group 2 (-2.08 ± 6.59 millions/ml).

The mean percentage motility was not different between the control, group 1 and group 2 ($91.80\% \pm 7.50$; $93.60\% \pm 5.01$; $91.00\% \pm 9.60$, $P > 0.05$).

The mean differences in sperm recovery rate

showed significant differences in group 1 ($8.97\% \pm 14.04$) and group 2 ($-4.85\% \pm 17.92$) compared to the control. It was an increase in group 1 and a reduction in the sperm recovery rate in group 2.

Changes in mean sperm motility were assessed following incubation of the processed sperm sample in Zn added media. The group 1 and 2 results are shown in Table 2. A significant reduction in mean sperm motility was observed in group 2 ($67.33\% \pm 18.52$ vs. $91.00\% \pm 9.60$, $P < 0.05$). Though a reduction was observed in group 1 it was not statistically significant ($83.33\% \pm 8.72$ vs. $93.60\% \pm 5.01$, $P > 0.05$).

The motility reduction was significantly greater in group 2 compared to group 1 ($26.01\% \pm 20.24$ vs. $10.97\% \pm 8.35$, $P < 0.05$).

The chromosomal integrity was not affected significantly by the addition of either concentration of Zn as evident by an absence of any difference in the number of sperms with red heads in either sample at the AO test. A similar observation was made with regard to cell membrane integrity as evident by the percentage of curve tailed sperms (Table 3).

Table 1. Comparison of sperm recovery rate, post wash count and motility with different Zn levels (mean \pm SD), (n = 20)

Parameter	Pre wash	Post wash		
		Control	Group 1	Group 2
Volume (ml)	3.04 ± 1.01	0.5	0.5	0.5
Concentration (millions/ml)	68.30 ± 26.75	18.34 ± 19.73	$21.87 \pm 21.61^*$	16.25 ± 17.73
Sperm recovery rate (%)		61.87 ± 37.64	$72.82 \pm 27.01^*$	52.64 ± 38.33
Motility (%)	61.60 ± 10.02	91.80 ± 7.50	93.60 ± 5.01	91.00 ± 9.60

* $p < 0.05$, compared to control

SD = Standard deviation

Table 2. Change in sperm motility following incubation in media containing different levels of Zn. (mean \pm SD), (n = 20)

	Control	Group 1	Group 2
Motility % (0 hr)	91.80 \pm 7.50	93.60 \pm 5.01	91.00 \pm 9.60
Motility % (4 hr)	78.83 \pm 14.31	83.33 \pm 8.72	67.33 \pm 18.52
% motility reduction	14.13 \pm 10.24	10.97 \pm 8.35	26.01 \pm 20.24*

* $p < 0.05$, compared to 0 hour
SD = Standard deviation

Table 3. Results of Acridine Orange (AO) and Hypo-osmotic swelling test (HOS Test) in the three groups (mean \pm SD), (n = 20)

	Control	Group 1	Group 2
AO test (green cells %)	92.38 \pm 9.25	94.92 \pm 6.29	88.46 \pm 16.42
HOS test (swollen sperms %)	81.83 \pm 13.92	80.00 \pm 10.50	84.14 \pm 9.13

SD = Standard deviation

Discussion

The WHO reference level of Zn in human semen ejaculate is 2.4 μ mol or more per total ejaculate (16).

The major contributor of Zn in the ejaculate is the prostate gland which plays a minor role in determining the total seminal fluid volume. The seminal fluid volume is mainly made of secretions from the seminal vesicles.

In this study the three groups make three seminal fluid environments with different concentrations of Zn. The control samples did not have any Zn while group 1 had a Zn concentration of 1.2 μ mol/ml. This is the same as the physiological Zn concentration of 1.2 μ mol/ml. Group 2 contained a higher concentration of Zn.

The sperm recovery rate was optimal in

group 1 whereas in the control (without Zn) and in group 2 this was significantly reduced. The reduction in the sperm recovery rate as the Zn concentrations deviate from the physiological levels is a novel finding which may have a clinical significance.

Reason for this increase in harvesting rate with addition of Zn could be attributed to increased antioxidant capacity of Zn. Antioxidants are reported to protect sperm membrane lipid from peroxidation and stabilize the membrane (13). As most of sperm functions are membrane functions Zn may help to improve their functional capacity by creating a favourable environment.

Progressive motility of sperms may be regulated by some divalent cations (Ca^{++} , Mg^{++} and Zn^{++}). However, the inhibitory or stimulatory effect on motility by these

cations depends on the concentration of each cation (14). It is well known that Zn interacts with other cations simultaneously in some functions of spermatozoa eg. Zn/Cadmium ratio affects the sperm motility and levels of antisperm antibodies (18). Some metalloenzymes such as lactate dehydrogenase and sorbitol dehydrogenase are known to contain Zn and they may help to the increase in sperm motility (19).

Favourable environment created by balanced ionic composition and increase in sperm motility through some mechanisms are other possible explanations for increase in harvesting rate in group 1.

Excess free Zn ion concentration in the media may act as a mechanical barrier to swim up is the reasonable explanation for reduction of the harvesting rate seen with high Zn levels in group 2. Addition of high levels of Zn to EBSS media caused a significant reduction in the percentage of motile sperms following four hours of incubation. The motility reduction could be due to elevated free Zn fraction and subsequent uptake by spermatozoa (20) and reduction of oxygen consumption, since high levels of Zn in semen impairs the oxygen consumption of sperms (21).

The chromosome integrated or green cells percentage was >70% in all samples and means were not significantly different between the Zn treated groups and the control. Similarly in Hypo-osmotic swelling test (HOS Test), the number of swollen sperms did not significantly differ at different Zn levels. AO test and HOS Test confirmed that semen preparation procedure (subjected to centrifugation force and elimination of most of Zn from the spermatozoa) and addition of extra Zn to the culture media does not alter the chromosome integrity and functional integrity of plasma membrane of sperm cells.

In conclusion, this study suggests that incorporation of Zn into the EBSS in concentrations close to the physiological range is beneficial in harvesting motile sperms in swim up procedure. But the optimum level favourable for sperms should be determined by testing different concentrations taking the sperm functional status also into consideration.

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References

1. Berkovitz A., Eltes F., Soffer Y., Zabludovsky N., Beyth Y., Farhi J., Levran D., Bartoove B. ART success and in vivo sperm cell selection depend on the ultra morphological status of spermatozoa. *Andrologia*. 1999; 31: 357-361.
2. Hammadeh H.E., Kuhnen A., Amer A.S., Rosenbauna P., Schmidt W. Comparison sperm preparation methods: effect on chromatin and morphology recovery rates and their consequences on the clinical outcome after in vitro fertilization embryo transfer. *International Journal of Andrology*. 2001; 24: 360-364.
3. Biefeld P., Babbo J., Ozornek H.M., Krussel J.S., Schuppe H.C., Hecht B.R., Jayendran R.S. A new Centri Swim procedure to increase the recovery of motile spermatozoa. *Andrologia*. 2001; 33: 356-358.
4. Babbo C.J., Hecht B.R., Jayendran R.S. Increased recovery of swim-up spermatozoa by application of "antigravitational" centrifugation. *Fertility and Sterility*. 1999; 72: 556-558.

5. Morales P., Vantman D., Barros C., Vigil P. Human spermatozoa selected by percoll gradient or swim-up are equally capable of binding to human zona pellucida and undergoing the acrosome reaction. *Human Reproduction*. 1991; 6: 401-404.
6. Brijinder S., Minhas B.A., Ripps B.S. Methods for enhancement of sperm function. *Frontiers in Bioscience*. 1996; 1: 65-71.
7. Morales P., Lianos M., Yovich J.L., Cummin J.M., Vigil P. Pentoxifylline increases sperm penetration in to zona free hamster oocytes without increasing the acrosome reaction. *Andrologia*. 1993; 25: 359-362.
8. Krausz C., Gervasi G., Forti G., Baldj E. Effect of platelet activating factor on motility and acrosome reaction on human spermatozoa. *Human Reproduction*. 1994; 9: 471-476.
9. Grigoriou O., Makrakis E., Konidaris S., Hassiacus D., Papadias K., Baka S., Creatsas G. Effect of sperm treatment with exogenous platelet activating factor on the outcome of intrauterine insemination. *Fertility Sterility*. 2005; 83: 618-621.
10. Ombelet W., Deblaere K., Bosmans E., Cox A., Jacobs P., Jonssen M., Nijs M. Semen quality and intrauterine insemination. *Reproductive Biomedicine*. 2003; 7: 485-492.
11. Kruse W.E., Zwick E.M., Batschulat K., Rohr G., Ambuster F.P., Petzoldt D., Strowitzki T. Are zinc levels in seminal plasma associated with seminal leucocytes and other determinants of semen quality. *Fertility Sterility* 2002; 77: 260-269.
12. Chia S.E., Ong C.N., Chua L.H., Ho L.M., Tay K.S. Comparison of Zinc concentrations in blood and seminal plasma and the various sperm parameters between fertile and infertile men. *Journal of Andrology*. 2000; 21: 53-7.
13. Hidirolou M., Knipfel J.E. Zinc in mammalian sperm: a review. *Dairy Science*. 1984; 67: 1147-1156.
14. Umeyama T., Ishikawa H., Takeshima H., Yoshii S., Koiso K. A comparative study of seminal trace elements in the fertile and infertile men. *Fertility and Sterility*. 1986; 46: 494-499.
15. Ykocg O.L., Ozdal M., Celik S., Balaban N. Antioxident activity of seminal plasma in infertile and fertile men. *Andrologia*; 2003; 35: 233-237.
16. World Health Organization. Laboratory manual for the examination of human semen and sperm-cervical mucus interaction. Fourth edition. Cambridge; Cambridge University Press, 1999.
17. Tejada R.I., Mitchel J.C., Norman A., Marik J.J., Friedman S. A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. *Fertility and Sterility*. 1984; 42: 87-91.
18. Omu A.E., Fernandes S. The relationship between zinc/ cadmium ratio in human semen: Effect on immune response. *The Kuwait Medical Journal* 2001; 33: 38-43.
19. Elzanaty S., Richthoff J., Malm J., Giwerzman A. The impact of epididymal and accessory sex gland function on sperm motility. *Human Reproduction*. 2002; 17: 2904-2911.
20. Carpino A., Siciliano L., Petrone M., Stefano C., Aquila S., Ando S. Low seminal Zinc bound to high molecular weight proteins in asthenozoospermic patients: evidence of increased sperm Zn content in oligoasthenozoospermic patients. *Human Reproduction*. 1998; 13: 111-114.
21. Wong W.Y., Flik G., Groenen P.M.W., Swinkels D.W., Thomas C.M.G., Copius-Peereboom J.H.J., Merkus H.M. W.M., Steegers-Theunissen R.P.M. The impact of calcium, magnesium, zinc and copper in blood and seminal plasma on semen parameters in men. *Reproductive Toxicology*. 2001; 15: 131-136.