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The importance of trace minerals copper, manganese, selenium and zinc in bovine sperm–zona pellucida binding

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Summary

Sperm–zona pellucida (ZP) binding is a necessary event for successful fertilization. The aim of this study was to determine the effect of trace minerals such as copper (Cu), manganese (Mn), selenium (Se) and zinc (Zn) on bovine spermatozoa binding to ZP. Sperm viability, functional membrane integrity, acrosomal status (AS), total antioxidant capacity (TAC) and sperm lipid peroxidation (LPO) were also evaluated. For the present study, *in vitro* fertilization (IVF) medium was supplemented with Cu (0.4 µg/ml Cu), Mn (5 ng/ml Mn), Se (100 ng/ml Se), Zn (0.8 µg/ml Zn), all minerals (Cu + Mn + Se + Zn), or tested without supplement (Control). Considerably more sperm bound to ZP when Cu, Se or Zn were added to the IVF medium, but there were no difference compared with the Control, Mn and Cu + Mn + Se + Zn groups. After 1 h of incubation, viability was increased by the addition of Cu, Mn and Se with respect to the Control but, after 2 h, viability was higher only with the addition of Mn to IVF medium. Functional membrane integrity improved in sperm treated with Cu. Acrosome integrity was higher in sperm treated with Zn after 1 h of incubation. LPO was significantly higher in sperm treated with Cu or Cu + Mn + Se + Zn. The mean TACs of sperm treated with Cu, Mn, Zn or Cu + Mn + Se + Zn were lower than in the Control. In conclusion, the results obtained in the present study determined that the presence of Cu, Se and Zn in the IVF medium increased the number of spermatozoa bound to the ZP, highlighting the importance of these minerals in the fertilization process.

Introduction

Binding of sperm to the zona pellucida (ZP) is one of the key events in the process of fertilization. In the ampulla of the oviduct, sperm penetrate the cumulus oophorus and bind to the ZP, which is the last barrier that sperm have to overcome before fertilizing the oocyte (Coutinho da Silva *et al.*, 2012). Sperm–ZP binding dysfunction is associated with male infertility because it produces a failed or sub-optimal sperm fertilizing capacity (Overstreet *et al.*, 1980; Oehninger *et al.*, 1997). For those reasons, ZP-binding assays are used to estimate sperm fertilizing capacity in several species including bovine (Ivanova and Mollova, 1993; Fazeli *et al.*, 1995; Zhang *et al.*, 1999; Coutinho da Silva *et al.*, 2012).

Small physiological concentrations of reactive oxygen species (ROS) are implicated in the control of normal sperm function, such as the ability of spermatozoa to bind to ZP (Aitken *et al.*, 2012). However, excessive ROS production induces DNA damage, impairs sperm motility and membrane permeability and may eventually induce sperm cell death (Barroso *et al.*, 2000; Gil-Guzman *et al.*, 2001; Aitken and Baker, 2004; Shamsi *et al.*, 2008; Aitken *et al.*, 2015). Copper (Cu), manganese (Mn), selenium (Se) and zinc (Zn) are present in all mammalian tissues and protect cells against ROS-induced damage (Underwood and Suttle, 1999; Chihuailaf *et al.*, 2002). Cu, Mn, Se and Zn improve sperm quality parameters (Lapointe *et al.*, 1996; Ursini *et al.*, 1999; Colagar *et al.*, 2009; Anchordoquy *et al.*, 2017), but very little information is known about their effects on sperm–ZP binding.

The objective of this study was to determine the effect of trace minerals Cu, Mn, Se and Zn on binding of bovine spermatozoa to the ZP. Moreover, sperm viability, sperm membrane integrity, acrosomal status (AS), total antioxidant capacity (TAC) and sperm lipid peroxidation were evaluated.

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Materials and methods

Reagents and media

Reagents for culture media and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (CAS 57360-69-7) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Standards as water solutions of copper sulphate, zinc sulphate, manganese sulphate and sodium selenite were purchased from Merck (Tokyo, Japan). The *in vitro* fertilization (IVF) medium used was TALP (Parrish *et al.*, 1986) supplemented with 6 mg/ml bovine serum albumin-fatty acid free (BSA-FAF), 20 µM penicillamine, 10 µM hypotaurine and 100 µg/ml heparin sulphate.

Oocyte recovery

Bovine ovaries obtained from slaughterhouse were transported within 3 h to the laboratory in NaCl solution (9 g/l NaCl) with streptomycin and penicillin at 37°C. Ovaries were used regardless of the stage of the oestrus cycle of the donor. Cumulus – oocyte complexes (COC) were aspirated from 3–8 mm follicles, using an 18-G needle connected to a sterile syringe. Only cumulus-intact complexes with evenly granulated cytoplasm were selected, using a low power (×20–30 magnification) stereomicroscope.

Semen preparation

In all experiments, frozen semen from a fertile bull, tested both *in vivo* and *in vitro*, was used. Straws containing 40×10^6 spermatozoa were thawed in a 37°C water bath and washed in a discontinuous Percoll gradient prepared by depositing 2 ml 90% Percoll under 2 ml 45% Percoll in a 15-ml centrifuge tube. Semen samples were deposited on the top of the Percoll gradient and centrifuged for 20 min at 500 *g*. The pellet was removed and resuspended in 300 µl of HEPES-TALP solution and centrifuged at 300 *g* for 10 min. For the binding assay, hypo-osmotic swelling test (HOST) and AS supernatant were discarded and spermatozoa were resuspended in IVF medium, counted in a haemocytometer chamber and further diluted. The final sperm concentration in IVF was 2×10^6 sperm/ml The incubation was conducted at 38.5°C in 5% CO_2 in air with saturated humidity. For the MTT assay, the final sperm concentration in IVF medium was 10×10^6 sperm/ml and 40×10^6 sperm/ml for TAC and the thiobarbituric acid-reactive substances (TBARS) assay.

Binding assays

Before each experiment, immature COCs were denuded by vortexing with 0.1% (w/v) hyaluronidase in HEPES-TALP, washed twice and incubated in droplets of 40 µl IVF medium for at least 2 h at 38.5°C in an atmosphere of 5% CO_2 in air. After incubation, oocytes were co-incubated with 2×10^6 spermatozoa/ml at 38.5°C in an atmosphere of 5% CO_2 in air for 2 h. Afterwards, the oocytes were washed five times in phosphate-buffered saline (PBS) to remove loosely bound spermatozoa and then fixed and stained with Hoechst 33342. The number of spermatozoa bound to each egg was determined by observation at ×400 magnification under an epifluorescence microscope Olympus BX40 (Olympus, Tokyo, Japan) equipped with a 365 nm excitation filter, a 400 nm barrier filter and a 400 nm emission filter.

MTT reduction assay

MTT (3[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide) is a yellow dye that is converted to purple formazan by the succinate dehydrogenase system of active mitochondria (Slater *et al.*, 1963). Therefore, the amount of formazan formed can be determined spectrophotometrically and serves as an estimate of the number of mitochondria and hence the number of living cells in the sample (Denizot and Lang, 1986). The MTT assay was performed according to the method of Mosmann (1983). For each sample, six wells of a 96-well microplate were used. In total, 100 µl sperm suspension (described above) plus 10 µl MTT stock solution (5 mg MTT/ml PBS) were placed in each well. According to Aziz (2006), the optical density of samples should be measured immediately after 1 and 2 h incubation at 37°C using a spectrophotometer (Biotek Instruments Inc., Bedfordshire, UK) at a wavelength of 550 nm. MTT reduction rate (optical density) for each sample was calculated by calculating the difference between the first and second readings and the first and third readings of the spectrophotometer, for the reduction rate after 1 h and 2 h incubation, respectively. Results for mitochondrial activity were expressed by normalizing the data in relation to the Control, in which mitochondrial activity was considered to be 100%.

The hypo-osmotic swelling test

HOST was used to evaluate functional sperm membrane integrity (Revell and Mrode, 1994). The test was performed by incubation of 25 µl semen with 200 µl HOST solution (100 mOsm/l, 57.6 mM fructose and 19.2 mM sodium citrate) for 30 min at room temperature (RT, 20°C). A wet mount was made using a 10 µl drop of homogenized mixture placed directly on a microscopic slide and covered with a coverslip. In total, 200 spermatozoa were counted in at least five different microscopic fields. The percentages of spermatozoa with swollen and curved tails were recorded.

Acrosomal status

The AS was assessed using *Pisum sativum* agglutinin conjugated to fluorescein isothiocyanate (PSA – FITC; Sigma Chemical Company, St Louis, MO, USA), as described earlier (Mendoza *et al.*, 1992). Briefly, sperm smears were fixed in methanol for 30 s after air drying and then stained using 50 mg/ml PSA – FITC in PBS for 30 min in a humidified chamber (HC) at RT. The slides were washed with distilled water and mounted. In total, 200 spermatozoa per sample were counted using an Olympus BX40 epifluorescence microscope (Olympus, Tokyo, Japan) using excitation wavelengths of 450–490 nm and a magnification of ×1000. The acrosomal region of the acrosome-intact spermatozoa was PSA – FITC positive and labelled green, while the acrosome-reacted spermatozoa retained only an equatorial labelled band with little or no labelling of the anterior head region.

Measurement of lipid peroxidation

Lipid peroxidation levels were measured using the TBARS method. The TBARS concentration in sperm suspension (described above) was measured spectrophotometrically and expressed as the malondialdehyde (MDA) level. The TBARS concentration was expressed as nmol MDA/10^6 sperm using tetramethoxypropane (TMP) as a standard. An aliquot of 100 µl of sperm suspension was mixed with 100 µl 8.1% SDS solution and 750 µl 20% acetic acid solution. After adding 750 µl 0.8% TBA solution and 2 ml distilled water to this mixture, it was heated for 1 h in a 95°C oven. The mixture was then cooled at RT and centrifuged at 4220 *g* for 15 min, after which the absorbance of the supernatant was measured at 532 nm using a spectrophotometer.

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The value was subsequently determined based on comparison with a TMP standard curve.

Total antioxidant status

Total antioxidant capacity was measured using a colorimetric method using the Randox total antioxidant status kit (cat no. NX2332, Randox Laboratories, Ltd, Crumlin, UK) with slight modifications. Briefly, 20 µl of sperm suspension (described above) was added to 1 ml of the chromogen, ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]). In total, 20 µl Trolox (6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid) at a concentration of 2.27 mmol/l was used as standard, whereas 20 µl deionized water was used as the blank. Chromogen (1 ml) was added to standard and blank samples. The absorbance was measured 3 min after substrate addition at 600 nm with a spectrophotometer. Results were expressed as mmol/l. Measurements in duplicate were used to calculate intra-assay variability.

Experimental design

Experiment 1: Effect of Cu, Mn, Se and Zn on the sperm–ZP binding

In Experiment 1, immature denuded oocytes and sperm were co-incubated for 2 h (as described above) in IVF medium supplemented with:

(i) 0, 2, 5 or 6 ng/ml Mn ($n = 86$ COC). The concentrations used are according to Kincaid (1999) and Underwood and Suttle (1999) classifications for Mn status in cattle (Experiment 1a);
(ii) 0, 10, 50 or 100 ng/ml Se ($n = 120$ COC). Concentration used were according the classification for Se status in cattle reported by Kincaid (1999) and Underwood and Suttle (1999) (Experiment 1b);
(iii) Cu + Mn + Se + Zn. Copper (0.4 µg/ml Cu; Anchordoquy *et al*., 2017); and Zn (0.8 µg/ml Zn; unpublished data) were established previously in our laboratory.

Mn and Se concentrations depended on the results of Experiments 1(a) and 1(b), respectively. A Control group without mineral supplementation was also analyzed ($n = 354$ COC) (Experiment 1c). All experiments were performed with COC obtained in separate batches of ovaries from 3 different days. The number of sperm bound to ZP was expressed as mean ± standard error of the mean (SEM).

Experiment 2: Effect of Cu, Mn, Se and Zn on sperm viability by MTT assay

In Experiment 2, the viabilities of sperm incubated for 1 or 2 h in IVF medium supplemented with Cu, Mn, Se, Zn, Cu + Mn + Se + Zn, or without supplement (Control) were investigated by MTT reduction assay (described above). In each replicate, 12 semen samples were pooled. Results were expressed as the percentage of mitochondrial activity and MTT reduction rate (mean ± SEM) from three independent replicates.

Experiment 3: Effect of Cu, Mn, Se and Zn on functional sperm membrane integrity

In Experiment 3, the functional sperm membrane integrity of sperm incubated for 0, 1 or 2 h in IVF medium supplemented with: Cu, Mn, Se, Zn, Cu + Mn + Se + Zn, or without supplement (Control) was investigated using HOST (described above). In each replicate, three semen samples were pooled. Results were expressed as the percentage of HOST positive sperm from three independent replicates.

Experiment 4: Effect of Cu, Mn, Se and Zn on acrosomal status

In Experiment 4, acrosomal status of sperm incubated for 1 or 2 h in IVF medium supplemented with Cu, Mn, Se, Zn, Cu + Mn + Se + Zn, or without supplement (Control) was investigated by PSA – FITC staining (described above). In each replicate, three semen samples were pooled. Results were expressed as the percentage from three independent replicates.

Experiment 5: Effect of Cu, Mn, Se and Zn on sperm lipid peroxidation

In Experiment 5, lipid peroxidation status of sperm incubated for 2 h in IVF medium supplemented with Cu, Mn, Se, Zn, Cu + Mn + Se + Zn, or without supplement (Control) was investigated using the TBARS method (described above). In each replicate, 12 semen samples were pooled. Results were expressed as the mean ± SEM from three independent replicates.

Experiment 6: Effect of Cu, Mn, Se and Zn on sperm total antioxidant capacity

In Experiment 6, TAC of sperm incubated for 2 h in IVF medium supplemented with Cu, Mn, Se, Zn, Cu + Mn + Se + Zn, or without supplement (Control) was investigated by total antioxidant status kit (described above). In each replicate, 12 semen samples were pooled. Results were expressed as the mean ± SEM from three independent replicates.

Statistical analysis

A completely randomized block design was used. The statistical model included the random effects of block ($n = 3$) and the fixed effect of treatment [0 vs 2 vs 5 vs 6 ng/ml Mn in Experiment 1(a); 0 vs 10 vs 50 vs 100 ng/ml Se in Experiment 1(b); and Control vs Cu vs Mn vs Se vs Zn vs Cu + Mn + Se + Zn in Experiments 1(c), 2, 3, 4, 5 and 6]. Variables such as number of sperm bound to ZP and MTT were analyzed with linear models using the MIXED procedure of SAS (SAS Institute, Cary, NC, USA). HOST and AS were analyzed by logistic regression using the GENMOD procedure (SAS Institute). TBARS and TAC were analyzed using the GLIMIX procedure (SAS Institute) with gamma distribution. Statistical significance was set at $P < 0.05$, while a trend for statistical significance was set between $P > 0.05$ and ≤ 0.10.

Results

Experiment 1: Effect of Cu, Mn, Se and Zn on sperm–ZP binding

In Experiment 1(a), there were no significant differences in number of sperm bound to ZP when Mn was added to IVF medium ($P > 0.05$; Fig. 1A). Manganese at a concentration of 5 ng/ml showed the highest number of ZPs and consequently this concentration was chosen for the next experiments. In Experiment 1(b), considerably more sperm bound to ZP were observed when Se was added to IVF medium at all concentrations studied ($P < 0.01$), but the difference was highest when using 50 and 100 ng/ml Se (Fig. 1B). For the next experiments 100 ng/ml Se

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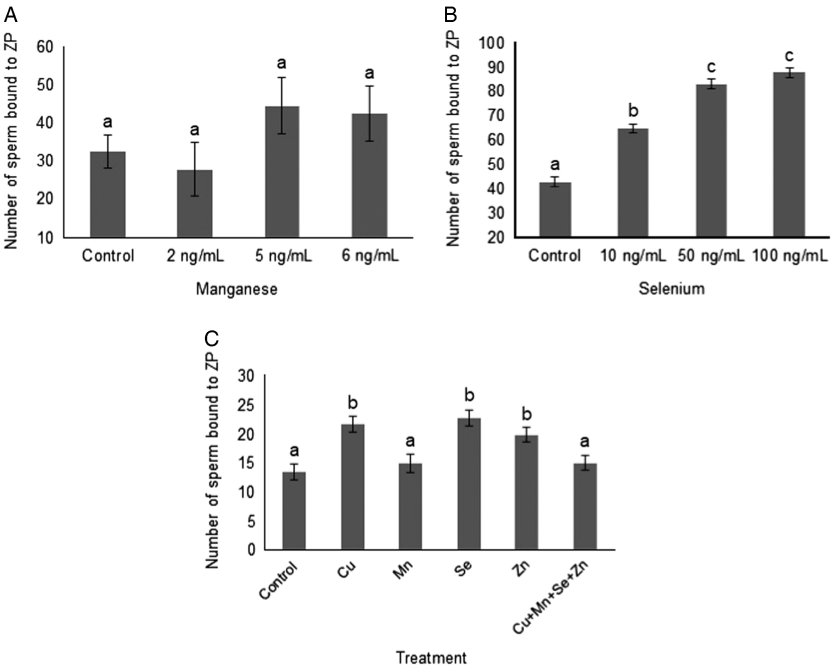

Figure 1. Effect of trace minerals Cu, Mn, Se and Zn on the sperm–ZP binding. The number of sperm bound to the zona pellucida (ZP) is expressed as mean ± standard error of the mean (SEM) (three replicates on different days). The figure shows the number of sperm bound to ZP after 2 h of incubation in IVF medium supplemented with 0 (Control), 2, 5 or 6 ng/ml Mn (*A*); 0 (Control), 10, 50 or 100 ng/ml Se (*B*); and Cu (0.4 µg/ml Cu), Mn (5 ng/ml Mn), Se (100 ng/ml Se), Zn (0.8 µg/ml Zn), Cu + Mn + Se + Zn, or without supplement (Control; *C*). [a,b,c]Bars with different letters differ statistically ($P < 0.05$).

was used. In Experiment 1(c), sperm bound to ZP was higher when Cu, Se or Zn were added to IVF medium ($P < 0.01$), but there were no differences among the Control, Mn and Cu + Mn + Se + Zn samples ($P > 0.05$; Fig. 1C).

Experiment 2: Effect of Cu, Mn, Se and Zn on sperm viability by MTT assay

In Experiment 2, spermatozoa incubated with Cu, Mn or Se showed a significant increase in mitochondrial activity after 1 h of incubation ($P < 0.05$), but there were no differences among Control, Zn and Cu + Mn + Se + Zn ($P > 0.05$). However, after 2 h of incubation the spermatozoa mitochondrial activity was significantly higher only for Mn addition to IVF medium with respect to the Control ($P < 0.05$; Table 1).

Experiment 3: Effect of Cu, Mn, Se and Zn on the functional sperm membrane integrity

In Experiment 3, percentage of HOST positive sperm at 0 and 1 h were similar among all treatments ($P > 0.05$). However, functional membrane integrity was increased after 2 h of sperm incubation with Cu compared with the Control ($P < 0.05$). Moreover, Se supplementation after 2 h of incubation tended to increase HOST positive sperm with respect to the Control ($P = 0.08$; Table 2).

Experiment 4: Effect of Cu, Mn, Se and Zn on acrosomal status

In Experiment 4, percentages of sperm with intact acrosome at 0 h were higher with the addition of Cu, Mn, or Se to IVF medium with respect to the Control ($P < 0.05$). However, after 1 h of incubation, acrosome integrity were significantly higher in sperm treated with Zn or Cu + Mn + Se + Zn ($P < 0.05$). After 2 h of incubation, Se and Zn supplementation tended to increase the percentage of acrosome-intact sperm compared with the Control ($P = 0.08$; Table 3).

Experiment 5: Effect of Cu, Mn, Se and Zn on lipid peroxidation

In Experiment 5, after 2 h of incubation, lipid peroxidation expressed by MDA level was significantly higher in sperm treated with Cu or Cu + Mn + Se + Zn in comparison with the Control ($P < 0.05$), but there were no differences among Control, Mn and Zn ($P > 0.05$). Moreover, Se supplementation tended to increase MDA levels respect to Control ($P = 0.07$; Fig. 2).

Experiment 6: Effect of Cu, Mn, Se and Zn on sperm total antioxidant capacity

In Experiment 6, after 2 h of incubation the mean TAC of sperm treated with Cu, Mn, Zn or Cu + Mn + Se + Zn were significantly lower than the Control ($P < 0.05$), but there was no difference between Control and Se ($P > 0.05$; Fig. 3).

Discussion

The objective of this study was to determine the effect of trace minerals Cu, Mn, Se and Zn on sperm–ZP binding. Our results demonstrated that: (a) Cu, Se and Zn added to IVF medium bound more sperm to ZP; (b) Cu, Mn and Se augmented sperm viability; (c) Cu improved functional membrane integrity; (d) Cu and Mn increased intact acrosome sperm percentages; (e) Cu and Cu + Mn + Se + Zn intensified lipid peroxidation; and (f) Cu, Mn, Zn and Cu + Mn + Se + Zn diminished TAC in sperm.

The ZP surrounds mammalian oocytes, which is a permeable extracellular glycoprotein matrix (Wassarman, 1990; Sinowatz *et al.*, 2001; Wassarman and Litscher, 2008). The ability of spermatozoa to bind ZP is achieved after processes of

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Table 1. Effect of trace minerals Cu, Mn, Se and Zn on sperm viability based on MTT assay

	After 1 h of incubation		After 2 h of incubation	
Treatment	MTT reduction rate (optical density)	Mitochondrial activity (%)	MTT reduction rate (optical density)	Mitochondrial activity (%)
Control	0.1242 ± 0.004^{a}	100.0^{a}	0.1540 ± 0.003^{ac}	$100.0^{a,c}$
Cu	0.1359 ± 0.004^{b}	109.4^{b}	$0.1619 \pm 0.003^{a,b}$	$105.1^{a,b}$
Mn	0.1376 ± 0.004^{b}	110.7^{b}	0.1642 ± 0.003^{b}	106.6^{b}
Se	0.1383 ± 0.004^{b}	111.3^{b}	$0.1602 \pm 0.003^{a,b}$	$104.0^{a,b}$
Zn	0.1319 ± 0.004^{a}	106.1^{a}	$0.1473 \pm 0.003^{a,c}$	$95.6^{a,c}$
Cu + Mn + Se + Zn	0.1276 ± 0.004^{a}	102.7^{a}	0.1529 ± 0.003^{c}	99.2^{c}

Mitochondrial activity is expressed as percentages and reduction rate as mean ± standard error of the mean (SEM) (three replicates on different days). For mitochondrial activity, data were normalized to measurements from Control cultures, which were as considered 100%. Viability based on MTT assay of sperm cultured in IVF medium supplemented with Cu (0.4 µg/ml Cu), Mn (5 ng/ml Mn), Se (100 ng/ml Se), Zn (0.8 µg/ml Zn), Cu + Mn + Se + Zn, or without supplement (Control) were evaluated after 1 or 2 h of incubation. [a,b,c]Values with different superscripts within a column differ significantly ($P < 0.05$).

Table 2. Effect of trace minerals Cu, Mn, Se and Zn on functional sperm membrane integrity

	HOST positive (%)		
Treatment	0 h	1 h	2 h
Control	47.5^{a}	24.2^{a}	$15.2^{a,c}$
Cu	49.5^{a}	19.2^{a}	19.6^{b}
Mn	45.3^{a}	20.9^{a}	$17.2^{a,b}$
Se	47.6^{a}	21.7^{a}	$18.9^{a,b}$
Zn	55.1^{a}	18.0^{a}	$15.8^{a,b,c}$

HOST, hypo-osmotic swelling test (functional sperm membrane integrity) is expressed as percentages (three replicates on different days). HOST of sperm cultured in IVF medium supplemented with Cu (0.4 µg/ml Cu), Mn (5 ng/ml Mn), Se (100 ng/ml Se), Zn (0.8 µg/ml Zn), Cu + Mn + Se + Zn, or without supplement (Control) were evaluated after 0, 1 or 2 h of incubation. [a,b,c]Values with different superscripts within a column differ significantly ($P < 0.05$). After 2 h of incubation, Se supplementation tended to increase the HOST positive sperm compared with the Control ($P = 0.08$).

Table 3. Effect of trace minerals Cu, Mn, Se and Zn on acrosomal status

	Acrosome integrity (%)		
Treatment	0 h	1 h	2 h
Control	75.3^{a}	69.8^{a}	69.0^{a}
Cu	$81.3^{b,c}$	$71.3^{a,b}$	69.8^{a}
Mn	84.6^{b}	$75.6^{b,c}$	73.0^{a}
Se	80.3^{c}	$74.0^{a,b,c}$	73.6^{a}
Zn	75.6^{a}	77.5^{c}	75.8^{a}
Cu + Mn + Se + Zn	74.7^{a}	76.5^{c}	72.6^{a}

Acrosome integrity is expressed as percentages (three replicates on different days). Acrosomal status of sperm cultured in IVF medium supplemented with Cu (0.4 µg/ml Cu), Mn (5 ng/ml Mn), Se (100 ng/ml Se), Zn (0.8 µg/ml Zn), Cu + Mn + Se + Zn, or without supplement (Control) were evaluated after 0, 1 or 2 h of incubation. [a,b,c]Values with different superscripts within a column differ significantly ($P < 0.05$). After 2 h of incubation, Se and Zn supplementation tended to increase the percentage of acrosome-intact sperm compared with the Control ($P = 0.08$).

spermatogenesis, epididymal maturation and capacitation (Reid *et al.*, 2011). This binding is mediated by a number of putative ZP sperm receptor, including glycosyl enzymes, such as β-1,4-galactosyltransferase, fucosyltransferase-5 and α-D-mannosidase, which requires a metal ion as a cofactor, preferentially Mn (Shur and Neely, 1988; Cornwall *et al.*, 1991; Miller *et al.*, 1992; Gong *et al.*, 1995; Chiu *et al.*, 2007). In the present study, Cu, Se and Zn supplementation increased the number of sperm bound to ZP; for Mn, there was some increase but not significant. This finding is in disagreement with Liu *et al.* (2009) who reported that supplementation of culture media with Zn had no effect on spermatozoa–ZP binding. However, the Zn concentration used by Liu and colleagues (2009) was 400 times higher than that used in the present study. The improvement in sperm–ZP binding with Cu addition to IVF medium is in agreement with our previous work (Anchordoquy *et al.*, 2017). To the best of our knowledge, this is the first time that the effects of Se, Mn or the combination of Cu + Mn + Se + Zn on sperm–ZP binding have been evaluated.

To determine whether the increase in sperm–ZP binding observed in this study was due to greater sperm viability, an MTT assay was performed. Therefore, after 1 h of incubation, an increase on sperm viability was observed with Cu, Mn and Se supplementation, whereas after 2 h, only Mn showed an improvement. Se has a favourable influence on sperm viability providing protection against oxidative stress (Ahsan *et al.*, 2014). Moreover, dietary supplementation with Se improves sperm viability in rams (Marai *et al.*, 2009) and buffalos (El-Sharawy *et al.*, 2017). Rise in bovine sperm viability has also been observed after 3 h of incubation with Cu and after 6 h with Mn (Lapointe *et al.*, 1996; Anchordoquy *et al*, 2017). Chia and colleagues (2000) demonstrated a relationship between Zn concentration in seminal plasma and sperm viability. However, the addition of Zn sulphate to semen extender in concentrations similar to those used in this study had a detrimental effect on the viability and membrane integrity of buffalo spermatozoa (Dorostkar *et al.*, 2014).

The integrity and functional activity of the sperm membrane are key factors that influence zona-binding ability and fertilization of mammalian spermatozoa (Varghese *et al.*, 2005). In the present study, HOST showed an increase in functional membrane integrity when sperm were incubated with Cu and Se. This finding is in agreement with a previous study in which we observed an increase in the percentage of HOST positive sperm with Cu supplementation to IVF medium (Anchordoquy *et al.*, 2017). For Se, addition of this mineral to semen extender

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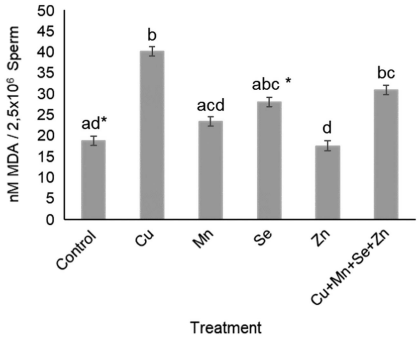

Figure 2. Effect of trace minerals Cu, Mn, Se and Zn on lipid peroxidation. Lipid peroxidation is expressed as malondialdehyde (MDA) level. Data are expressed as mean ± standard error of the mean (SEM) (three replicates on different days). Lipid peroxidation of sperm cultured in IVF medium supplemented with Cu (0.4 µg/ml Cu), Mn (5 ng/ml Mn), Se (100 ng/ml Se), Zn (0.8 µg/ml Zn), Cu + Mn + Se + Zn, or without supplement (Control) was evaluated after 2 h of incubation. [a,b,c,d]Bars with different letters differ statistically ($P < 0.05$). Bars with (*) tended to be different ($P = 0.07$).

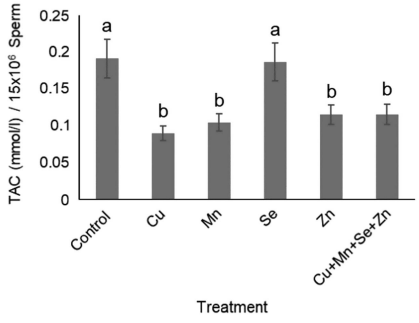

Figure 3. Effect of trace minerals Cu, Mn, Se and Zn on sperm total antioxidant capacity. Total antioxidant capacity is expressed as mmol/l. Data are expressed as mean ± standard error of the mean (SEM) (three replicates on different days). Total antioxidant capacity of sperm cultured in IVF medium supplemented with Cu (0.4 µg/ml Cu), Mn (5 ng/ml Mn), Se (100 ng/ml Se), Zn (0.8 µg/ml Zn), Cu + Mn + Se + Zn, or without supplement (Control) was evaluated after 2 h of incubation. [a,b]Bars with different letters differ statistically ($P < 0.05$).

increased functional membrane integrity of frozen–thawed buffalo sperm (Dorostkar *et al.*, 2012). Recently, Reis *et al.* (2014) showed that membrane integrity of Nellore sperm was increased by dietary Mn supplementation. *In vitro*, Mn supplementation of bovine semen during cryopreservation showed a protective effect, growing the percentage of HOS-positive spermatozoa (Cheema *et al.*, 2009). However, in the present study, Mn addition to IVF medium did not modify the functional integrity of the sperm's plasma membrane.

Only motile acrosome-intact sperm bind to the ZP (Hoodbhoy and Dean, 2004). In the present study, Cu, Mn or Se supplementation increased percentages of acrosome-intact sperm at 0 h, suggesting a protective effect of these minerals against spontaneous acrosome reaction (AR). Although, after 1 h of incubation Zn and Cu + Mn + Se + Zn increased the percentages of intact acrosomes, after 2 h that improvement was only maintained by Zn and Se treatments. These results are consistent with that observed in a previous study in which Cu supplementation did not modify AS after 3 or 6 h of incubation (Anchordoquy *et al.*, 2017). In addition, Roblero *et al.* (1996) found that AR was not affected when sperm were incubated for 5 h in medium containing 1 µg/dl to 1 mg/dl Cu. With respect to Se, Marai *et al.* (2009) reported that dietary supplementation with sodium selenite improved semen quality, decreasing acrosome damage. The relationship between Zn and AR has already been studied. Michailov *et al.* (2014) showed that Zn stimulated AR in capacitated sperm through the epidermal growth factor receptor. In the present study, Zn maintained the percentages of acrosome-intact sperm during the 2 h of incubation.

Oxidative stress is a major contributor to defective sperm function including the competence for fertilization. These effects include LPO, ending in cytotoxic aldehydes generation such as MDA and 4-hydroxynonenal (Jones *et al.*, 1979; Nair *et al.*, 2006; Kasimanickam *et al.*, 2007; Aitken and Curry, 2011). However, abundant evidence suggests that ROS produced by mammalian sperm play a physiologically role promoting capacitation process through redox regulation of tyrosine phosphorylation (Baumber *et al.*, 2003; Ecroyd *et al.*, 2003; Rivlin *et al.*, 2004; Roy and Atreja, 2008; Basim *et al.*, 2009; Gonçalves *et al.*, 2010). Mammalian spermatozoa including bovine have the capacity to generate ROS, mainly hydrogen peroxide (Tosic and Walton, 1946). Mitochondria in the sperm midpiece are the major source of oxygen metabolites (Storey, 2008). Transition metals such as Fe, Cu, Pb, or Cd have been described to be among several causes of mitochondrial ROS generation (Jones *et al.*, 1979; Kiziler *et al.*, 2007). In the present study, MDA production was increased when Cu, Se and Cu + Mn + Se + Zn were added to IVF medium, but not with Mn or Zn supplementation. These results are in agreement with Kaushik and colleagues (2015) who demonstrated that Mn is a strong ion inhibitor of LPO in human semen that is far superior compared with Zn. The Mn inhibitory role on LPO has been described *in vivo* and *in vitro* (Aitken, 1997). Manganese is required for mitochondrial superoxide dismutase synthesis (Sikka, 1996) and has an important role as an antioxidant, regulating peroxyl radicals (Coassin *et al.*, 1992). Gavella and Lipovac (1998) studied the inhibitory effect of Zn on superoxide anion generation in human spermatozoa, demonstrating that Zn ions themselves are not able to act as inducers of LPO. This is in agreement with the results obtained in this study. Conversely, an interesting fact is that the metals that produced greater LPO such as Cu and Se are those that increased the number of sperm bound to ZP. These results are consistent with that observed by Aitken *et al.* (1989) and Kodama *et al.* (1996) who demonstrated that the induction of mild lipid peroxidation increases sperm–zona binding and sperm fertilizing capacity. This unpredicted peroxidative effect is an unexpected positive, although the mechanisms involved are not yet clarified (Aitken *et al.*, 2012).

Transitions metals such as Mn, Cu and Zn are involved in essential biological processes, as they are cofactors of metalloproteins, many of these with antioxidant activity (Underwood and Suttle, 1999; Chihuailaf *et al.*, 2002). Although essential in trace amounts, at higher levels these metals may have pro-oxidant effect (Underwood and Suttle, 1999; Chihuailaf *et al.*, 2002). The decrease in TAC observed after supplementation of IVF medium with Cu, Mn or Zn suggested that these trace minerals might increase the spermatic levels of ROS.

Although spermatozoa from a single bull were used in this study, the results were similar when frozen semen from other bulls were used (unpublished observations). In a previous study using another bull, we observed comparable results with Cu supplementation (Anchordoquy *et al.*, 2017).

In conclusion, the results from the present study showed that the presence of Cu, Se and Zn in the IVF medium increased the number of spermatozoa bound to ZP. This may, at least in part, be due to an increase in: (i) the viability and functional membrane integrity of sperm (Cu and Se); (ii) the number of spermatozoa

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with intact acrosome (Se and Zn); and (iii) lipid peroxidation (Cu and Se). We inferred that Cu, Se and Zn might play an important role in sperm–ZP binding, highlighting the importance of these minerals in the fertilization process.

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Conflicts of interest. The authors declare that there are no conflicts of interest.

Ethical standards. Not applicable.

References

Ahsan U, Kamran Z, Raza I and Iqbal Z (2014) Role of selenium in male reproduction—a review. *Anim Reprod Sci* **146**, 55–62.

Aitken RJ (1997) Molecular mechanisms regulating human sperm function. *Mol Hum Reprod* **3**, 169–73.

Aitken RJ and Baker MA (2004) Oxidative stress and male reproductive biology. *Reprod Fertil Dev* **16**, 581–8.

Aitken RJ and Curry BJ (2011) Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. *Antioxid Redox Signal* **14**, 367–81.

Aitken RJ, Clarkson JS and Fishel S (1989) Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol Reprod* **41**, 183–97.

Aitken RJ, Jones KT and Robertson SA (2012) Reactive oxygen species and sperm function – in sickness and in health. *J Androl* **33**, 1096–106.

Aitken RJ, Baker MA and Nixon B (2015) Are sperm capacitation and apoptosis the opposite ends of a continuum driven by oxidative stress? *Asian J Androl* **17**, 633–9.

Anchordoquy JP, Anchordoquy JM, Pascua AM, Nikoloff N, Peral-García P and Furnus CC (2017) The copper transporter (SLC31A1/CTR1) is expressed in bovine spermatozoa and oocytes: copper in IVF medium improves sperm quality. *Theriogenology* **97**, 124–33.

Aziz DM (2006) Assessment of bovine sperm viability by MTT reduction assay. *Anim Reprod Sci*, **92**(1–2), 1–8.

Barroso G, Morshedi M and Oehninger S (2000) Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Hum Reprod* **15**, 1338–44.

Basim J Mackenzie-Bell AM and Buhr MM (2009) Reactive oxygen species and boar sperm function. *Biol Reprod* **81**, 553–61.

Baumber J, Sabeur K, Vo A and Ball BA (2003) Reactive oxygen species promote tyrosine phosphorylation and capacitation in equine spermatozoa. *Theriogenology* **60**, 1239–47.

Cheema RS, Bansal AK and Bilaspuri GS (2009) Manganese provides antioxidant protection for sperm cryopreservation that may offer new consideration for clinical fertility. *Oxid Med Cell Longev* **2**, 152–9.

Chia SE, Ong CN, Chua LH, Ho LM and Tay SK (2000) Comparison of zinc concentrations in blood and seminal plasma and the various sperm parameters between fertile and infertile men. *J Androl* **21**, 53–7.

Chihuailaf RH, Contreras PA and Wittwer FG (2002) Patogénesis del estrés oxidativo: Consecuencias y evaluación en salud animal pathogenesis of oxidative stress: consequences and evaluation in animal health. *Vet Méx* **33**, 265.

Chiu PCN, Chung M-K, Koistinen R and Yeung WSB (2007) Glycodelin-A interacts with fucosyltransferase on human sperm plasma membrane to inhibit spermatozoa–zona pellucida binding. *J Cell Sci* **120**(Pt 1), 33–44.

Coassin M, Ursini F and Bindoli A (1992) Antioxidant effect of manganese. *Arch Biochem Biophys* **299**, 330–3.

Colagar AH, Marzony ET and Chaichi MJ (2009) Zinc levels in seminal plasma are associated with sperm quality in fertile and infertile men. *Nutr Res* **29**, 82–8.

Cornwall GA, Tulsiani DR and Orgebin-Crist MC (1991) Inhibition of the mouse sperm surface alpha-D-mannosidase inhibits sperm-egg binding *in vitro*. *Biol Reprod* **44**, 913–21.

Coutinho da Silva MA, Seidel GE, Squires EL, Graham JK and Carnevale EM (2012) Effects of components of semen extenders on the binding of stallion spermatozoa to bovine or equine zonae pellucidae. *Reproduction* **143**, 577–85.

Denizot F and Lang R (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods*, **89**, 271–7.

Dorostkar K, Alavi-Shoushtari SM and Mokarizadeh A (2012) Effects of *in vitro* selenium addition to the semen extender on the spermatozoa characteristics before and after freezing in water buffaloes (*Bubalus bubalis*). *Vet Res Forum* **3**, 263–8.

Dorostkar K, Alavi Shoushtari SM and Khaki A (2014) Effects of *in vitro* zinc sulphate additive to the semen extender on water buffalo (*Bubalus bubalis*) spermatozoa before and after freezing. *Int J Fertil Steril* **8**, 325–32.

Ecroyd HW, Jones RC and Aitken RJ (2003) Endogenous redox activity in mouse spermatozoa and its role in regulating the tyrosine phosphorylation events associated with sperm capacitation. *Biol Reprod* **69**, 347–54.

El-Sharawy M, Eid E, Darwish S, Abdel-Razek I, Islam MR, Kubota K, Yamauchi N and El-Shamaa I (2017) Effect of organic and inorganic selenium supplementation on semen quality and blood enzymes in buffalo bulls: selenium effects on buffalo semen quality. *Anim Sci J* **88**, 999–1005.

Fazeli AR, Steenweg W, Bevers Mvan den Broek J, Bracher V, Parlevliet J and Colenbrander B (1995) Relation between stallion sperm binding to homologous hemizonae and fertility. *Theriogenology* **44**, 751–60.

Gavella M and Lipovac V (1998) *In vitro* effect of zinc on oxidative changes in human semen. *Andrologia*, **30**, 317–23.

Gil-Guzman E, Ollero M, Lopez MC, Sharma RK, Alvarez JG, Thomas AJ Jr and Agarwal A (2001) Differential production of reactive oxygen species by subsets of human spermatozoa at different stages of maturation. *Hum Reprod* **16**, 1922–30.

Gonçalves FS, Barretto LS, Arruda RP, Perri SH and Mingoti GZ (2010) Effect of antioxidants during bovine *in vitro* fertilization procedures on spermatozoa and embryo development. *Reprod Domest Anim*, **45**, 129–35.

Gong X, Dubois DH, Miller DJ and Shur BD (1995) Activation of a G protein complex by aggregation of beta-1,4-galactosyltransferase on the surface of sperm. *Science* **269**(5231), 1718–21.

Hoodbhoy T and Dean J (2004) Insights into the molecular basis of sperm-egg recognition in mammals. *Reproduction* **127**, 417–22.

Ivanova M and Mollova M (1993) Zona-penetration *in vitro* test for evaluating boar sperm fertility. *Theriogenology* **40** 397–410.

Jones R, Mann T and Sherins R (1979) Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. *Fertil Steril* **31**, 531–7.

Kasimanickam R, Kasimanickam V, Thatcher CD, Nebel RL and Cassell BG (2007) Relationships among lipid peroxidation, glutathione peroxidase, superoxide dismutase, sperm parameters, and competitive index in dairy bulls. *Theriogenology* **67**, 1004–12.

Kaushik K, Mittal PK and Kalla NR (2015) Antioxidant potential of Mn2 + in the human ejaculated spermatozoa under oxidative stress. *Int J Pharm Sci Res* **42**, 2153–62.

Kincaid RL (1999) Assessment of trace mineral status of ruminants: a review. *J Anim Sci 77(E-Suppl)*, 1–10.

Kiziler AR, Aydemir B, Onaran I, Alici B, Ozkara H, Gulyasar T and Akyolcu MC (2007) High levels of cadmium and lead in seminal fluid and blood of smoking men are associated with high oxidative stress and damage in infertile subjects. *Biol Trace Elem Res* **120**(1–3), 82–91.

Kodama H, Kuribayashi Y and Gagnon C (1996) Effect of sperm lipid peroxidation on fertilization. *J Androl* **17**, 151–7.

Lapointe S, Ahmad I, Buhr MM and Sirard MA (1996) Modulation of postthaw motility, survival, calcium uptake, and fertility of bovine sperm by magnesium and manganese. *J Dairy Sci* **79**, 2163–9.

Liu D-Y, Sie B-S, Liu M-L, Agresta F and Baker HWG (2009) Relationship between seminal plasma zinc concentration and spermatozoa–zona pellucida binding and the ZP-induced acrosome reaction in subfertile men. *Asian J Androl* **11**, 499–507.

Marai IFM, El-Darawany A-HA, Ismail E-SA-F and Abdel-Hafez MAM (2009) Reproductive and physiological traits of Egyptian Suffolk rams as affected by selenium dietary supplementation and housing heat radiation effects during winter of the sub-tropical environment of Egypt. *Arch Anim Breed* **52**, 402–9.

Mendoza C, Carreras A, Moos J and Tesarik J (1992) Distinction between true acrosome reaction and degenerative acrosome loss by a one-step staining method using *Pisum sativum* agglutinin. *J Reprod Fertil* **95**, 755–63.

Michailov Y, Ickowicz D and Breitbart H (2014) Zn^{2+}-stimulation of sperm capacitation and of the acrosome reaction is mediated by EGFR activation. *Dev Biol* **396**, 246–55.

Miller DJ, Macek MB and Shur BD (1992) Complementarity between sperm surface beta-1,4-galactosyltransferase and egg-coat ZP3 mediates sperm-egg binding. *Nature* **357**(6379), 589–93.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**(1–2), 55–63.

Nair SJ, Brar AS, Ahuja CS, Sangha SP and Chaudhary KC (2006) A comparative study on lipid peroxidation, activities of antioxidant enzymes and viability of cattle and buffalo bull spermatozoa during storage at refrigeration temperature. *Anim Reprod Sci* **96**(1–2), 21–9.

Oehninger S, Mahony M, Ozgür K, Kolm P, Kruger T and Franken D (1997) Clinical significance of human sperm–zona pellucida binding. *Fertil Steril* **67**, 1121–7.

Overstreet JW, Yanagimachi R, Katz DF, Hayashi K and Hanson FW (1980) Penetration of human spermatozoa into the human zona pellucida and the zona-free hamster egg: a study of fertile donors and infertile patients. *Fertil Steril* **33**, 534–42.

Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Critser ES, Eyestone WH and First NL (1986) Bovine *in vitro* fertilization with frozen–thawed semen. *Theriogenology* **25**, 591–600.

Reid AT, Redgrove K, Aitken RJ and Nixon B (2011) Cellular mechanisms regulating sperm–zona pellucida interaction. *Asian J Androl* **13**, 88–96.

Reis LSLS, Ramos AA, Camargos AS and Oba E (2014) Effect of manganese supplementation on the membrane integrity and the mitochondrial potential of the sperm of grazing Nellore bulls. *Anim Reprod Sci* **150**(1–2), 1–6.

Revell SG and Mrode RA (1994) An osmotic resistance test for bovine semen. *Anim Reprod Sci* **36**, 77–86.

Rivlin J, Mendel J, Rubinstein S, Etkovitz N and Breitbart H (2004) Role of hydrogen peroxide in sperm capacitation and acrosome reaction. *Biol Reprod* **70**, 518–22.

Roblero L, Guadarrama A, Lopez T and Zegers-Hochschild F (1996) Effect of copper ion on the motility, viability, acrosome reaction and fertilizing capacity of human spermatozoa *in vitro*. *Reprod Fertil Dev* **8**, 871–4.

Roy SC and Atreja SK (2008) Effect of reactive oxygen species on capacitation and associated protein tyrosine phosphorylation in buffalo (*Bubalus bubalis*) spermatozoa. *Anim Reprod Sci* **107**(1–2), 68–84.

Shamsi MB, Kumar R and Dada R (2008) Evaluation of nuclear DNA damage in human spermatozoa in men opting for assisted reproduction. *Indian J Med Res* **127**, 115–23.

Shur BD and Neely CA (1988) Plasma membrane association, purification, and partial characterization of mouse sperm beta-1,4-galactosyltransferase. *J Biol Chem* **263**(33), 17706–14.

Sikka SC (1996) Oxidative stress and role of antioxidants in normal and abnormal sperm function. *Front Biosci* **1**, e78–86.

Sinowatz F, Töpfer-Petersen E, Kölle S and Palma G (2001) Functional morphology of the zona pellucida. *Anat Histol Embryol* **30**, 257–63.

Slater TF, Sawyer B and Sträuli U (1963) Studies on succinate-tetrazolium reductase systems. *Biochim Biophys Acta* **77**, 383–93.

Storey BT (2008) Mammalian sperm metabolism: oxygen and sugar, friend and foe. *Int J Dev Biol* **52**(5–6), 427–37.

Tosic J and Walton A (1946) Formation of hydrogen peroxide by spermatozoa and its inhibitory effect of respiration. *Nature* **158**, 485.

Underwood EJ and Suttle NF (1999) *The Mineral Nutrition of Livestock*, 3rd edn. Wallingford: CABI.

Ursini F, Heim S, Kiess M and Flohé L (1999) Dual function of the selenoprotein PHGPx during sperm maturation. *Science* **285**(5432), 1393–6.

Varghese AC Sinha B and Bhattacharyya AK (2005) Current trends in evaluation of sperm function: *in vitro* selection and manipulation of male gametes for assisted conception. *Indian J Exp Biol* **43**, 1023–31.

Wassarman PM (1990) Regulation of mammalian fertilization by zona pellucida glycoproteins. *J Reprod Fertil Suppl* **42**, 79–87.

Wassarman PM and Litscher ES (2008) Mammalian fertilization: the egg's multifunctional zona pellucida. *Int J Dev Biol* **52**(5–6), 665–76.

Zhang BR, Larsson B, Lundeheim N, Håård MG and Rodriguez-Martinez H (1999) Prediction of bull fertility by combined in vitro assessments of frozen–thawed semen from young dairy bulls entering an AI-programme. *Int J Androl* **22**, 253–60.

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