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ZINC AFFECTS RABBIT SPERMATOZOA *IN VITRO*: EFFECTS ON MOTILITY AND VIABILITY

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ABSTRACT

Zinc is an essential trace element, which plays important roles in different biological activities. The aim of this study was to evaluate the effect of zinc on rabbit spermatozoa during *in vitro* conditions. Fresh semen was obtained from 8 sexually mature New Zealand White rabbits, which were bred at National Agricultural and Food Centre in Lužianky. Experimental groups were prepared by diluting the semen with two forms of zinc ($ZnCl_2$, $ZnSO_4$ .7 H_2O) in five different concentration: ZA, ZA1 (4.883 mg $Zn.l^{-1}$), ZB, ZB1 (9.766 mg $Zn.l^{-1}$), ZC, ZC1 (19.531 mg $Zn.l^{-1}$), ZD, ZD1 (39.063 mg $Zn.l^{-1}$), ZE, ZE1 (78.125 mg $Zn.l^{-1}$). Experimental samples were compared against control groups (ZK, ZK1). Semen was evaluated using the Computer Assisted Semen Analysis (CASA) at time intervals 0, 60, 120 and 180 minutes after incubation at 39°C. MTT test was used for determination of cell viability. Results of monitored motility parameters (motility, progressive motility and velocity curved line) showed decreasing trend in experimental samples. Significantly lower values of motility were observed in sample ZE, where percentage of motility decreased to 9.18±1.34% and after 180 minutes of incubation. Evaluation of spermatozoa velocity curved line detected significant ($P<0.001$) decrease at time intervals 120 and 180 minutes in samples with the highest concentration (ZE) of zinc. Measurement observed results with significant counteractive effect on viability in samples ZA, ZB, ZC1, ZD, ZD1 ($P<0.05$) and ZB1, ZC, ZE, ZE1 ($P<0.01$) after 180 minutes of incubation. Detected data confirm negative effect of zinc in each concentration on spermatozoa motility parameters and viability. Supplemented zinc in form $ZnCl_2$ to rabbit semen had higher negative impact on rabbit spermatozoa motility and viability parameters than zinc in form $ZnSO^4.7H_2O$.

Keywords: zinc, rabbit, CASA, MTT, spermatozoa

INTRODUCTION

Farmers effort to make rabbit production more profitable was in recent years reached mainly by the use of artificial insemination (AI) **(Castellini, 2007)**. The successful insemination requires fully functional spermatozoa with a normal membrane status diluted in extender rich in nourishing and beneficial substances **(Fik, 2018; Lukac *et al.*, 2010)**. Measurement of sperm motility is important tool to determine the potential for successful production of rabbit offspring and monitor the male reproductive performance **(Kime *et al.*, 1996; Slanina *et al.*, 2014)**.
Zinc is an essential trace element, which plays important roles in different biological processes **(Yamaguchi *et at.*, 2009)**. Its multifaceted role is known in spermatogenesis, testes development, sperm physiologic functions and also has effect on sperm motility **(Colagar *et al.*, 2009)**. Zinc deficiency results in gonadal dysfunction on histological level, however its content is varying throughout the life of the individual. Juvenile testes contain the lowest amount of zinc with increasing trend during puberty. Zinc along with calcium and magnesium can decrease or increase progressive motility with the concentration being the deciding factor **(Bedwal and Bahuguna, 1994)**. Zinc is highly present in prostatic fluid which therefore maintain ideal environment for spermatozoa until the fertilization **(Riffo *et al.*, 1992)**. Furthermore zinc has also antioxidant activity and may decrease the production of reactive oxygen species **(Kendall *et al.*, 2000; Valko *et al.*, 2005)**. The function of zinc is also to be a co-factor for most enzymatic reactions **(Kasperczyk *et al.*, 2015)**. **Kerns *et al.*(2018)** recently reported that zinc ions (Zn^{2+}) take part in mammalian sperm capacitation.. Reduced zinc content in seminal plasma can impair antioxidant defence, however increased zinc content has positive effect on motility and spermatozoa production **(Nenkova *et al.*, 2017)**. Sufficient zinc content in male body support production of testosterone **(Favier, 1992)**. Toxic effect of different toxic elements may be diminished by zinc presence **(Massányi *et al.*, 2003)** therefore its supplementation is recommended with positive outcome in spermatozoa motility and concentration **(Narasimhaiah *et al.*, 2017)**. Comparatively little attention has been drawn towards the toxic properties of zinc originated from industrial fume or environmental pollution **(Fosmire, 1990)**. Further zinc toxicity may come from food and beverages contaminated with zinc from galvanized containers **(Duncan *et al.*, 1992)**.
The aim of this study was to evaluate and compare the effect of different forms and concentrations of zinc on rabbit spermatozoa motility and viability parameters during *in vitro* incubation.

MATERIAL AND METHODS

Semen collection and processing

Fresh semen was obtained from 8 sexually mature New Zealand White rabbits, which were bred at National Agricultural and Food Centre in Lužianky. Semen collection was realised by the use of pre-warmed artificial vagina after sexual stimulation by a rabbit doe. Consequently, semen was stored at 37°C during transport. Further in lab, aliquots of 8 ejaculates used as controls (ZK, ZK1) were diluted with physiological solution (NaCl 0.9% Braun, B. Braun Melsungen AG, Germany) in ratio 1:7. Experimental samples were prepared according to the same dilution rate, using two forms of zinc ($ZnCl_2$, $ZnSO_4.7H_2O$) in five concentrations (Table 1, Table 2) dissolved in the physiological solution. Solutions of zinc were prepared in advance to ensure the proper dissolution of zinc.

Table 1 Concentration of zinc ($ZnCl_2$) used in this study

Sample	Concentration of Zn ($mg.l^{-1}$)	$ZnCl_2$ ($mg.50ml^{-1}$)
ZK	0	0
ZA	4.883	0.244
ZB	9.766	0.488
ZC	19.531	0.977
ZD	39.063	1.953
ZE	78.125	3.906

Table 2 Concentration of zinc ($ZnSO_4.7H_2O$) used in this study

Sample	Concentration of Zn ($mg.l^{-1}$)	$ZnSO_4.7\ H_2O$ ($mg.50ml^{-1}$)
ZK1	0	0
ZA1	4.883	0.373
ZB1	9.766	0.747
ZC1	19.531	1.494
ZD1	39.063	2.987
ZE1	78.125	5.975

Motility analyses

Semen was evaluated using the Computer assisted semen analysis (CASA) method with SpermVision software (Minitube, Tiefenbach, Germany) and the microscope Olympus BX 51 (Olympus, Japan). Each sample was placed into Makler counting chamber (10 µm, Sefi-Medical Instruments, Germany). Analyses were realised at four different time periods (0, 60, 120, 180 minutes). Samples were stored in incubator at 39°C during the whole experiment. In each sample following parameters were evaluated: MOT (motility), PRO (progressive motility), VCL (velocity curved line) **(Tirpák *et al.*, 2017; Halo Jr. *et al.*, 2018)**.

Assessment of mitochondrial toxicity

The viability of the spermatozoa exposed to both forms of zinc was assessed using the mitochondrial toxicity test (MTT). This colorimetric assay measures the conversion of 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich, St. Louis, USA) to purple formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria of living cells. Optimal density was determined spectrophotometrically at a wavelength of 570 against 620 nm as reference by a microplate ELISA reader (Multiskan FC, ThermoFisher Scientific, Finland). The data were expressed as percentage against the control which was recalculated to 100% **(Jambor *et al.*, 2017)**.

Statistical analysis

All data were analysed by the Statistical Analyses System (SAS 9.2. using of application Enterprise guide 5.1). Means of control and experimental groups (%, $\mu m.s^{-1}$) were analysed by one-way factor analyses applying the Tukey´s test. All statistical tests were carried out at levels of significance at P<0.05, P<0.01 and P<0.001 and results were interpreted as means and expressed with SD.

RESULTS AND DISCUSSION

Spermatozoa motility analysis at time intervals 0 (79.67±9.18%) and 60 min (81.27±4.11%) showed approximately the same percentage of motility compared to control groups. Motility in experimental groups (ZA, ZB, ZC, ZD, ZE) with addition of zinc ($ZnCl_2$) was decreased compared to control group. Significantly decreased motility was found in sample ZE at Time 60 (P<0.01), 120 and 180 (P<0.001). Also significant drop-off was measured at Time 180 in samples ZD (P<0.001) and ZB (P<0.01). Same negative effect on motility was found in samples (ZA1, ZB1, ZC1, ZD1, ZE1) with addition of zinc ($ZnSO_4.7\ H_2O$) except ZA at interval 120 minutes, where motility was moderately increased against control group. Significantly lower values were observed in sample ZE1 at interval 120 min (P<0.05) and in 180 minutes (P<0.001). Rapid lower values of motility were observed in sample with the highest concentration of zinc (ZE), where the percentage of motility decreased to 9.18±1.34% (Figure 1).

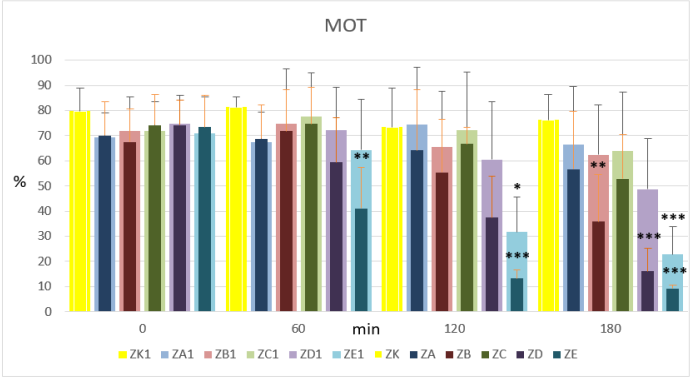

Figure 1 The effect of two forms of zinc with various doses of zinc on the total motility (%) of rabbit spermatozoa (n=8) in 0 min, 60 min, 120min and 180 min. Each bar represents the mean (±SD). The level of significance was set at *P<0.05; **P<0.01; ***P<0.001.

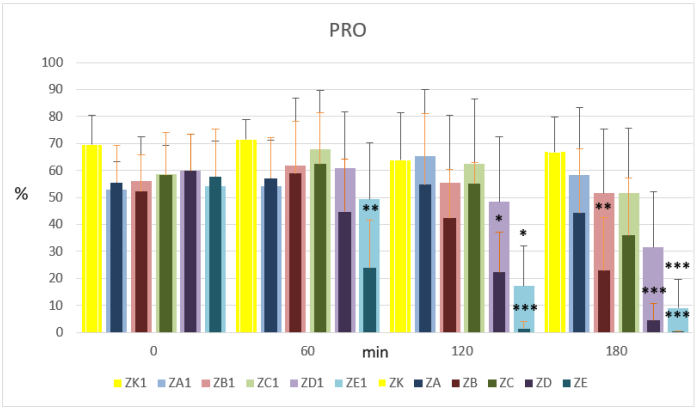

Figure 2 The effect of two forms of zinc with various doses of zinc on the progressive motility (%) of rabbit spermatozoa (n=8) in 0 min, 60 min, 120min, 180 min. Each bar represents the mean (±SD). The level of significance was set at *P<0.05; **P<0.01; ***P<0.001.

Reflecting the trend of total motility, adverse effect of zinc was detected in progressive motility. Experimental sample ZE in times 120 and 180 min had progressive motility on 4.49±6.28% and 0.15±0.25%. The lower progressive motility in comparison to control group was measured in each interval, only in sample ZA1 were observed very similar results as in control group after 120 minutes of incubation (Figure 2). Significant results were found in sample ZE after 60 minutes (P<0.01), 120 and 180 minutes (P<0.001) of cultivation. Further significant results were monitored at interval 120 min in samples ZE1, ZD (P<0.05), at interval 180 minutes in samples ZB (P<0.01), ZD and ZE1 (P<0.001).

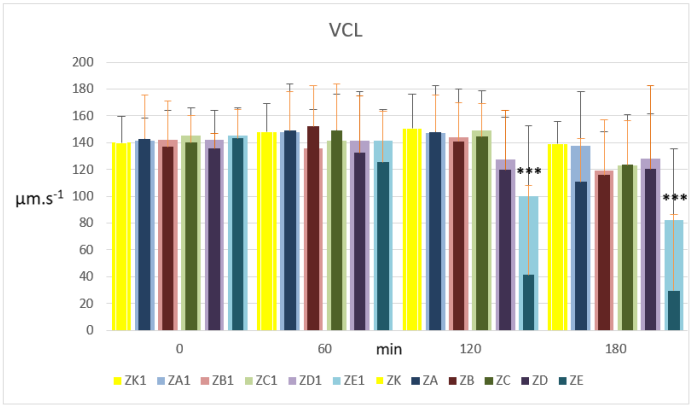

Figure 3 The effect of two forms of zinc with various doses of zinc on the velocity curved line ($\mu m.s^{-1}$) of rabbit spermatozoa (n=8) in 0 min, 60 min, 120min, 180 min. Each bar represents the mean (±SD). The level of significance was set at *P<0.05; **P<0.01; ***P<0.001.

Groups with addition of zinc in inceptive interval had approximately same values of velocity curved line as controls groups. Negative effect of zinc on spermatozoa velocity was represented especially by sample ZE (Figure 3). Evaluation of spermatozoa velocity curved line detected significant (P<0.001) decrease after 120 and 180 min of incubation in ZE. The lowest speed of rabbit spermatozoa

was determined after 180 minutes of incubation in sample ZE with speed 29.57±57.00µm.s^{-1} compared to control group with 138.96±16.87 µm.s^{-1}.

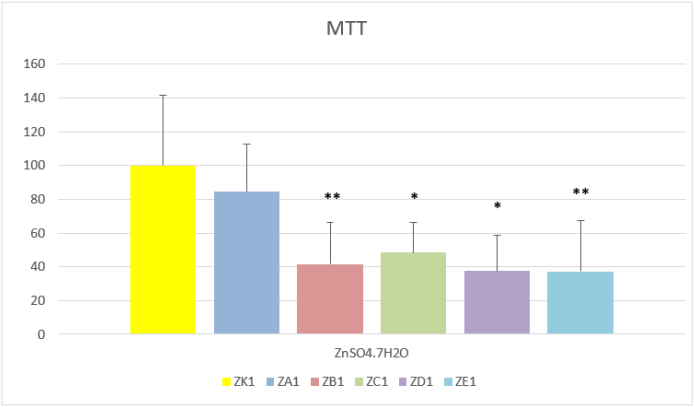

Figure 4 The effect of various doses of zinc ($ZnSO^4.7H_2O$) on the viability (%) of rabbit spermatozoa (n=8) assessed after 180 minutes of incubation. Each bar represents the mean (±SD) of measured optical density and converted to percents. The control was recalculated to 100%. The level of significance was set at *P<0.05; **P<0.01; ***P<0.001.

MTT test, which monitors the production of succinate dehydrogenase, was used for determination of cell viability. According to the MMT assay, supplementation of zinc ($ZnSO_4.7H_2O$) to fresh rabbit spermatozoa after 180 minutes of incubation had significantly negative effect on the cell viability in the experimental groups ZB1, ZE1 (P<0.01) and ZC, ZD1 (P<0.05; Figure 4).

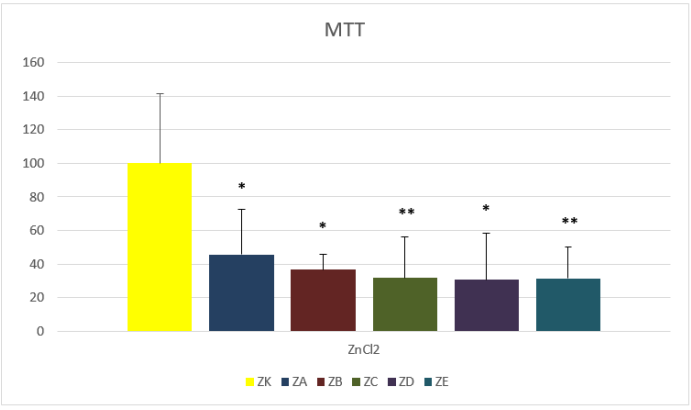

Figure 5 The effect of various doses of zinc ($ZnCl_2$) on the viability (%) of rabbit spermatozoa (n=8) assessed after 180 minutes of incubation. Each bar represents the mean (±SD) of measured optical density and converted to percents. The control was recalculated to 100%. The level of significance was set at *P<0.05; **P<0.01; ***P<0.001.

Similar negative effect on cell viability was also recorded in the other form of zinc ($ZnCl_2$). Measurement showed results with significantly counteractive effect on viability in samples ZA, ZB, ZD (P<0.05) and ZC, ZE (P<0.01; Figure 5).

The present study was design to study the effect of zinc supplementation to rabbit spermatozoa *in* vitro. Its presence is important in testes development **(Merker and Günther, 1997)**, spermatogenesis **(Yamaguchi *et al*., 2009)** but some studies assert that influence of zinc on spermatozoa motility is still controversial **(Gavella and Lipovac, 2009)**.

Few studies **(Omu *et al*., 1998; Chia *et al*., 2000)** show that a high concentration of zinc is connected with increased sperm motility. Results of other research paper **(Colagar *et al*., 2009; Yamaguchi *et al*., 2009)** suggest association between low zinc concentration and poor sperm quality. Also no significant correlation was indicated between level of seminal zinc and sperm motility **(Lewis-Jones *et al*., 1996; Kotdawala *et al*., 2012)**. Association between lower spermatozoa motility and higher concentration of zinc in seminal plasma was observed in study focused on seminal plasma of older men **(Henkel *et al*., 2006)**.

Results of **Narasimhaiah *et al*., (2018)**, who studied effect of various concentration of organic zinc supplemented to goat bucks feed showed significantly higher progressive motility compared to control group without feed supplemented with zinc.

Despite the fact that many studies evaluated seminal concentration of zinc and its effect on reproduction **(Massányi *et al*., 2003, 2008; Krakowski *et al*., 2015; Kasperczyk, 2016)**, only few information are accessible for *in vitro* effect **(Riffo *et al*., 1992; Gavella and Lipovac, 2009; Talevi *et al*., 2013)**. **Gavella and Lipovac (2009)**, who studied *in vitro* effect of zinc on oxidative changes in human semen observed that zinc participates in oxidative changes occurring after ejaculation. Positive protective effect of *in vitro* treatment with zinc ($ZnCl_2$), d-aspartate and coenzyme q10 on human sperm motility, lipid peroxidation and DNA fragmentation were observed by **Taveli *et al*. (2013)**. **Riffo *et al*. (1992)** confirmed effect of zinc ($ZnSO_4$) on human sperm motility, acrosome reaction as well as on capacitation. **Wu *et al*. (2015)** supplemented freshly ejaculated human spermatozoa with $ZnCl_2$ along with H_2O_2. Added zinc was able to diminish H_2O_2 induced oxidative stress however zinc in semen samples without H_2O_2 caused decreased motility. In terms of motility, **Barbato *et al*. (2017)** confirmed positive effect of zinc in combination with D-aspartate and co-enzyme Q10 on spermatozoa with induced oxidative stress. Moreover also embryos, produced of oxidative stress affected spermatozoa and fresh oocytes, developed better in culture mediums treated with mentioned supplements.

The negative influence of zinc was detected also in carp spermatozoa. **Chyb *et al*. (2000)** in their study evaluated how zinc in different concentrations affects motility parameters, VCL (velocity curved line), VAP (average path velocity) and VSL (straight line velocity). Carp semen with added zinc in concentrations of 100 and 200 ppm showed significantly decreased motility parameters.

Results of several studies indicate that extracellular zinc truly effect sperm motility, whether effects positively or negatively depends on species and the dose **(Stoltenberg, 1997; Yamaguchi *et al*., 2009)**.

CONCLUSION

The results of this study indicate that both forms of zinc used in present study have not beneficial effect on rabbit spermatozoa after incubation in different time periods. Supplemented zinc in form $ZnCl_2$ to rabbit semen had higher negative impact on rabbit spermatozoa motility and viability parameters than zinc in form $ZnSO^4.7H_2O$. However, further studies with *in vitro* zinc supplementation may confirm the toxic effect of selected concentrations. Possibly, the addition of smaller concentrations of zinc might appear as beneficial.

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