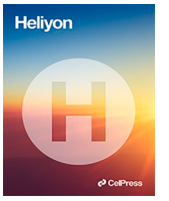
Heliyon 10 (2024) e35879

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Calcium affects stallion spermatozoa parameters in different incubation temperatures

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ARTICLE INFO

Keywords:
Stallion
Spermatozoa
$CaCl_2$
Motility
Viability

ABSTRACT

This study was aimed to determine the effect of $CaCl_2$ on the motility and viability of stallion spermatozoa during different incubation temperatures. Experimental samples were prepared by diluting the ejaculates (n = 10) from three uniformly housed and fed breeding stallions with six different concentrations of $CaCl_2$ (A: 0.1125, B: 0.225, C: 0.45, D: 0.938, E: 1.25, and F: 1.875 mg/mL). The control samples (CON) were prepared by diluting ejaculate only with physiological solution. Samples were divided into two aliquots for analyses at different storage temperatures (5 °C and 37 °C). The motility parameters were analysed by Computer Assisted Semen Analysis system at several time intervals (0, 1, 2 and 3 h) and the viability was assessed using a mitochondrial toxicity test (MTT) realized at the end of incubation at both temperatures. Addition of $CaCl_2$ to stallion semen showed significant effect on motility parameters, especially in the highest concentrations at 5 °C. Significant objectionable effect of $CaCl_2$ on both total and progressive motility was observed at temperature 37 °C compared to control sample. However, results of velocity curved line in samples C, D and F at time 1 h and also at time 2 h in sample F showed significant positive effect of $CaCl_2$. Sperm viability in experimental samples did not show a significant difference compared to the control at either 5 °C or 37 °C. The results of this study did not confirm essential effect of calcium on reproductive parameters of stallion. To conclude, our study demonstrated that the effect of $CaCl_2$ on stallion sperm motility differs in a dose-dependent manner; however, the overall impact on motility parameters does not seem to be beneficial.

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https://doi.org/10.1016/j.heliyon.2024.e35879
Received 27 April 2023; Received in revised form 1 August 2024; Accepted 5 August 2024
Available online 8 August 2024

1. Introduction

Cold storage of semen intended for artificial insemination is widely used in horse breeding. This type of storage is preferred over frozen storage due to practical reasons, such as the need for highly specific freezing techniques and the compromised fertility outcomes of frozen-thawed semen [1,2]. Gibb and Aitken [3] reviewed developments in stallion semen preservation and underlined the continuous need for optimization of semen processing and storage, as equine reproductive biotechnology doesn't quite meet the effectiveness recorded in other farm animal species.

One of the ways of how to improve the quality of stored semen is enrichment of insemination doses with beneficial substances of natural or artificial origin [4]. Nutraceuticals are dietary supplements of plant or animal origin providing health benefits. They are natural alternatives to pharmacological substances and are nowadays frequently used in human and veterinary medicine. Comparable advantage of nutraceuticals arises from their bioavailability. Efforts in the supplementation of nutraceuticals have shown promising positive effect on stallion spermatozoa under both *in vitro* and *in vivo* conditions [5,6]. However, the content of biologically active compounds varies due to geographical origin, environmental factors, specimen varieties, and storage types, making it difficult to determine the most effective concentrations of these substances [7,8]. Uniform, highly pure and stable composition under defined conditions is the biggest advantage of synthetized compounds. Abundant number of studies demonstrates how these compounds positively affect stallion spermatozoa during cold storage. There is evidence for these substances to act solely [9,10] or synergistically [11,12].

Calcium is an essential element that is decisive regulator of many physiological processes in any living cell, including sperm. Extracellular Ca provides calcium ion to maintain intracellular Ca, bone mineralization, blood clotting and plasma membrane potential. Further, calcium stabilizes the plasma membrane and affects its permeability [13,14]. In this way, while the calcium in the head of spermatozoa could be involved in the modulation of progesterone-induced acrosomal exocytosis, the calcium in the midpiece could be more related to mitochondria-modulated processes such as sperm motility, mitochondria-based energy production and early capacitation steps [15]. Calcium is an important modulator for capacitation, acrosome reaction and is probably a key messenger in the exchange of information between sperm and egg. Albumin present in the female reproductive tract removes cholesterol from the sperm plasma membrane thus allowing better permeability. In addition to tyrosine phosphorylation, the capacitation includes elevation of intracellular pH and Ca^{2+} [16].

Calcium is involved in the activation of proacrosin to acrosin happening during the acrosome reaction [17]. The proteolytical machinery enabled by acrosome reaction converts exposed acrosin precursor into acrosin [18]. External calcium accelerates this conversion in the concentration-dependent manner [19]. Calcium also activates the adenylate cyclase of the AMP system, thereby increasing intracellular concentrations of cAMP. Increased levels of intracellular calcium are also necessary for the processes associated with the acrosome reaction. However, high intracellular calcium levels over a longer period of time also lead to cell death if fertilization does not occur [17]. Calcium ions (Ca^{2+}) are the trigger for the acrosome reaction in mammalian sperm, and there is considerable evidence that calcium ions also affect sperm motility. The prostate, seminal vesicles and epididymis. Have been shown to be also very rich in calcium, which is why several studies have investigated the association between calcium and male fertility [14]. The role of calcium is also in the maintenance of spermatogenesis, sperm maturation, DNA metabolism and gene expression in germ cells [20].

The aim of the present study was to assess the effects of various $CaCl_2$ concentrations on the kinetic parameters and viability of stallion spermatozoa during incubation at 5 °C and 37 °C as a potential component of stallion semen extenders, which have been used in common practice to protect and nourish semen during the semen storage until the artificial insemination service.

2. Materials and methods

2.1. Collection and processing of semen

Samples of semen (n = 10) were obtained from breeding stallions that were clinically healthy and located in western part of Slovakia (District of Nitra) between the ages of 3 and 18. Ejaculates were obtained from the following breeds: one Oldenburger stallion and two Holsteiner stallions. Stallions were fed and housed with the same circumstances and the semen collection procedure was carried out to their routine collection schedule (three times a week in the early morning). The stallions were meticulously handled in accordance with the ethical guidelines of the Animal Protection Regulation of the Slovak Republic RD 377/12, following with the European Union Regulation 2010/63. Experimental protocols no. 178/2002 were accepted by the committee at SUA in Nitra, Slovak Republic. Ejaculates were collected on a regular schedule utilizing a pre-heated, lubricated artificial vagina (Colorado model, Minitüb, Tiefenbach, Germany) following stimulation of stallion by a mare positioned near the phantom. Only the samples which met the given criteria (minimum 50 % motility and concentration of at least 1×10^9 sperm/mL) were used in the experiments that followed. Each ejaculate was instantly after the collection diluted with $CaCl_2$ ($CaCl_2$, SLAVUS s.r.o., Bratislava, Slovakia) dissolved in the saline in ratio 1:2 to a final concentration of around 50×10^6 cells/mL. Six experimental groups were prepared to the following final concentration (mg/mL): A: 0.1125, B: 0.225, C: 0.45, D: 0.938, E: 1.25, F: 1.875. The control sample (CON) was diluted without addition of $CaCl_2$. The diluted semen samples were divided into two aliquots for the two incubation temperatures (5 °C and 37 °C). Samples were incubated at 5 °C and 37 °C during whole experiment as previously described by Halo et al. [9].

2.2. Motility analysis

The motility analysis was assessed using a CASA system (Computer Assisted Semen Analysis) - Sperm Vision™ program (Minitüb, Tiefenbach, Germany), provided with a negative phase contrast microscope Olympus BX 51 (Olympus, Tokyo, Japan) with 20 × magnification at time intervals 0, 1, 2 and 3 h. Sample (10 μL) from each experimental/control group was transferred into a Makler Counting Chamber® (depth 10 μm, Semi–Medical Instruments, Haifa, Israel) and warmed up for 15 s to 37 °C before being analysed. To carry out a single CASA assessment, at least seven different fields within the counting chamber was performed. Using the set up unique to stallions and the chosen parameters listed below were assessed: sperm total motility (MOT; %), sperm progressive motility (PRO; %), velocity curved line (VCL; μm/s) as previously defined by Tirpák et al. [21] and Halo et al. [22].

2.3. Viability

The viability was measured using the mitochondrial toxicity test (MTT) after 3 h of incubation at 5 °C or 37 °C. The conversion of 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis,MO, USA) to purple formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria of living cells. Mitochondrial activity was realized by an ELISA reader (Multiskan FC, ThermoFisher Scientific, Vantaa, Finland) at wavelength of 570 nm against 620 nm. The data were expressed as a percentage, while the value of the control sample was standardized to 100 % [23].

2.4. Statistical analysis

All date were analysed by the GraphPad 9 software (GraphPad Software Inc., San Diego, CA, USA). All obtained data were examined for normal Gaussian distribution using a D'Agostino–Pearson normality test following Shapiro–Wilk normality test. Descriptive statistical parameters (mean, standard deviation) were determined using the one-way ANOVA and Dunnett's post-test. Three levels of statistical significance estimation were used: *** ($p < 0.001$), ** ($p < 0.01$), and * ($p < 0.05$). Results were interpreted as means and expressed with standard deviation (SD) [11].

3. Results

3.1. Incubation at 5°C

As the incubation time increased, a gradual percentage decrease in motility was observed in all samples. In sample F, demonstrably lower values were recorded at each observation time compared to the control sample: at 0 h ($p < 0.001$); at times 1, 2 and 3 h ($p < 0.01$). Also, in samples D and F, a significant decrease in motility was found (time 0, 2 and 3 h). Results are shown in Fig. 1A. During progressive motility monitoring, there was a significant demonstrable decrease ($p < 0.001$) in progressive mobility, especially in experimental samples with the highest concentration of $CaCl_2$ (D, E, F) at times of 0, 1 and 2 h. After 1 h of incubation, a significant increase in progressive motility ($p < 0.05$) was observed in sample B. At time 2 h, a significantly lower ($p < 0.001$) sperm progressive motility was presented across all samples compared to the control. Results are shown in Fig. 1B.

The analysis of velocity curved line showed that, an approximately balanced speed was observed among the experimental samples and the control at times 0, 1 and 2 h. The only significant decrease ($p < 0.01$) in velocity curved line was recorded at 3 h in sample F (Fig. 2). After 3 h of incubation at temperature 5 °C, results of viability in experimental samples showed very similar results compared to control samples, but without statistical significances (Fig. 3).

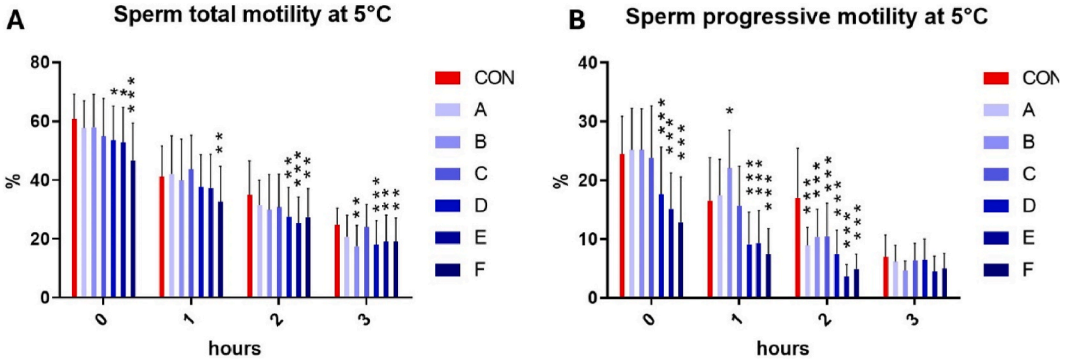

Fig. 1. (A) The effect of $CaCl_2$ on the sperm total motility (%) at 5 °C (n =10). (B) The effect of $CaCl_2$ on the sperm progressive motility (%) at 5 °C (n = 10). CON: 0, A: 0.1125, B: 0.225, C: 0.45, D: 0.938, E: 1.25, F: 1.875 mg/mL. The mean value of the observed samples is represented by each bar (±SD). The levels of significance were set at *** ($p < 0.001$), ** ($p < 0.01$) and * ($p < 0.05$).

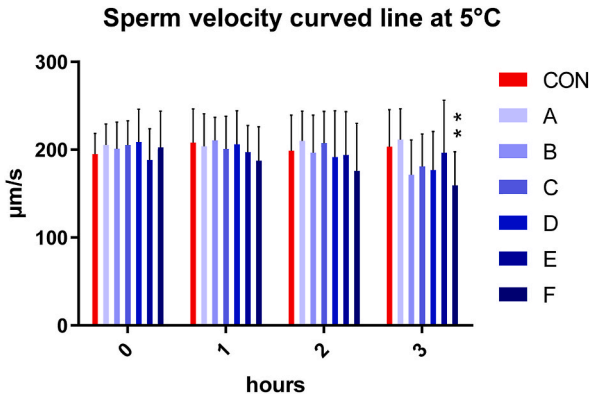

Fig. 2. The effect of $CaCl_2$ on the sperm velocity curved line (μm/s) at 5 °C ($n = 10$). CON: 0, A: 0.1125, B: 0.225, C: 0.45, D: 0.938, E: 1.25, F: 1.875 mg/mL. The mean value of the observed samples is represented by each bar (±SD). The levels of significance were set at *** ($p < 0.001$), ** ($p < 0.01$) and * ($p < 0.05$).

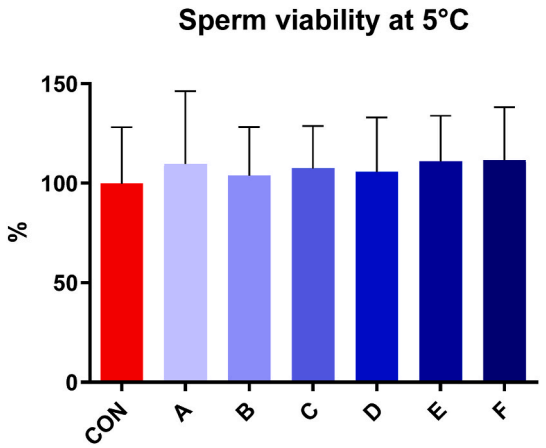

Fig. 3. The effect of $CaCl_2$ on the sperm viability (%) at 5 °C ($n = 10$) after 3 h of incubation. CON: 0, A: 0.1125, B: 0.225, C: 0.45, D: 0.938, E: 1.25, F: 1.875 mg/mL. The mean value of the observed samples is represented by each bar (±SD). The levels of significance were set at *** ($p < 0.001$), ** ($p < 0.01$) and * ($p < 0.05$).

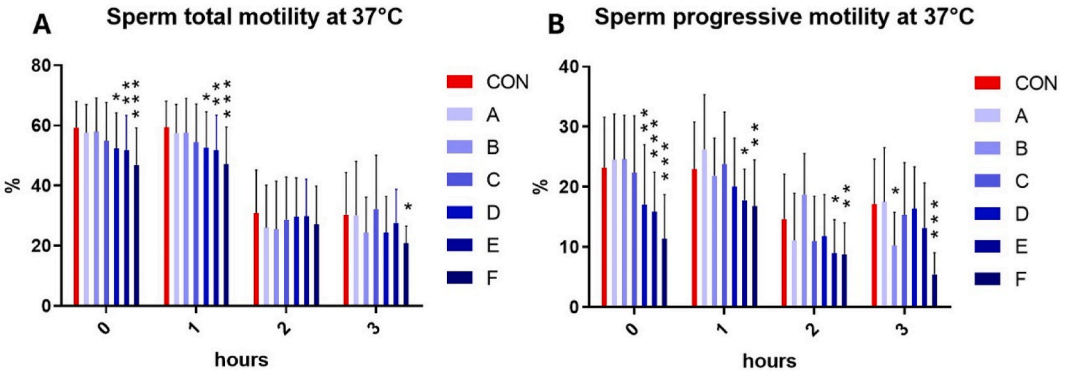

Fig. 4. (A) The effect of $CaCl_2$ on the sperm total motility (%) at 37 °C (n =10). (B) The effect of $CaCl_2$ on the sperm progressive motility (%) at 37 °C ($n = 10$). CON: 0, A: 0.1125, B: 0.225, C: 0.45, D: 0.938, E: 1.25, F: 1.875 mg/mL. The mean value of the observed samples is represented by each bar (±SD). The levels of significance were set at *** ($p < 0.001$), ** ($p < 0.01$) and * ($p < 0.05$).

3.2. Incubation at 37°C

The negative dose-dependent effect of $CaCl_2$ at higher temperature during *in vitro* incubation was similar to that at 5 °C. The highest concentrations of $CaCl_2$ demonstrated a negative effect on stallion motility throughout the entire incubation period. A significant decrease of motility was observed at times 0 and 1 in samples D ($p < 0.05$), E ($p < 0.01$) and F ($p < 0.001$). In initial time, a significant decrease in progressive motility in experimental samples D ($p < 0.01$) and E, F ($p < 0.001$) was observed. During the entire incubation period, there was reduced progressive motility in the samples with the highest $CaCl_2$ concentrations compared to the control sample: time 1 and 2 h E ($p < 0.05$) and F ($p < 0.01$), respectively, and time 3 h F ($p < 0.001$). The evaluation of stallion spermatozoa motility and progressive motility is illustrated in Fig. 4(A and B).

The values of the velocity curved line of stallion spermatozoa were significantly higher compared to the control sample after 1 h of incubation in samples C, D and F ($p < 0.05$) and after 2 h of incubation in sample F ($p < 0.001$). Results are shown in Fig. 5. After 3 h of incubation, the highest percentage of viable spermatozoa was in the sample A. Other experimental samples showed a reduced percentage of viability compared to the control sample, however this difference was not significant (Fig. 6).

4. Discussion

High concentrations of calcium in the preservative medium can reduce sperm motility, spermatozoa metabolism and "calcium intoxication" can be a factor in temperature shock. Lowering the temperature of the environment increases the absorption of calcium by the sperm, and the effect worsens if the cooling rate is higher [24].

The influence of a medium with a low calcium content was addressed in the work of Nishida et al. [25]. By preincubating human sperm in a medium with a low calcium content, they were able to enhance the acrosome reaction in spermatozoa and also increase the fertilizing ability of human sperm, thus proving simplicity and effectiveness of this treatment. This method was proposed as a method for preparing sperm from sub-fertile men for human assisted reproduction therapy. Nishida et al. [25] used light microscopy and triple-stain technique to evaluate the motility and acrosome reaction, while we used a more objective CASA system as our main focus was aimed onmotility-related effects. Furthermore, our motility analysis results are supported with colorimetric viability assay.

The protective effect of calcium against the negative effect of lead on sperm motility of Swiss white mice was described by Iranpour et al. [26]. The progressive motility of sperm washed out of the epididymis in a group of mice, which were injected lead (200 mg/kg) and subsequently injected calcium chloride (80 mg/kg) for three days had higher progressive sperm motility than the mice given only lead, implicating a potentially meliorative effect of calcium chloride on acute heavy metal poisoning.

The positive effect of Ca^{2+} has been found not only in livestock animals, humans but also in fish. Alavi et al. [27] in their work evaluated sperm concentration, volume, ion content, osmolality and the effect of the dilution ratio on sperm motility of European perch (Perca fluviatilis L.). Optimal sperm motility was observed when sperm was pre-diluted in an immobilization solution in a ratio of 1:50. The spermatozoa showed a maximum velocity, when activated in 2.5 mmol/L Ca^{2+}, 50 mmol/L K^{+} and sucrose with osmolality of 100 mOsm/kg.

The synergistic effect of calcium, magnesium and creatine phosphatase on the viability of human sperm has been studied by Fakih et al. [28]. Human spermatozoa were incubated with 1 mmol/L calcium, 5 mmol/L magnesium and 10 mmol/L creatine phosphatase for 0, 1, 5 and 10 h, during which spermatozoa motility and velocity were analysed. The results suggested that calcium and creatine phosphate can promote sperm motility and velocity at a significant level. Thus, the addition of calcium or creatine phosphate to the medium may increase the ability of sperm fertilization during *in vitro* fertilization. The lowest concentration of $CaCl_2$ used in our study is consistent with the work of Fakih et al. [28].

Optimal calcium concentration in the regulation of sperm motility under *in vitro* conditions investigated Bhoumik et al. [29]. In their work, they examined the effect of different concentrations of Ca^{2+} (0–1000 μmol/L) on the qualitative parameters of goat spermatozoa washed out of the epididymis. Based on the results, they concluded that the optimal concentration of Ca^{2+} that promotes sperm motility is 10 μmol/L. Conversely, with higher calcium concentrations, sperm motility decreased within a few minutes. They also concluded that high concentrations of Ca^{2+} in semen can be the reason of infertility in many patients. Therefore, it should be remembered that the level of extracellular Ca^{2+} is an essential parameter for regulating spermatozoa motility.

Another work dealing with the influence of Ca^{2+} on the motility of boar spermatozoa was carried out by Li et al. [30]. Immediately after collection and subsequent centrifugation, spermatozoa were incubated in non-capacitating (N-Cap) medium, capacitating (Cap) medium and capacitating medium with different Ca^{2+} concentrations (Cap-Ca^{2+}; 0.5, 1.0, 2.0, 3.0, 4.0 mmol/L). \ After 2.5 h of incubation, the motility was inhibited in a sample with a concentration of 4 mmol/L Ca^{2+}. Similar results were also recorded for other monitored velocity parameters (VCL, VAP, VSL). In our work, similar concentrations of calcium in samples A - C (1–4 mmol/L Ca^{2+}) were used. The results of the highest concentration used (4 mmol/L) correspond to the results of Li et al. [30]. The inhibition of sperm motility monitored in the present study may be connotated with findings of Meseguer et al. [31] who reported that presence of an extracellular Ca^{2+} is required to promote capacitation, hyperactivation and acrosome reaction, it is possible that the Ca^{2+} supplementation to seminal extenders containing egg yolk may be able to increase the capacitation and therefore the acrosome reaction with the consequent harmful effect on the viability of spermatozoa.

5. Conclusions

Based on the results of the analysis of the effect of $CaCl_2$, its inhibitory effect on the stallion spermatozoa movement parameters during cultivation at 5 °C was observed, however positive effects of $CaCl_2$ have also been noted; demonstrably higher values than the

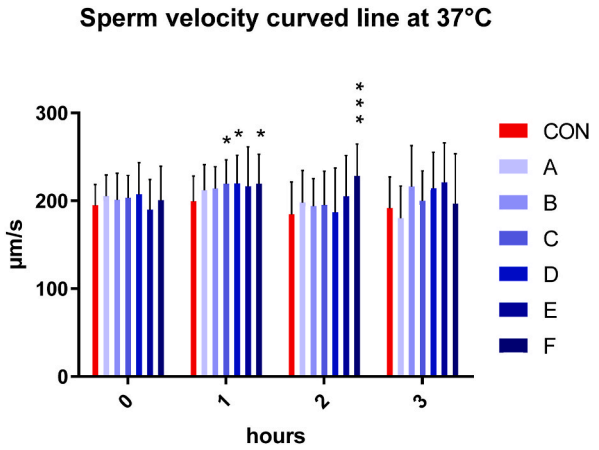

Fig. 5. The effect of $CaCl_2$ on the sperm velocity curved line (μm/s) at 37 °C ($n = 10$). CON: 0, A: 0.1125, B: 0.225, C: 0.45, D: 0.938, E: 1.25, F: 1.875 mg/mL. The mean value of the observed samples is represented by each bar (±SD). The levels of significance were set at *** ($p < 0.001$), ** ($p < 0.01$) and * ($p < 0.05$).

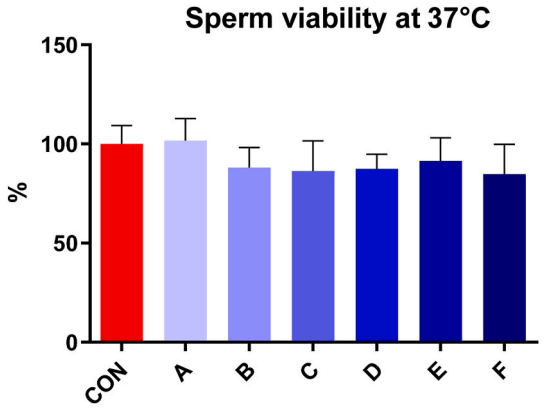

Fig. 6. The effect of $CaCl_2$ on the sperm viability (%) at 37 °C ($n = 10$) after 3 h of incubation. CON: 0, A: 0.1125, B: 0.225, C: 0.45, D: 0.938, E: 1.25, F: 1.875 mg/mL. The mean value of the observed samples is represented by each bar (±SD). The levels of significance were set at *** ($p < 0.001$), ** ($p < 0.01$) and * ($p < 0.05$).

control sample were found in association with velocity curved line at 37 °C. However, further studies with *in vitro* $CaCl_2$ supplementation may clarify its effect on stallion spermatozoa parameters and the mode of action.

Institutional review board statement

The stallions were meticulously handled in accordance with the ethical guidelines of the Animal Protection Regulation of the Slovak Republic RD 377/12, following with the European Union Regulation 2010/63. Experimental protocols no. 178/2002 were accepted by the committee at SUA in Nitra, Slovak Republic.

Informed consent statement

Not applicable.

Data availability statement

All the data is contained within the article and the Supplementary Materials.

CRediT authorship contribution statement

Marko Halo Jr: Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Data

curation, Conceptualization. **Filip Tirpák:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Formal analysis, Conceptualization. **Martin Massányi:** Formal analysis. **Lucia Dianová:** Formal analysis. **Michal Lenický:** Formal analysis. **Tomáš Slanina:** Methodology. **Jiřina Zemanová:** Supervision. **Alžběta Matušková:** Investigation. **Agnieszka Greń:** Supervision. **Marko Halo:** Funding acquisition. **Peter Massányi:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was funded by Scientific Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic, grant number VEGA 1/0698/22, VEGA 1/0437/24. This work was also supported by the project of the Slovak Research and Development Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic, grant number APVV-21-0168 and the project of the Grant Agency of the Slovak University of Agriculture in Nitra 11-GASPU-2021.

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