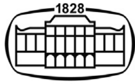

AKADÉMIAI KIADÓ

The effects of caffeine on the motility and viability of stallion spermatozoa at different temperature conditions

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RESEARCH ARTICLE

ABSTRACT

The purpose of this study was to evaluate the dose- and time-dependent effect of caffeine treatment on the motility and viability of stallion spermatozoa at different temperatures. Six dose groups (A to F) were established with changing caffeine concentrations (from 0.625 to 10 mg/mL). The control samples were prepared by diluting the ejaculate only with physiological salt solution. The samples were examined after 0, 1, 2 and 3 h of incubation at 5 °C and 37 °C. The motility parameters were evaluated by Computer Assisted Semen Analyzer (CASA) system, and the viability was assessed by the mitochondrial toxicity test at the end of the incubation. A positive effect of the lowest tested caffeine concentration on the motility parameters was observed throughout the incubation period at 5 °C. At the end of the 3h incubation, the viability in every sample in these groups, treated with any caffeine concentration, showed lower values compared to the control. At the higher incubation temperature (37 °C), caffeine positively affected the motility in samples B ($P < 0.05$) and D, E, F ($P < 0.001$) after 3 h of incubation; however, the viability showed a slightly decreasing tendency. Our results suggest that caffeine, in an optimal concentration, may be used as a component of stallion semen extenders.

KEYWORDS

caffeine, spermatozoa, stallion, CASA, viability

INTRODUCTION

Extenders for stallion ejaculates have been developed and modified for decades. Their main goal is to ensure and prolong the viability of spermatozoa during long-time storage and transport prior to artificial insemination (Ijaz and Ducharme, 1995). Suitable semen extenders must provide spermatozoa with a sufficient amount of energy sources and maintain pH and osmotic pressure on the natural levels monitored in the seminal plasma, thus offering a beneficial environment for the spermatozoa (Siudzińska and Łukaszewicz, 2008). Extended ejaculates should be transported and used within the shortest possible time, ideally not exceeding 24 h from the collection. Fertilising ability naturally declines along with the increasing time after collection, hence an extensive number of studies focusing on addition of stimulating substances and antioxidants has been conducted to minimise the impact of storage time on spermatozoa quality (Rossi et al., 2020; Tirpák et al., 2021a).

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Various natural substances are used to prolong viability, stimulate motility and protect spermatozoa against reactive oxygen species (Stephens et al., 2013; Pariz et al., 2019). Derivatives of pentoxifylline and methylxanthine are used for improving the motility. Caffeine as a phosphodiesterase inhibitor may increase the intracellular level of cyclic adenosine monophosphate (cAMP) in spermatozoa, which may affect their oxidative metabolism. Elevated cAMP levels accelerate glycolysis, which generates adenosine triphosphate (ATP), which moves spermatozoa. Thus the dilution of the ejaculate with caffeine increases the ATP levels in the cells, leading to increased sperm motility (Stephens et al., 2013). Increased level of cAMP in spermatozoa activates the protein kinase that eventually contributes to tyrosine phosphorylation, post-translational modification involved in the capacitation of spermatozoa. Moreover, caffeine may regulate the metabolism of spermatozoa and thus may lead to instant hyperactivation (Rossi et al., 2020).

Caffeine, as an important member of the methylxanthine class, has been shown to improve spermatozoa motility and viability of fresh or frozen semen in various animal species such as Turkey (Slanina et al., 2018a), stallion and donkey (Rota et al., 2019), bull (El-Gaafary et al., 1990), and also in man (Rees et al., 1990). Caffeine is an alkaloid and its catabolic products xanthine and theobromine show antioxidant properties, which are considered to increase the motility and fertilisation ability of spermatozoa (Azam et al., 2003). In some cases, caffeine was able to initiate movement of immotile spermatozoa, for example when isolated from the testes (El-Gaafary et al., 1990).

Based on the accessible literature, we assumed that the addition of caffeine to stallion spermatozoa should result in an improvement of semen quality throughout the incubation time. The overall goal of the present study was to assess the effects of various caffeine concentrations on the kinematic parameters and viability of stallion spermatozoa during *in vitro* incubation at 5 °C and 37 °C.

MATERIALS AND METHODS

Animals and semen collection

Semen was collected from 10 clinically healthy breeding stallions (Nitra region, Slovakia) of 4–19 years of age, composed of the following breeds: Lipizzaner, Hucul, Holsteiner, Oldenburger. Selected stallions were housed and fed under the same conditions. The animals were carefully handled in accordance with the ethical guidelines of the Slovak Animal Protection Regulation RD 377/12, conforming to the European Union Regulation 2010/63. The ejaculates were obtained according to a regular collection schedule using a lubricated pre-warmed artificial vagina (Colorado type, Minitube, Tiefenbach, Germany). Ejaculate collection was performed with the help of trained staff in the mornings during the breeding season (from February to September).

Semen processing

Immediately after collection, the fresh semen was filtered over a gauze layer to remove impurities. Then the semen was diluted at 1:2 with the caffeine solutions (Caffeine powder, Reagent Plus®, Sigma-Aldrich, USA dissolved in physiological saline). Six dose groups were prepared with the following final concentration (mg/mL) regarding caffeine: A: 0.625, B: 1.25, C: 2.5, D: 5, E: 7.5 and F: 10, all containing approximately 50×10^6 cells/mL. The volume of each sample was 1 mL in an Eppendorf tube, which was kept in standing position at 5 °C or 37 °C. The diluted semen samples were divided into two aliquots for the two incubation temperatures (5 °C and 37 °C) as described previously (Slanina et al., 2018a). The control samples (CON) were diluted with physiological salt solution without caffeine. Prior to the motility measurements, 10 µL of sample was placed into a Makler Chamber and kept on heating plate for 20 s to warm up to the appropriate physiological temperature.

Motility analysis

Semen motility was assessed using the Computer Assisted Semen Analyzer (CASA) method with SpermVision software (Minitube, Tiefenbach, Germany) equipped with a negative phase contrast microscope (Olympus BX 51, Olympus Corporation, Tokyo, Japan) with 20 × magnification (Halo et al., 2021a; Vizzari et al., 2021). At incubation times 0, 1, 2 and 3 h a set-up for stallion analysis was used and the most important parameters were selected: total motility (MOT; %), progressive motility (PRO; %), and velocity curved line (VCL; µm/s). Within each of the measurements by the CASA system, the motility parameters from minimum seven fields of the Makler Counting Chamber (10 µL, Sefi-Medical Instruments, Germany) were assessed (Massányi et al., 2020; Tirpák et al., 2021b).

Mitochondrial activity of spermatozoa

The viability of the cells treated with caffeine was assessed by the metabolic activity (MTT) test after 3 h of incubation. This colorimetric assay measures the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich) to purple formazan in viable cells. The optical density was determined at a wavelength of 570 nm against 620 nm as reference using a microplate spectrophotometer ELISA reader (Multiscan FC, ThermoFisher Scientific, Vantaa, Finland). The data were expressed as percentage of the control (Jambor et al., 2017; Halo et al., 2021b).

Statistical analysis

All the statistical analyses were performed using the GraphPad Prism 5 (GraphPad Software Inc., USA). The spermatozoa parameters were examined by one-way analysis of variance (ANOVA) with Dunnett's *post hoc* test. All obtained data were tested for normal Gaussian distribution using a Shapiro–Wilk normality test. The level of significance was set at $^{***}(P < 0.001)$, $^{**}(P < 0.01)$ and $^{*}(P < 0.05)$ and the results were interpreted as means and SD.

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RESULTS

Incubation at 5 °C

Analysis of the effect of various caffeine concentrations revealed that the highest spermatozoa motility (57.7%) was recorded at the initial period in sample A. A positive effect of the lowest tested caffeine concentration (0.625 mg/mL, sample A) was recorded throughout the incubation period. A significant increase ($P < 0.001$) in the spermatozoa motility in sample A compared to the control sample (CON) was detected after 2 h of storage at 5 °C. On the other hand, in samples E ($P < 0.05$) and F ($P < 0.001$) a significantly lower motility was found after 1 h of incubation (Fig. 1).

The progressive motility (PRO) of the stallion spermatozoa reflected the results of the total motility (MOT). Higher concentrations of caffeine (samples C, E, and F) showed significant ($P < 0.05$, $P < 0.01$, $P < 0.05$, respectively) detrimental effects after 1 h of storage at 5 °C. In contrast, the lower caffeine concentrations, samples A, C ($P < 0.001$) as well as B ($P < 0.01$) and D ($P < 0.05$) indicated a positive effect after 2 h of storage (Fig. 2).

The results of caffeine effect on VCL showed quite balanced values throughout the incubation period in the experimental samples compared to the control. Sample F, containing the highest concentration of caffeine (10 mg/mL) displayed a significantly higher ($P < 0.05$) VCL after 2 h of incubation (Fig. 3).

The viability of spermatozoa was investigated after 3 h of storage at 5 °C. The experimental samples with various caffeine concentrations showed lower values compared to the control group with no statistical significance (Fig. 4).

Incubation at 37 °C

At the initial time of incubation, the motility results of the experimental samples and the control sample were very balanced (Fig. 5). As the incubation time progressed, the caffeine-treated samples exhibited improved motility compared to the control. After 3 h of storage, a significantly increased motility in samples B ($P < 0.05$) and D, E, F ($P < 0.01$) was observed.

A similar tendency was observed in the progressive motility, as significantly increased spermatozoa motility was registered in samples B ($P < 0.001$) and D, E ($P < 0.05$) after 3 h of storage (Fig. 6).

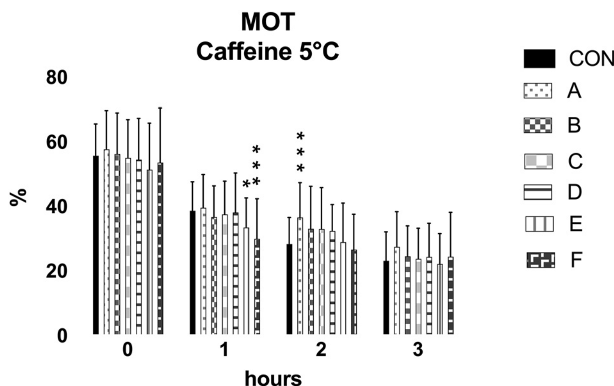


Fig. 1. The effect of caffeine on total spermatozoa motility at 5 °C. Each bar represents the mean value of samples examined ($n = 10$). A – 0.625 mg/mL; B – 1.25 mg/mL; C – 2.5mg/mL; D – 5mg/mL; E – 7.5 mg/mL; F – 10 mg/mL of caffeine. The level of significance was set at *** ($P < 0.001$), ** ($P < 0.01$) and * ($P < 0.05$)

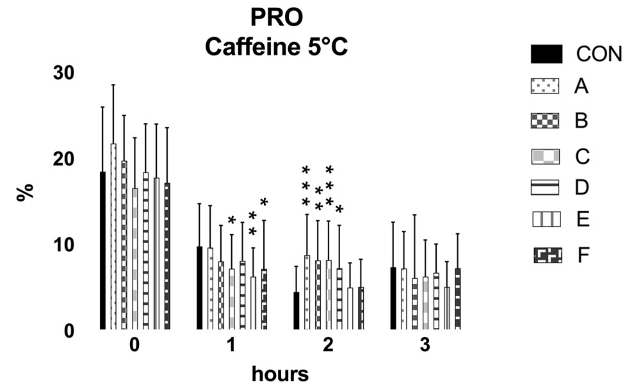


Fig. 2. The effect of caffeine on the progressive motility of spermatozoa (%) at 5 °C. For further information see legend to Fig. 1

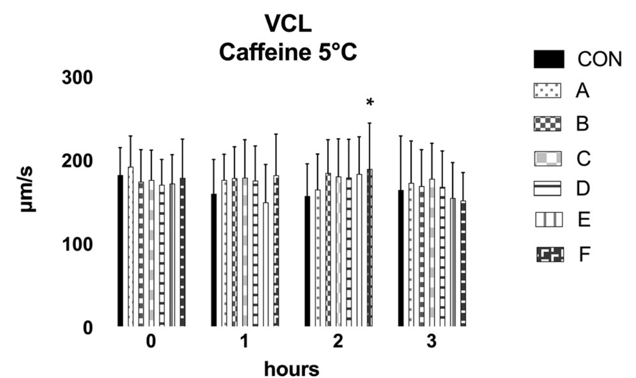


Fig. 3. The effect of caffeine on the velocity curved line (µm/s) at 5 °C. For further information see legend to Fig. 1

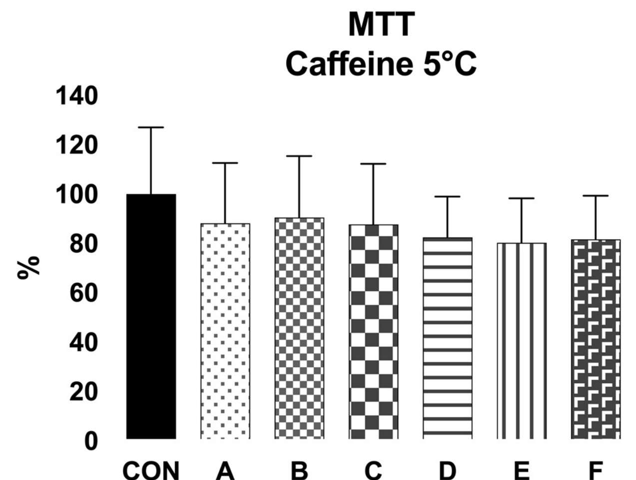
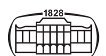


Fig. 4. The effect of caffeine on the viability (%) of stallion spermatozoa after 3 h of incubation at 5 °C. For further information see legend to Fig. 1



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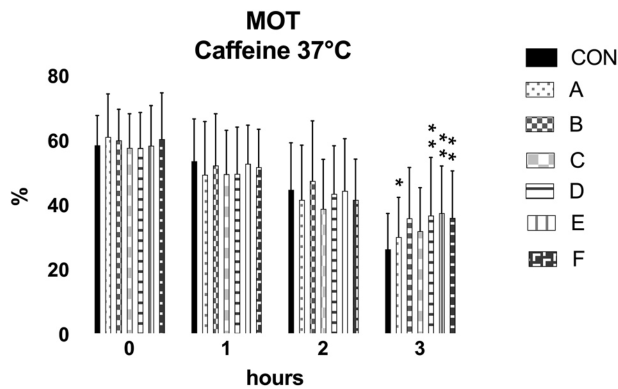

Fig. 5. The effect of caffeine on total spermatozoa motility (in %) at 37 °C. For further information see legend to Fig. 1

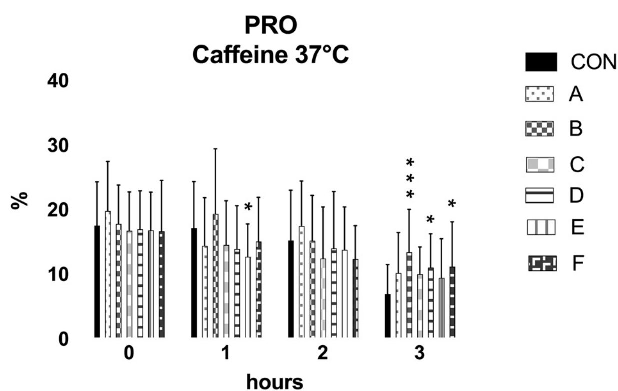

Fig. 6. The effect of caffeine on the progressive motility of spermatozoa (%) at 37 °C. For further information see legend to Fig. 1

The results of the VCL parameter showed significant decrease in sample C ($P < 0.01$) compared to the control sample after 2 h of incubation. A significant increase of VCL was observed in the sample with the highest caffeine concentration (F – 10 mg/mL) after 3 h of storage (Fig. 7).

The cell viability, assessed at the terminal period of incubation at 37 °C, exhibited a decreasing tendency in all experimental samples compared to the control, however the differences were not statistically significant (Fig. 8).

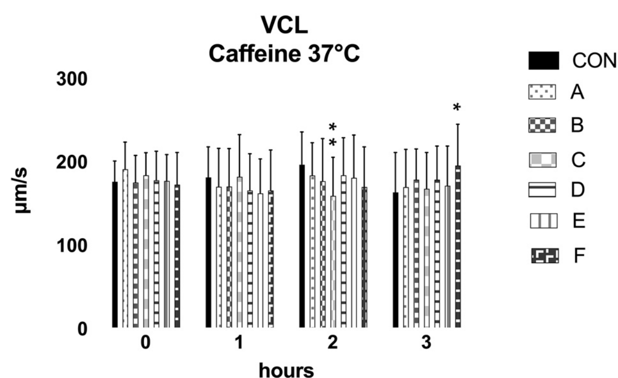

Fig. 7. The effect of caffeine on the velocity curved line (μm s^{-1}) at 37 °C. For further information see legend to Fig. 1

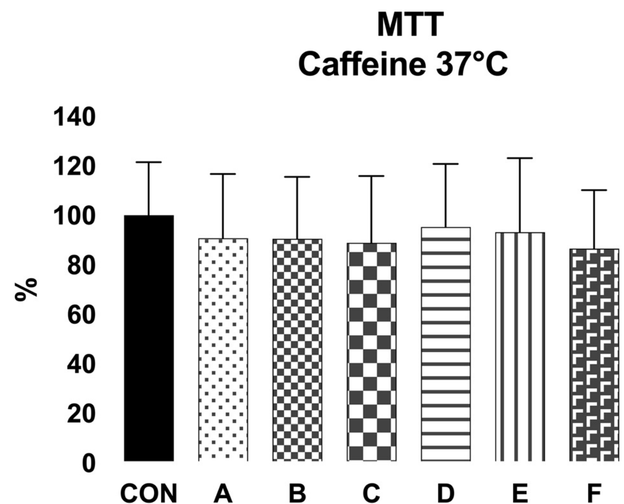

Fig. 8. The effect of caffeine on the viability (%) of stallion spermatozoa after 3 h of incubation at 37 °C. For further information see legend to Fig. 1

DISCUSSION

The present study examined the effects of stimulating agent caffeine on the movement characteristics of stallion spermatozoa with the overall aim to enhance the reproductive efficiency.

Derivatives of pentoxifylline and methylxanthine are studied as potential substances that can protect spermatozoa after thawing (Rota et al., 2019) or during incubation at 5 °C (Slanina et al., 2014). Stephens et al. (2013) thoroughly described the effect of addition of caffeine (2 mM), pentoxifylline (3.5 mM) and taurine (25 mM) to frozen-thawed equine semen. Addition of caffeine in the study of Stephens et al. (2013) had no significant effect on total and progressive motility. In our study we used quite higher concentrations of caffeine (3.2–51.5 mM), and also the incubation temperature was different (5 and 37 °C), while in the previously mentioned study the experiments were conducted at 'laboratory temperature'.

The positive effect of caffeine on the qualitative parameters of spermatozoa was observed following the treatment of thawed equine semen (Rota et al., 2019). Their results showed that immediate supplementation of caffeine to frozen-thawed stallion semen did not significantly differ from the control in terms of total and progressive motility; however, caffeine concentrations of 5 and 10 mM demonstrably increased VCL and the amplitude of lateral head displacement (ALH) compared to the control sample. Results for spermatozoa velocity curved line assessed using the CASA system in our study are consistent with the findings by Rota et al. (2019), where caffeine positively affected this parameter in both cases.

Recently, it has been reported that stallion spermatozoa treated with 5 mM of caffeine added prior to and after 24 h of storage at 4 °C improved neither the motility nor the other kinematic parameters (Rossi et al., 2020). These findings are

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not in close agreement with our results during storage at 5 °C. After 2 h of incubation, the percentage results of motility and progressive motility showed a significant increase in the experimental samples with the lowest caffeine concentration.

In another study, caffeine affected Turkey spermatozoa (Slanina et al., 2018b). The main goal of that research was to analyse the effects of various concentrations of caffeine on the viability and kinematic parameters of Turkey spermatozoa during *in vitro* incubation at 5 and 41 °C. It has been demonstrated that caffeine concentrations between 0.15625 and 7.5 mg/mL had a stimulating effect on the motility of Turkey spermatozoa. No significant effects on viability have been detected throughout *in vitro* incubation at 5 °C and 41 °C, which is consistent with our findings. We did not observe a positive stimulating effect on the viability of stallion spermatozoa at any of the storage temperatures (5 °C or 37 °C).

As in our study, Stachecki et al. (1994) used the CASA system for analysis of spermatozoa motility after caffeine administration. A negative effect of caffeine treatment was found on fresh epididymal spermatozoa of domestic cat. The velocity curved line and velocity straight line decreased after the addition of 10 and 20 mM of caffeine. However, the cryopreserved epididymal spermatozoa of the domestic cat, exposed to various caffeine concentrations, exhibited enhanced motility, velocity curved line, velocity straight line and amplitude of lateral head replacement.

Yamaguchi et al. (2009) have modified Beltsville thawing solution with caffeine (1.15 mM) and $CaCl_2$ (3.97 mM). Their results have demonstrated that this customised freeze-thawing solution for boar semen resulted in better pregnancy and farrowing rates. Following the addition of 10 mM of caffeine to the thawing solution caused enhanced progressive motility, linearity and straightness of boar spermatozoa compared to the untreated sample, which is in accordance with our results. Furthermore, the integrity of the spermatozoa head membranes was not influenced (Yamaguchi et al., 2013). The caffeine concentrations used in their study were comparable with our range of concentrations; however, they added it to the thawing solution while we used caffeine during incubation at 5 °C and 37 °C.

The effect of caffeine was also studied in humans. Significant findings have been obtained by Banihani and Khaled (2021) on men with normozoospermic and asthenozoospermic semen samples. The addition of 10 mM of caffeine resulted in increased progressive motility of spermatozoa. Furthermore, the presence of caffeine enhanced the activity of the seminal creatine kinase.

Alves et al. (2021) have enriched thawed equine insemination doses with caffeine in concentrations of 3 mM, 5 and 7.5 mM. *In vitro* assessments have shown a beneficial impact of 5 mM of caffeine displayed in improved total motility, elevated beat cross frequency and decreased concentration of nitrite. In a subsequent *in vivo* experiment, the laboratory results have been confirmed by recording a significant difference between the fertility rates obtained with conventional insemination doses and doses supplemented with 5 mM of caffeine. Our lowest caffeine concentrations correlate with the concentrations in the study of Alves et al. (2021), where they added caffeine after the thawing process.

In conclusion, we found that caffeine seems to be an effective additive to stallion spermatozoa during cold storage and also during incubation at 37 °C what simulates the actual intrauterine temperature of the mare. We conclude that caffeine treatment can improve the motility and kinematic parameters of stallion spermatozoa in certain measures but the application of caffeine without other nourishments, usually used in semen extenders, limits the potential of caffeine. In respect to spermatozoa viability, the samples were not affected by the caffeine addition. Further studies are needed to evaluate the effect of the implementation of stimulants in the conventional semen extenders in order to prolong the motility and viability of stallion spermatozoa and to increase the success of insemination of mares. Moreover, based on data of the literature, the beneficial properties of caffeine might be used not only in cold storage extenders but also in those intended for cryo-storage.

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