

## Influence of pentoxifylline and caffeine on stallion epididymal sperm motility after thawing

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### Abstract

Two substances at two concentrations each were added to frozen-thawed stallion epididymal spermatozoa to improve motility indicators, which were assessed 30, 60, 120, 180 and 240 min after thawing. Pentoxifylline was added at concentrations of 3.6 mM (1 mg/ml) and 7.18 mM (2 mg/ml). Both concentrations had a positive effect on total and progressive motility of spermatozoa throughout the study. The other substance, caffeine, was added at concentrations of 2 mM (0.4 mg/ml) and 5.5 mM (1 mg/ml). Both concentrations had a positive effect on total and progressive motility of spermatozoa only 30 and 60 min after thawing. Subsequently, 180 min after thawing, improvement was found only in total sperm motility.

*Equine, insemination doses, frozen spermatozoa*

Collection and cryopreservation of epididymal spermatozoa may be the only chance to preserve the unique genotype of a stallion in the case of a lethal injury or castration.

Besides the fact that sources of spermatozoa are limited, there is also the problem of quality of epididymal spermatozoa which is usually not as good as ejaculated sperm in all the commonly evaluated indicators (including fertility). In consideration of this issue, there is an effort to improve these indicators to enable the use of epididymal spermatozoa for regular artificial insemination. One possible way of doing this is by adding different substances to the commonly used cryopreservation media.

Pentoxifylline (PTX) and caffeine (CAF) are readily available and their effect on ejaculated sperm motility has been studied before (Gradil and Ball 2000; Melo-Oña et al. 2012; Stephens et al. 2013; Tsunoda et al. 2015). However, information about their effect on epididymal spermatozoa is scarce (Guasti et al. 2013). Considering the fact that sperm need around 4 h to reach the oviduct, it is important to evaluate the effect of the substances dynamically over that time period. The aim of this study was to determine the effect of different PTX and CAF concentrations on selected motility indicators of stallion epididymal sperm in time. To the authors' knowledge, there is only one study detailing the influence of PTX and CAF on ejaculated sperm over time (150 min only) (Stephens et al. 2013).

### Materials and Methods

For this research, 9 stallions were used. All of them were between 3 and 9 years old, of different breeds, clinically healthy and having both testicles descended in the scrotum.

They were castrated under general intravenous anaesthesia (xylazine, ketamine, diazepam, guaifenesin). The anaesthesia took place without any complications in all the stallions. The method of castration with covered ductus deferens and suture of incisions was chosen.

Collection of the epididymal spermatozoa was done as a retrograde flush of cauda epididymis and ductus deferens within 60 min post castration. The retrograde flushing technique requires careful removal of surrounding fascia of the cauda epididymis and ductus deferens. Subsequently a 14-gauge catheter was inserted in the proximal part of the ductus deferens, and prewarmed (38 °C) semen extender (Gent Extender, Minitübe International, Tiefenbach, Germany) was passed through the cauda and ductus deferens under gentle pressure. Spermatozoa were collected into a preheated beaker and merged together from both epididymides. The volume of the medium was 10 ml for each epididymis.

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Sample evaluation was done under a stereomicroscope. Total motility was estimated and the concentration was counted in a Bürker's chamber.

The samples were then transferred to 0.5 ml straws. After equilibration at 5 °C for 150 min, the samples were frozen using liquid nitrogen vapour in a semiautomatic freezing device and then stored in liquid nitrogen.

Samples were thawed in warm water (38 °C) for 30 s. Sperm from each stallion were diluted (Gent Extender, Minitübe International, Tiefenbach, Germany) to a final concentration of  $30\text{--}50 \times 10^6$ /ml, mixed and incubated for 10 min at 38 °C. Then sperm motility was evaluated. Epididymal spermatozoa collected from each stallion were divided into five tubes and PTX and CAF were added as follows:

- control
- pentoxifylline (final concentration of 3.6 mM, i.e. 1 mg/ml)
- pentoxifylline (final concentration of 7.18 mM, i.e. 2 mg/ml)
- caffeine (final concentration of 2 mM, i.e. 0.4 mg/ml)
- caffeine (final concentration of 5.5 mM, i.e. 1 mg/ml)

The PTX used was added as an injectable solution Trental® 100 mg/5 ml (Takeda GmbH, Konstanz, Germany). The CAF used was also added as an injectable solution, COFFEINUM BIOVETA 125 mg/ml injection solution (Bioveta a.s., Ivanovice na Hané, Czech Republic).

Sperm motility of samples (n=45) was evaluated at 30, 60, 120, 180 and 240 min after dilution. The studied indicators were measured by Computer-Assisted Sperm Analysis (CASA). The following indicators were evaluated: total motility (TM), progressive motility (PM), curvilinear velocity (VCL) a straightness (STR).

Due to the huge dispersion of the results, the data were analysed by non-parametric Wilcoxon pair test, where  $P \leq 0.01$  was considered highly significant and  $P \leq 0.05$  was considered significant.

## Results

### Total motility

Addition of PTX at both concentrations had a positive effect on TM of spermatozoa throughout the study. In some cases, the effect was highly significant, mainly at the PTX concentration of 7.18 mM. Caffeine showed a positive effect on sperm total motility in most cases. A significant effect was not proved only after 120 min at both concentrations and after 240 min at the higher concentration (Table 1).

The dynamics of total sperm motility within 240 min after thawing are shown in Fig. 1.

### Progressive motility

Pentoxifylline at both concentrations significantly improved PM throughout the whole study. Caffeine at both concentrations improved PM significantly but only for a short time period of 30 min after adding to the samples. At the lower concentration, the positive effect lasted for 60 min after the addition (Table 2).

The dynamics of progressive sperm motility within 240 min after thawing are shown in Fig. 2.

### Curvilinear velocity and straightness

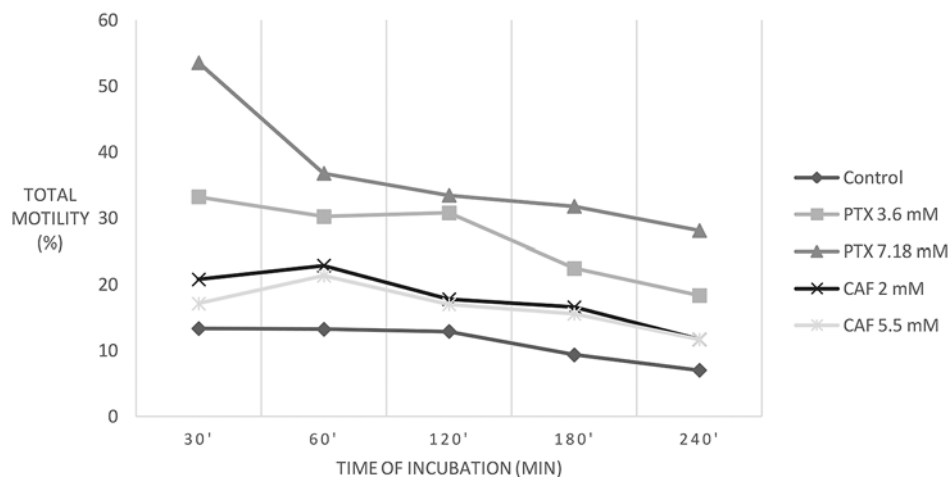
Neither PTX nor CAF had any significant effect on the VCL and STR values. The mean VCL values are shown in Table 3; the mean STR values are shown in Table 4.

Table 1. Mean values (%) of total motility during incubation with the addition of pentoxifylline and caffeine.

Duration (min)	Control	PTX 3.6 mM	PTX 7.18 mM	CAF 2 mM	CAF 5.5 mM
30	13.33	33.22*	53.56**	20.78*	17.11*
60	13.22	30.22**	36.78**	22.89*	21.34**
120	12.89	30.78**	33.45*	17.78	17.00
180	9.33	22.44*	31.78**	16.56*	15.56*
240	7.00	18.34*	28.11**	11.67*	11.67

\* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; significant differences in the experimental groups versus control in a row  
PTX – pentoxifylline, CAF – caffeine

Fig. 1. Total motility changes during incubation with the addition of pentoxifylline and caffeine



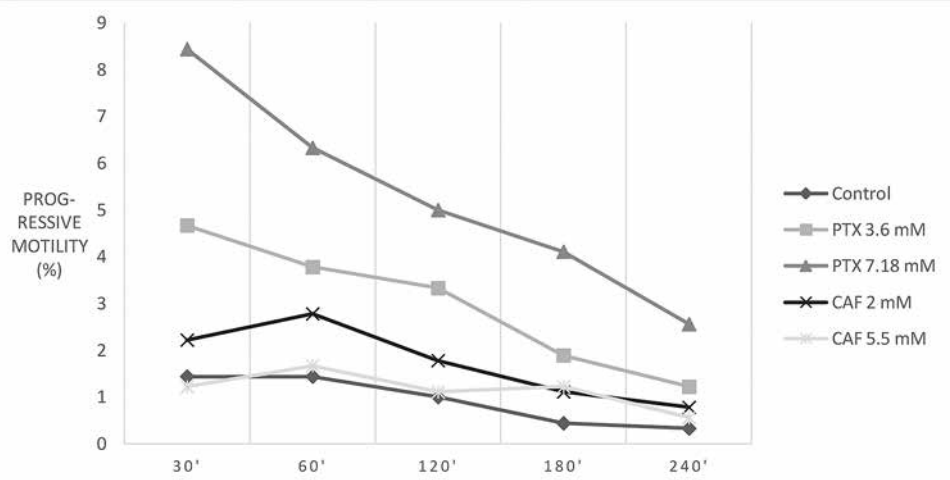
PTX – pentoxifylline; CAF – caffeine

Table 2. Mean values (%) of progressive motility during incubation with the addition of pentoxifylline and caffeine.

Duration (min)	Control	PTX 3.6 mM	PTX 7.18 mM	CAF 2 mM	CAF 5.5 mM
30	1.44	4.67*	8.44**	2.22*	1.22*
60	1.44	3.78*	6.33**	2.78*	1.67
120	1.00	3.33*	5.00*	1.78	1.11
180	0.44	1.89*	4.11**	1.11	1.22
240	0.33	1.22*	2.56**	0.78	0.56

\* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; significant differences in the experimental groups versus control in a row  
PTX – pentoxifylline, CAF – caffeine

Fig. 2. Progressive motility changes during incubation with the addition of pentoxifylline and caffeine



PTX – pentoxifylline; CAF – caffeine

Table 3. Mean values of curvilinear velocity ( $\mu\text{m/s}$ ) during incubation with the addition of pentoxifylline and caffeine.

Duration (min)	Control	PTX 3.6 mM	PTX 7.18 mM	CAF 2 mM	CAF 5.5 mM
30	107.90	101.13	106.40	104.07	86.53
60	108.34	99.96	109.73	102.67	98.10
120	107.72	95.67	103.26	81.30	87.96
180	110.65	85.64	104.82	91.95	81.98
240	92.99	86.27	89.51	68.37	69.37

PTX – pentoxifylline, CAF – caffeine

Table 4. Mean values of straightness (%) during incubation with the addition of pentoxifylline and caffeine.

Duration (min)	Control	PTX 3.6 mM	PTX 7.18 mM	CAF 2 mM	CAF 5.5 mM
30	62.56	69.67	69.00	67.44	68.33
60	67.22	70.00	70.44	71.11	70.33
120	64.67	72.33	71.11	66.89	69.22
180	61.89	64.11	70.11	72.00	59.44
240	48.33	67.78	72.11	56.22	54.78

PTX – pentoxifylline, CAF – caffeine

## Discussion

Pentoxifylline has a positive effect on motility in studies of ejaculated stallion spermatozoa (Gradil and Ball 2000; Stephens et al. 2013) and in other species (Mirshokraei et al. 2001; Barakat et al. 2015). However, there are not many studies about using PTX with stallion epididymal spermatozoa. Guasti et al. (2013) found that adding PTX had a positive effect on motility of stallion epididymal spermatozoa. One study found no increase of motility in ejaculated spermatozoa after adding PTX (Tsunoda et al. 2015). The dosage of PTX in the studies mentioned above was around 3.6 mM and 7.18 mM, which is equal to a concentration of 1 mg/ml and 2 mg/ml, respectively. We decided to use both concentrations in our study in order to compare the results.

Caffeine is another substance used for the purpose of increasing sperm motility indicators in different animal species (Fayed 1996; Barakat et al. 2015) but its effect on ejaculated or epididymal stallion spermatozoa was not investigated very deeply and the results differed (Melo-Oña et al. 2012; Stephens et al. 2013).

We decided to use concentrations of 2 mM and 5.5 mM, which is equal to 0.4 mg/ml and 1 mg/ml, respectively. The concentrations were set according to other authors. Stephens et al. (2013) reported the most effective concentration for stallion ejaculate to be 2 mM. Fayed (1996) reported 5.5 mM to be the best for bull epididymal spermatozoa. Study of bull semen reported similar results (Barakat et al. 2015).

The influence of PTX and CAF on equine sperm indicators over a longer time period is known only in ejaculated spermatozoa (Stephens et al. 2013). Furthermore, the maximum elapsed time period in the study was only 150 min. In our research, we decided to examine the spermatozoa for up to 4 h which is approximately the time needed to reach the oviduct from the uterus (Katila et al. 2000).

In our study, PTX had a significantly positive effect on TM and PM of stallion epididymal spermatozoa throughout the whole measured time period. That corresponds with previous studies conducted on ejaculated (Gradil and Ball 2000; Stephens et al. 2013) and epididymal (Guasti et al. 2013) spermatozoa in stallions.

Due to easier manipulation of the insemination dose, it would be better to add PTX to the spermatozoa before cryopreservation. However, studies carried out in this field showed that adding PTX before freezing negatively influenced motility (Gradil and Ball 2000). The authors of this study assume that it was caused by the exhaustion of spermatozoa before freezing which resulted in less motile spermatozoa after thawing.

A different study reported that adding PTX to equine epididymal spermatozoa before freezing had neither a negative nor a positive effect on motility (Guasti et al. 2013). However, the methods used in that study differed considerably from the previous one by Gradil and Ball (2000). Pentoxifylline was added to the flushing medium and spermatozoa were incubated for 15 min only. Then the samples were centrifuged and rediluted with a freezing medium without PTX. The reason why a decrease in motility was not observed might be: 1) new medium provided new substrates for glycolysis and ATP production or 2) the spermatozoa were not exhausted due to short incubation with PTX. This procedure could be used in the future, however, it is necessary to check whether the effect of PTX on equine epididymal spermatozoa is positive.

There are no known studies regarding the effect of PTX on fertility. One study explored the embryotoxicity of PTX (in human medicine PTX is used to induce motility in nonviable spermatozoa prior to the intracytoplasmic sperm injection) showing that PTX does not have a negative effect on embryo development (Cook et al. 2002).

Although PTX had a positive effect on TM and PM, it did not improve VCL. This result conflicts with other research on equine epididymal (Guasti et al. 2013) and ejaculated (Gradil and Ball 2000; Tsunoda et al. 2015) spermatozoa. It may have been caused by leaving epididymal plasma and its immobilization factors with the spermatozoa during cryopreservation (the samples were not centrifuged after flushing the epididymis).

Another reason may have been high sperm concentrations in some samples which may have prevented the free motion of sperm cells.

A significant effect of PTX on STR was not observed. In this case, our results correspond with the studies of Tsunoda et al. (2015) and Gradil and Ball (2000), which were done with ejaculated spermatozoa. The effect of PTX on STR in epididymal spermatozoa has not been explored yet.

It appears that better results were reached with the higher PTX concentrations of 7.18 mM compared to the lower concentrations of 3.6 mM.

In our study, CAF had a significantly positive effect on TM of epididymal spermatozoa throughout 30, 60 and 180 min after thawing; PM was significantly increased only during the first 30 min of incubation. This might be caused by the fact that some of the sperm cells showed a pathological movement (for example circular movement, spasmodic movement, backward movement etc.). The reason for that could be the fact that CAF can induce hyperactive motility in sperm cells (Barakat et al. 2015) which manifests itself as a sharp movement of the tail and irregular tortuous movement of the sperm cell.

Compared to PTX, CAF seemed to have a lower effect on TM and PM. That corresponds with the studies done on ejaculated spermatozoa (Stephens et al. 2013), nevertheless, the exact mechanism has not been explained yet.

Melo-Oña et al. (2012) reported positive results on equine ejaculated and epididymal spermatozoa that were reached when CAF was mixed with other substances (medium containing heparin, CAF, and bovine serum albumin). Fertility was not affected, so we can conclude that CAF has no deleterious effect on fertility. Unfortunately, the authors did not mention the concentration of CAF used.

Interestingly, when used with bull semen, CAF showed a better effect on TM and PM compared to PTX. It is possible that its effect differs among species. The use of CAF had a negative effect on ram and human ejaculates whereas in bulls and rabbits the effect was positive (Barakat et al. 2015).

Adding CAF did not have a significantly positive effect on VCL; even the highest concentration of CAF (5.5 mM) influenced VCL negatively during the 60–120 min of incubation.

This could be caused by a toxic effect of the higher CAF concentration on the sperm cells. Although PM decreased after 60 min and TM also decreased after 120 min, the effect was not significantly negative compared to the control.

As mentioned above, the use of CAF did not significantly influence STR even though we observed many sperm cells with a pathological movement. It has been described that CAF can induce hyperactive movement by influencing calcium canals (Barakat et al. 2015). However, in our case, the change of movement was not so severe as to change the STR value. We were unable to find any report of the effect of CAF on STR; therefore, we do not have any data to compare our results with.

In conclusion, adding PTX to frozen-thawed equine epididymal spermatozoa significantly improves total and progressive sperm motility for up to 4 h. The addition of CAF improves total sperm motility, but not PM, because many types of pathological movement were observed. Based on these results we can conclude that the addition of PTX to insemination doses prepared from stallion epididymal sperm can improve their quality.

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