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An Innovative Method for Cryopreservation of Horse Semen

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1. Introduction

Cryopreservation of semen is an important tool for the horse breeding industry. It provides an evident possibility for global transport, exchange, and sale of genetic material; moreover, this is the technology that ensures gene storage and banking. Horse breeding is getting more and more focus with increased demand by the professionals; breeding festivals and championships draw an ever-growing number of visitors each year. An example of such an event is the Salon du Cheval hosting competitions like the Longines Masters of Paris in show jumping and national competitions in dressage, show jumping and western riding, as well as the Arabian Horse World Championships (*Discover the Show*, n.d.). The show attracts several hundred thousand visitors each year with more than two thousand horses and riders entered in the many competitions, including several hundred Arabian horses in the World Championship (Caroline J., 2019; *Arabian Horse World Championship*, n.d.). The World Arabian Horse Organization (WAHO) has 82 countries affiliated with the organization though studbooks, all working on promoting the breed and increasing the Arabian horse population worldwide (WAHO, n.d.) The Arabian horse is one of the oldest horse breeds and is known for its versatility, the breed can be seen competing in many different disciplines all over the world as well as being used for leisure riding. The Arabian horse is still remaining popular to this age and there is a demand for continuously breeding new foals.

The demand from the breeding industry for gene banking and transport of semen globally poses great challenges for breeding professionals. The individual response of different stallions to the cryopreservation protocols makes frozen semen production challenging and difficult to plan. In the equestrian world now, there is a high demand for top quality breeding and production of horses with good genetic potential. Whether the horse is intended for sport competitions or races where the athletic potential is important or if it is for the purpose of leisure riding where a good temperament is an important trait, the breeder wants good quality genetic material from good stallions. Therefore, many breeders want to be able to choose between stallions from all over the world to produce offspring with the preferred traits and athletic potential from proven sires. This need results in high quality frozen semen to be in great demand worldwide. However, with current cryopreservation techniques there are a considerable amount of fluctuations regarding the quality and fertility

of frozen semen. Some of the reasons for this is due to individual variations between the stallions as well as seasonal changes in the semen quality and characteristics (Graham, 1996; Janett et al., 2003a, 2003b; Loomis and Graham 2008; Kuhl et al., 2016; Greiser et al., 2020; Suliman et al., 2020). To meet the demands of the industry a cryopreservation method which produces semen of stable and good quality is needed to improve the fertility rates of artificial inseminations (AI) using frozen semen.

The assisted reproductive technique of applying sublethal stress to spermatozoa, embryos or oocytes have shown great results in species like cattle, pigs, sheep, mice, and fish. (Pribenszky et al., 2010; Faragó et al., 2017). The theory behind it is that sublethal stress applied to the cells will induce a response with a temporary increase in a general, non-specific resistance to further stresses. When stress enhances the cells function it may be considered as eustress (Seyle, 1975). However, if the stress level applied is over the cells tolerance limit apoptosis will occur. This increased tolerance to stress will prepare the cells for the stresses associated with cryopreservation (Pribenszky and Vajta, 2011). One of the techniques using this preconditioning with sublethal stress is PTAT (Pressure Triggered Acquisition of Tolerance) or HHP (High Hydrostatic Pressure) as it has been formerly called. This technique uses hydrostatic pressure as a stressor to prepare the cells for further reproductive procedures.

In the present study we hypothesized that a fine-tuned sperm preconditioning protocol will improve the efficacy of sperm cryopreservation and will reduce quality fluctuations of frozen semen production in the Arabian horse. We also hypothesized that an individual PTAT treatment protocol set for a stallion would provide a significant improvement in frozen semen quality both in the spring and autumn breeding seasons. Altogether we aimed to define a semen treatment protocol to improve frozen semen quality in the Arabian horse.

2. Literature review

2.1. Artificial insemination in the horse

Artificial insemination (AI) and cryopreservation of semen have become more and more important in the equine industry during the last decades. Most studbooks today, with the exception of the Thoroughbred racehorse, allows for the use of AI and cryopreserved semen. In German sport horse breeds the use of AI compared to natural covering was close to 90% in 2010 compared to only 1.4% in 1985, most done by fresh or chilled semen. While in France the percentage of AI was lower than in Germany, 56% in 2010, the use of cryopreserved semen was higher; with 4 663 cases of frozen semen compared to 5 033 cases of fresh or cooled semen (Aurich, 2012).

Newer numbers have been reported by Kowalczyk et al. (2019), where they report that the percentage of foal being born into the world as a result of insemination with either chilled or frozen semen have reached ~90%. However, in Europe this proportion is only 43%. In 2017, 66% of AI in sport mares was performed with chilled semen, while in 34% of the cases frozen semen was used. In breeding mares, the ratio of chilled versus frozen semen used in AI is more or less 50/50, with frozen semen accounting for 51% and chilled semen for 49% of the total AI performed. In the remaining mare stock not fitting into the two previous groups, 75% of all AI was performed with chilled semen while frozen semen only represented 5% of the cases.

In Germany there was reported that in 2019, 26 705 mares were inseminated with chilled semen and 1 769 with frozen semen, 873 mares had embryo transfers performed on them and 1 347 was covered with the use of natural mating (German Equestrian Federation: Annual report 2019 Appendix: Breeding Statistics).

All these numbers and statistics show a development in the equine industry where AI is favoured over natural mating. However, AI with chilled semen is still preferred over frozen semen, but with improvement in cryopreserving methods resulting in frozen semen with more stable qualities frozen semen might increase in interest in the future.

2.2. Seasonal changes in semen quality

Due to the increased interest for AI in the equine breeding industry because of reasons like: hindering transmission of infectious diseases, decreasing trauma during mating and the possibility to select from a bigger pool of top stallions, the need for research in this field has increased. Such as two studies by Janett et al. (2003a, 2003b) studying the presence of seasonal variations concerning semen quality and freezability in Warmblood and Franches-Montagnes stallions. Their results showed a clear seasonal pattern for all semen quality parameters; volume, concentration and motility, and the number of morphologically normal sperm and sperm with major defects. Volume and total sperm count for fresh semen showed to be higher in summer in both studies, while motility showed to be higher in summer for Warmblood stallions and lower in summer for Franches-Montagnes stallions. Sperm concentration was significantly lower during summer for Warmblood stallions, while for Franches-Montagnes stallions the sperm concentration was significantly lower in the spring compared to autumn and winter. Concerning sperm morphology, the number of normal sperm was significantly higher in autumn than summer and winter and with a lower incidence of major sperm defects in autumn compared to the spring, summer, and winter months for both breeds. For frozen-thawed semen, motility and viability was tested and the Hypoosmotic Swelling test (HOS) was performed. Post-thaw motility was highest in autumn compared to spring and summer, and viability was lowest in summer clearly differing from other seasons in the study with the Warmblood stallions, while viability showed no difference for the Franches-Montages stallions. The HOS test revealed a significantly higher occurrence of membrane damaged spermatozoa in winter compared to the other three seasons. A highly significant correlation between pre- and post-freeze motility observed in the Franches-Montagnes stallions shows that semen with higher motility will freeze better. Another important finding was that progressive motility, which have been linked to stallion fertility, were significantly higher in fresh semen in summer and autumn compared to winter. Even though the study's results differ from each other to some degree it clearly shows the presence of seasonal variations regarding semen quality and freezability.

A new study by Greiser et al. (2020) also found an effect of season on semen traits in as study on seven different breeds and a total of 121 fertile stallions. Seasonal changes could be seen in gel-free volume, sperm concentration, progressive motility of fresh semen, DNA fragmentation index (DFI), total number of sperm, total number of progressively

motile sperm and non-viable sperm. Total number of sperm and total number of progressively motile sperm were observed the highest in the breeding season (spring and summer) which is in agreement with the study above by Janett et al. (2003a). Progressive motility of fresh semen was significantly lower in the breeding season compared to autumn and winter, which again agrees with the study done in Franches-Montagnes stallions by Janett et al. (2003b). In the study by Greiser et al. (2020) progressive motility of fresh semen was shown to have significant negative correlation with DFI and non-viable sperm. However, seasonal changes of motility did not correlate with DFI. Post-thaw progressive motility however did not differ significantly among seasons in this study, while it in both studies by Janett et al. (2003a, 2003b) was shown to be higher in the in autumn. The gel-free volume decreased in the breeding season compared to the autumn and winter months, which is in contrast with the previous results in the above-mentioned study where volume was shown to be higher in summer.

Another study done with a larger case number of 180 stallions from several different horse breeds showed a seasonal effect on cryopreservation, where the post-thaw progressive motility differed according to season. In the study semen was classified as acceptable if the post-thaw progressive motility was ≥35%. The proportion of acceptable ejaculates was 48% in winter (December-February), 65% in spring (March-May), 77% summer (June-August) and 64% in autumn (September-November) (Kuhl et al., 2016).

A study on the seasonal changes in semen quality in fertile and subfertile stallions was performed by Suliman et al. (2020). The case number was smaller in this study than the previous ones, with 8 stallions being observed. The study observed no seasonal changes in ejaculate volume, sperm concentration and total sperm number in chilled semen between the fertile and subfertile stallions. However, in the breeding season the percentage of spermatozoa with normal morphology was significantly higher in the fertile stallions compared to the subfertile ones. Total and progressive motility was also shown to be higher in the fertile stallions than the subfertile in the breeding season. Sperm movement characteristics derived with the use of CASA (computer assisted semen analysis) showed a difference in average path velocity (VAP) and curvilinear velocity (VCL) between the two groups of stallions in the breeding season, where fertile stallions had a higher percentage of VAP and VCL than the subfertile. In conclusion the study demonstrated that there is a

seasonal effect on sperm production and quality despite some conflicting results on which parameters that are affected. Nevertheless, it was shown that fertile stallions were more influenced by season, and that the seasonal effect is lost in subfertile stallions.

Although the results from studies about the seasonal effect on semen quality parameters shows varied results, it can be concluded that the seasons affect the semen of stallions. However, which parameters that are affected in which season are more unclear.

2.3. Causes of seasonal changes in equine semen quality

2.3.1. Seasonal changes in hormones and testicular function

Possible reasons for the before-mentioned seasonal changes in semen quality can be the seasonal change in testicular function in stallions. Although the stallion is fertile all year round and not only in the breeding season, there are differences between the breeding and non-breeding season. Such changes are gonadotropin, testosterone and estradiol release, testicular size, sperm production and sexual behaviour, which are clearly reduced outside the breeding season (Aurich, 2016).

Suliman et al. (2020) also discuss seasonal hormonal changes. They concluded that a possible reason for the absence of seasonal effect in subfertile stallions, as described earlier, may be because of the lack of seasonal increase of the basal levels of testosterone, FSH (Follicle-Stimulating Hormone) and LH (Luteinizing Hormone). These hormones showed a significant increase in fertile stallions during the breeding season. This also may reflect the lack of seasonal regulation of the synthesis of gonadotropins in the subfertile stallions. Furthermore, the significant increase in basal hormone concentration in the fertile stallions between the breeding and the non-breeding season may be a trigger for processes which lead to the increased proportion of morphologically normal spermatozoa in the breeding season compared to the non-breeding season.

2.3.2. Seasonal changes in the seminal plasma and sperm plasma membrane

Another reason for this seasonal variation in sperm quality may be the seasonal changes in seminal plasma composition. Seminal plasma effect both the cooled-stored and cryopreserved stallion semen. Therefore, seasonal changes in the seminal plasma composition may contribute to changes of semen quality of processed semen. However, changes in the equine sperm plasma membrane must be considered as a reason as well. In bulls, season influences the fatty acid composition of the sperm plasma membrane and this influences the time period bulls semen is collected and cryopreserved. Cryopreserved semen defined as good quality differed in lipid concentration and fatty acid composition of the

seminal plasma and cell compartment from semen defined as bad quality in bulls. Changes of the fatty acid composition of equine spermatozoa has also been demonstrated. Of 14 fatty acids determined, 11 of them changes from January to June, such as docosapentaenoic acid. The percentage docosapentaenoic acid, which is a major component of the polar lipids in the equine spermatozoa, was shown to increase from January to June. Docosapentaenoic acid also varies amongst stallions and is believed to cause differences in sperm cryosurvival between stallions categorized as good or bad freezers. Therefore, like in bulls, the composition of seminal plasma as well as the sperm plasma membrane is probably involved in the seasonal changes of processed semen quality in stallions (Aurich, 2016).

2.4. Individual stallion variations of semen quality

This individual variation between stallions have also been reported by Graham (1996) and Loomis and Graham (2008). Their studies showed that the post-thaw quality of spermatozoa of stallions could be improved with utilizing split-ejaculate test freeze procedures to determine the preferred freezing protocol of the individual stallion. This meant that stallions, that had semen that previously did not freeze well and had poor post-thaw quality, now could be accepted for commercial semen cryopreservation by adjusting the freezing protocol to the individual stallion. However, the semen from some stallions will not cryopreserve well regardless of the efforts invested.

Loomis and Graham (2008) further concluded that this individual customization of freezing protocols to the stallions should become a part of the everyday cryopreservation procedure to maximize the sperm cryosurvival. These inter-species differences can be found in other species as well, such as bulls. The solution to this in the dairy industry, where frozen semen AI have become the new norm, is that the bulls that have semen that do not freeze well with the standard protocol are replaced with new bulls. However, this solution is unlikely to ever be acceptable to the horse breeding industry, where specific genotypes are desired from the sire (Loomis and Graham, 2008). Therefore, the possibility to improve the sperm quality of frozen semen from certain top stallions by individualizing the freezing protocols is of great value to the equine industry.

The findings of semen quality variations between individual stallions is also supported by a study from Greiser et al. (2020) which observed that variance components among stallions within a breed were significant for semen quality.

2.5. Sublethal stress as a preconditioning treatment

A different approach to optimize freezing protocols is to prepare the cells for the detrimental procedure of cryopreservation. Several studies have discussed a preconditioning treatment using a sublethal stressor, such as osmotic stress, heat stress and hydrostatic pressure (HHP/PTAT). The concept is that a sublethal stressor will elicit a response with a temporary increase in a general, non-specific resistance to further stress, such as cryopreservation (Pribenszky and Vajta, 2011).

Studies researching osmotic stress have shown varied results. One research team got positive results for their experiment on treatment of porcine oocytes with sublethal concentrations of NaCl, after a recovery period followed by vitrification and parthenogenic activation or somatic cell nuclear transfer, the blastocyst rates were significantly increased in the hyperosmotic NaCl-treated group versus the controls. Another research studying the effect of NaCl, sucrose and trehalose on oocytes found out that after recovery, vitrification and parthenogenic activation all treatment groups resulted in increased blastocyst rates compared to the controls. While when parthenogenic activation was replaced by somatic cell nuclear transfer all hyperosmotic treatments increased blastocyst rates, but treatments with sucrose or trehalose significantly reduced the cell number within the blastocysts. However, other studies using similar osmotic pressure values, but different treatment times found osmotic stress to be detrimental to the oocytes (Lin et al., 2009a, 2009b).

Studies experimenting on the effect of heat stress have shown that even a slight increase in temperature, by as little as 3.5°-4.5°C, of the embryo holding media for 60-120min resulted in reduced blastocyst and hatching rates after in vitro fertilization and culturing in both bovine and porcine embryos (Ju et al., 1999; Tseng et al., 2006) A different study found out that a milder elevated temperature (2.5°C) for a longer period of time of 6 hours caused mitochondrial damage and developmental arrest in bovine embryos (Rivera et al., 2004).

2.6. Hydrostatic pressure as a sublethal stressor

The use of hydrostatic pressure as a sublethal stressor have been researched in several species and on different types of cells, including spermatozoa, embryos, and oocytes. The features that makes hydrostatic pressure treatment a good choice for a sublethal stressor are: 1) it acts instantly and uniformly on the sample, 2) hydrostatic pressure has zero penetration problems or gradient effects, and 3) hydrostatic pressure can be applied with the highest precision, consistency, reliability, and safety (Pribenszky et al., 2010).

2.6.1. PTAT treatment in embryos and oocytes

PTAT treatment has been studied in embryos of several mammalian species such as mouse, cattle, and sheep. The common outcome of those studies was a significant increased cryosurvival rate and faster resumption of normal in vitro development after cryopreservation and thawing. The optimal treatment parameters for expanded blastocysts were in the range of 40-60 MPa for 30-70 minutes regardless of the species (Pribenszky and Vajta, 2011).

Pribenszky et al. (2008a) found out that PTAT treatment with applying 60 MPa followed by 60 minutes of recovery significantly improved the in vitro survival, hatching rates and the speed of resumption of normal in vitro development in vitrified bovine blastocysts. Further studies by Jiang et al. (2016) revealed that PTAT treatment at 40 MPa and 60 MPa followed by 2 hours of recovery significantly improved the re-expansion rates in vitrified bovine blastocysts, while 80 MPa significantly reduced the re-expansion rates. They also tested if temperature had any effect and tested samples treated on 24°C and 39°C, the re-expansion rates were not affected by temperature. Gene ontology analysis revealed that PTAT treatment at 40 and 60 MPa induced down-regulation of genes involved in cell death and apoptosis, while genes in RNA processing, cellular growth and proliferation were up-regulated. In contrast, PTAT treatment at 80 MPa induced an up-regulation of apoptosis and a down-regulated protein folding and cell cycle-related genes. These changes in gene expression induced by treatment at 40 and 60 MPa appears to have prepared the embryo for the upcoming stress applied during vitrification. However, when too much pressure was applied, 80 MPa for bovine blastocysts, the induced changes of the gene expression was lethal to the embryo (Jiang et al., 2016).

For ovine blastocysts PTAT treatment at 40 MPa for 70 minutes followed by immediate vitrification or 120 minutes recovery before the vitrification were tested. After 3 hours of incubation re-expansion rates were significantly increased in the PTAT treated embryos with zero recovery time compared to embryos with 120 minutes recovery time and the controls. Similar re-expansion and hatching rates between the groups were recorded after 8 and 24 hours of incubation as well. Nevertheless, blastocysts vitrified immediately after pressure treatment showed an increased cell number compared to the group with 120 minutes recovery time and the controls, making the PTAT treatment with no recovery time the best option for preconditioning for ovine blastocysts (Ledda et al., 2010).

A study on zebrafish embryos by Faragó et al. (2017) using preconditioning with PTAT treatment at 5 MPa for 90 minutes recorded that after 24 hours of chilling (which have proved to be detrimental to zebrafish embryos) the survival of embryos in the PTAT group was significantly higher than the control group. Embryo morphology was evaluated on day 10 after fertilization, where more morphologically normal embryos were detected in the PTAT chilled group versus the chilled control group. Further on in the study there was recorded that all chilled controls died by day 30 after fertilization, while several embryos in the PTAT chilled group survived and were later on able to reproduce. The PTAT chilled embryos continued to survive to maturity and fertility, viability and morphology was compared to the unchilled control group. There was no significant differentiation between the unchilled groups and the PTAT chilled groups regarding fertilization rates, viability of offspring or the occurrence of normal morphology. These results conclude that with the use of preconditioning the embryos gained an increased tolerance to the effects of chilling, and therefore there is a possibility to ship and trade zebrafish embryos, as well as gene preservation, with further improvement of the PTAT-chilling/cryopreservation procedure in the future.

Pribenszky et al. (2008b) researched the effect of hydrostatic pressure preconditioning on porcine oocytes and found the upper tolerance limit to be in the range of 40 MPa. When 60-80 MPa was applied to the oocytes they did not cleave after activation. Further experimenting concluded that PTAT treatment at 20-40 MPa increased the developmental competence of the oocytes and cleavage rates of oocytes treated with PTAT before vitrification were higher than the untreated control group.

2.6.2. PTAT treatment in spermatozoa

The use of sublethal hydrostatic pressure in semen cryopreservation has also been investigated during the last decades. For boar spermatozoa the treatment with 80 MPa was found to reduce semen motility considerably, and treatment in the range of 10-40 MPa was suggested as the best option for porcine semen. Experiments using PTAT treatment at 30 MPa for 90 minutes at room temperature resulted in and increases post-thaw sperm motility compared to the control group (Pribenszky et al., 2011). PTAT treatment of boar semen has shown to increase the number of live piglets born per sow (Pribenszky et al., 2008c) and increase the litter size when semen was pretreated with 30 MPa (Kou et al., 2008). These results were confirmed by Horváth et al. (2016) who also recorded a larger litter size and higher number of live-born piglets when PTAT treated semen was used for insemination compared to untreated semen. Further experiments done by Horváth et al. (2016) revealed that there were significant differences regarding the rates of non-return to estrus between PTAT treated semen cases and the untreated semen cases. The occurrence of non-return to estrus was significantly higher in sows inseminated with PTAT treated semen, as well as the odds for sows to become pregnant and farrowing were greater when PTAT treated semen was used compared to untreated semen. Both total and progressive motility was also recorded to be significantly increased in PTAT treated semen.

Huang et al. (2009) studied the changes in the protein profile of boar semen before and after cryopreservation with both untreated and pressure treated samples. There were seven proteins that differed between the treatment groups, three proteins representing PTAT-specific response proteins and four proteins related to cooling and freezing. The levels of ubiquinol-cytochrome c reductase complex core protein 1, perilipin and carbohydrate-binding protein AWN precursor were significantly elevated in PTAT-treated samples after cooling and freezing-thawing, which suggest that these proteins may be PTAT response related proteins. Spermadhesin AWN are found in the boar's seminal plasma and are believed to assist in the interaction between the sperm and the zona pellucida at fertilization, perilipin has a role in regulating triacylglycerol hydrolysis and ubiquinol cytochrome c reductase is part of the oxidative phosphorylation system. In both treated and untreated samples the levels of testis-specific glyceraldehyde 3-phosphate dehydrogenase and cytosolic 5'-nucleotidase 1B were significantly decreased after cooling and cryopreservation, while outer dense fiber of sperm tails 2 and quinone oxidoreductase were

elevated. These may then be related to the response in porcine sperm to the cooling and freezing process. Glyceraldehyde 3-phosphate dehydrogenase is required for sperm motility and disrupting its activity could lead to male infertility. Outer dense fibers are a main component of the sperm tail in mammals and may play an important role in sperm morphology, integrity, and function. Inhibition of 5'-nucleotidase resulted in sperm motility inhibition, and therefore it is suggested that 5'-nucleotidase plays an important role in the regulation of sperm motility and its decrease after cooling and freezing may suggest it is associated with the decrease of sperm motility after cryopreservation. However, the exact role and physiological function of all these differing proteins in the cryopreservation procedure is still unclear and need further exploration.

In bulls, PTAT treatment of semen was found to significantly increase the post-thaw motility and the proportion of spermatozoa with intact tail, head and acrosome increased significantly. Cows inseminated with PTAT treated semen had at 60 days post-insemination a non-return to estrus rate of ~90%, while the 60 days non-return rate in the same population and time without treatment was ~83% (Pribenszky et al., 2007).

Different PTAT treatment protocols have been published to benefit different cell types and species. Krümpel (2014) has tested a general PTAT preconditioning protocol with treatment at 20 MPa to treat equine semen before cryopreservation and found no significant effect on post-thaw motility. In later experiments we found that the optimal treatment parameters for the treatment of equine semen is $\leq$ 10 MPa. We have also found that optimal PTAT treatment parameters might be individual to each stallion (unpublished data).

3. Materials and methods

3.1. Animals and semen preparation

All experiments were performed at the Bábolna National Stud Farm Ltd. (Arabian Stud, Hungary) in the spring breeding season in March and April as well as the autumn breeding season in September and October. Semen samples, 4-16 samples from each stallion, were collected from four purebred Arabian stallions and two Shagya-Arabian horses (age: 10.7 ± 7.0 years; mean ± SD), regularly used as semen donors in the stud farm.

The actual semen-processing schedule and cryopreservation protocol of the stud farm were followed during the experiments. Three stallions were examined in the autumn, and four in the spring breeding season. One purebred Arabian stallion, aged 19, in the particular interest of the stud farm, was investigated in both seasons (5 semen samples in the autumn, and 11 samples in the spring breeding season) (Table 1).

TABLE 1: STALLIONS USED IN THE EXPERIMENT:

Stallion	*Breed*	*Age*	*Season*	*Optimal treatment parameters*	*Number of repetititons*
No. I.	Arabian thoroughbread	19 years	Spring and autumn	5 MPa / 10 min	16
No. II.	Arabian thoroughbread	4 years	Spring	5 MPa / 10 min	14
No. III.	Arabian thoroughbread	9 years	Spring	5 MPa / 10 min	11
No. IV.	Arabian thoroughbread	2 years	Spring	5 MPa / 30 min	4
No. V.	Shagya - Arabian	18 years	Autumn	5 MPa / 10 min	4
No. VI.	Shagya - Arabian	12 years	Autumn	10 Mpa / 10 min	6

Table 1: Information about the 6 stallions used in the experiment

Semen was collected twice weekly with the use of a phantom and a teaser mare. Collection was done with the use of an artificial vagina fitted with a filter to separate the gel fraction from the ejaculate. After semen collection and an initial subjective semen assessment, samples were diluted in a 1:1 ratio with Centrifuge Medium for Equine semen without antibiotic (Minitüb GmbH, Tiefenbach, Germany) warmed up in a water bath to 35°C. Afterwards sperm density and motility (total and progressive motilities, %) were evaluated using the AndroVision®MiniTube CASA system (Minitüb GmbH, Tiefenbach, Germany) at 37°C, according to the evaluation protocol recommended by the manufacturer. After centrifugation for 10 minutes at 450xg the supernatant was discarded, and the pellet was re-suspended to the concentration of 6 x 10^8 motile sperm / 0.5 ml in Gent extender (Minitüb GmbH, Tiefenbach, Germany), controlled with the help of CASA.

3.2. PTAT treatment

The PTAT treatment was inserted into the stud farm's standard procedure of stallion sperm processing and freezing, as a single additional step after the semen centrifugation and re-suspension, without further modifying or adjusting the routine protocol of the lab, as shown in Figure 2 later on. The PTAT treatment protocol for each participating stallion's semen was individually adjusted before the start of the experiment to the protocol benefitting the stallion the most.

3.2.1. Pre-adjustment of treatment parameters

To identify the individual optimal PTAT treatment parameters the re-extended semen of each stallion was split to five parts and simultaneously four different treatment protocols were applied; 5 MPa for 10/30 minutes and 10 MPa for 10/30 minutes. The fifth part was the untreated control sample. The four PTAT samples and the control sample were cryopreserved and then thawed and investigated for motility parameters as described below. The experiments were repeated 2-3 times and the treatment parameters that provided the largest effect size for a given stallion were used for that stallion throughout the rest of the experiments.

3.2.2. PTAT treatment

During the next experiments, after the optimal PTAT treatment parameters had been identified for each stallion, re-extended semen of each stallion was split into two groups. The first group was the untreated semen control, and the second group was the PTAT treated semen. Following the re-extension and splitting, the semen was then aspirated in Luer-lock syringes (B. Braun Melsungen AB, Melsungen, Germany). Afterwards the air was emptied from syringes and they were locked by a Luer-lock cap.

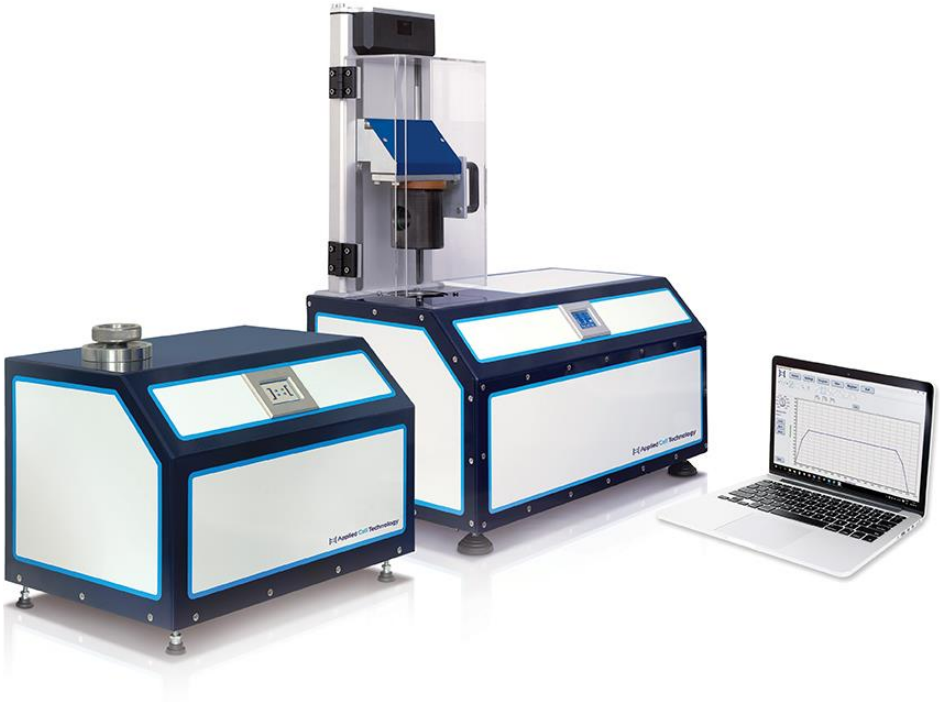

Figure 1: Illustration of the hydrostatic pressure devices used for semen (front left) and for embryos and oocytes (back right). Photo courtesy of Applied Cell Technology Ltd.

The syringes of the different PTAT groups, depending on the different stallions' optimal PTAT parameters, were placed into the pressure chambers of computer-controlled, programmable hydrostatic pressure devices (GBOX 2010, Applied Cell Technology Ltd., Gyöngyös, Hungary) (Figure 1). Water was used in the chambers as pressure medium. The devices were set to perform the individual treatment protocol of 5 or 10 MPa (one pascal (Pa), equals to one newton per square metre [N/m^2 or $kg \cdot m^{-1} \cdot s^{-2}$] or $145{,}04 \cdot 10^{-6}$ psi) hydrostatic pressure for 10 or 30 minutes, at 21°C. The devices controlled the pressure build-up, holding and depressurization to atmospheric pressure. During the treatment, the control sample was kept at atmospheric pressure and at 21°C, right next to the pressure chambers. Following the treatments, the samples from the optimal treatment group plus one control group were loaded into 0.5 ml straws of two different colours (one for each group) and marked with the date and the stallion and stud farm's identification information. Four identical straws represented one group, so altogether eight straws represented one repetition (4 straws from the optimal treatment group and 4 straws from the control group).

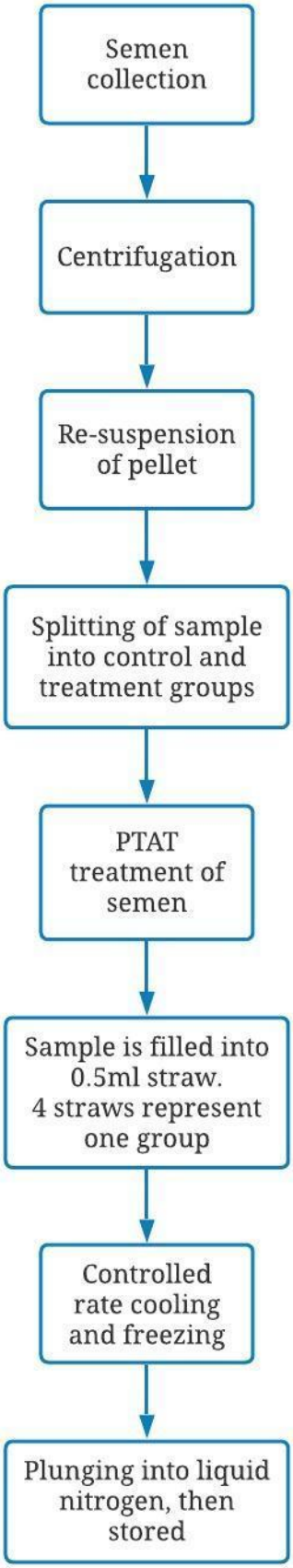

Figure 2: Illustration showing the different steps of the experiment

3.3. Semen cryopreservation

After the PTAT treatments were completed all the samples were cryopreserved using a standard freezing protocol by a Sy-Lab IceCube 14S-A controlled rate freezer (Sy-Lab Gerate GmbH, Neupurkersdorf, Austria). Samples were cooled from 21°C to 5°C with a cooling rate of -0.1°C/minute, then further cooled to -10°C with a rate of -15°C/minute, and finally to -140°C with a rate of -40°C/minute. (Figure 3). Straws were then plunged into liquid nitrogen (LN_2) before they were transported to and stored in LN_2 storage containers until thawing.

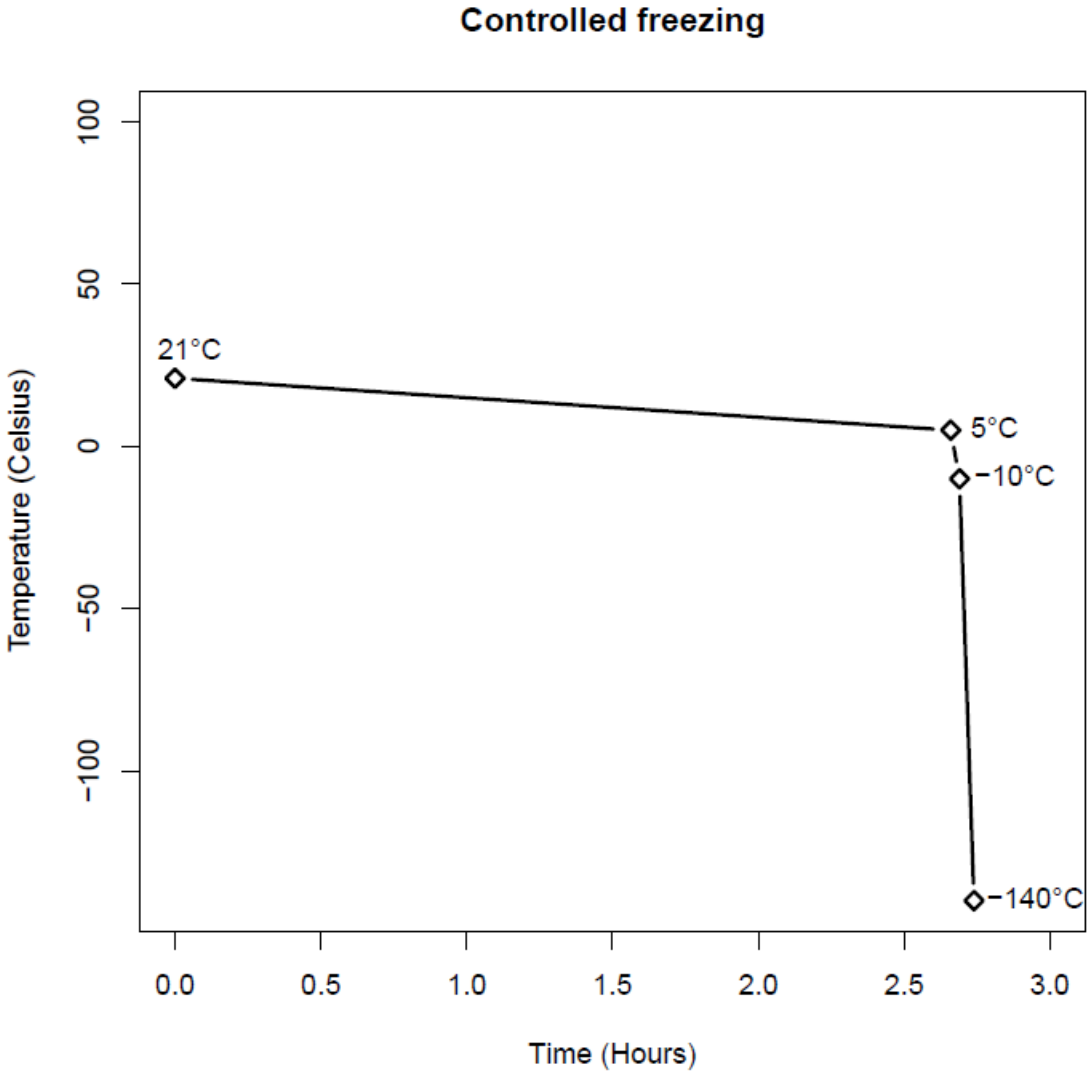

Figure 3: Illustration of the controlled cooling and freezing process used in the experiment

Thawing was performed by merging 4 straws, representing one experimental group, into 37°C water bath for 30 seconds. Afterwards straws were emptied into an Eppendorf tube pre-warmed to 37°C in a warming block. After careful but thorough mixing of the semen, 6 µL sample was loaded into Makler chamber and 10 fields of the chamber (at least 100 sperms / field, altogether >1000 spermatozoa per sample) were assessed on a heated stage at 37°C, using the AndroVision®MiniTube CASA system (Minitüb GmbH, Tiefenbach, Germany). The analysis of the semen was performed following the evaluation protocol recommended by the manufacturer.

3.4. Statistical analysis

All data were analyzed using the GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). The total and progressive motility data of the individual PTAT-protocols were compared to the matching controls using the Student's paired t-test, as all data sets passed the D'Agostino & Pearson omnibus normality test.

Values are presented as the mean ± SEM, accompanied with the 95% confidence intervals (95%CI). A probability value (p) of less than 0.05 was considered significant.

4. Results

4.1. Post-thaw motility

The PTAT-preconditioning parameters for each stallion enrolled in the study were optimized earlier (data not shown in detail): among the six stallions three different protocols were identified. The protocols were; 5 MPa for 10 minutes, 5 MPa for 30 minutes and 10 MPa for 10 minutes. Optimized protocols were found to be specific for the individual stallion and the same optimal protocol was used for the individual stallion throughout the experiments.

The initial fresh (TM and PM native) and post-thaw total and progressive motilities of the PTAT group (PTAT) and untreated controls (CTRL) in both breeding seasons are presented in Table 2 and Table 3.

TABLE 2: TOTAL MOTILITY BEFORE AND AFTER CRYOPRESERVATION

TM (%)	SPRING (n=40)			AUTUMN (n=15)			EFFECT SIZE		
	TM native	CTRL	PTAT	TM native	CTRL	PTAT	Spring	Autumn	Spring + Autumn
Mean	**74.6**	**57.4**	**64.0**	**76.8**	**44.6**	**56.9**	**6.59**	**12.3**	**8.14**
SEM	2.14	2.59	2.52	2.34	4.96	4.99	1.41	1.74	1.17
Lower 95% CI	70.2	52.1	58.9	71.8	34.0	46.2	3.73	8.57	5.79
Upper 95% CI	78.9	62.6	69.1	81.9	55.3	67.6	9.44	16.0	10.5
p= (CRTL vs. PTAT)	<0.0001****			<0.0001****					

Table 2: Total motility (TM) of fresh (TM native) and cryopreserved-thawed semen in the control (CTRL) and preconditioned (PTAT) groups.

TABLE 3: PROGRESSIVE MOTILITY BEFORE AND AFTER CRYOPRESERVATION

PM (%)	SPRING (n=40)			AUTUMN (n=15)			EFFECT SIZE		
	PM native	CTRL	PTAT	PM native	CTRL	PTAT	Spring	Autumn	Spring + Autumn
Mean	**66.9**	**46.1**	**53.3**	**67.1**	**31.9**	**43.3**	**7.15**	**11.4**	**8.31**
SEM	2.38	2.52	2.58	2.26	4.3	4.94	1.34	2.24	1.17
Lower 95% CI	62.0	41.0	48.0	62.2	22.6	32.7	4.44	6.6	5.97
Upper 95% CI	71.7	51.2	58.5	71.9	41.1	53.8	9.87	16.2	10.7
p= (CRTL vs. PTAT)	<0.0001****			<0.0002***					

Table 3: Progressive motility (PM) of fresh (PM native) and cryopreserved-thawed semen in the control (CTRL) and preconditioned (PTAT) groups.

In the spring breeding season 40 samples collected from 4 horses were analyzed. The mean total motility (TM) of fresh samples were 74.6 ± 2.14%. The post-thaw motility of the PTAT samples were significantly higher compared to the control (64.0 ± 2.52% vs. 57.4 ± 2.59%, $p<0.0001$). The mean progressive motility (PM) of fresh samples were 66.9 ± 2.38%. And here as well the post-thaw motility of the PTAT samples were significantly higher compared to the control (53.3 ± 2.58% vs. 46.1 ± 2.52%, $p<0.0001$).

In the autumn breeding season, there were collected 15 semen samples from 3 stallions. Average total motility (TM) of the fresh samples were 76.8 ± 2.34%. The mean post-thaw motility in the PTAT group were significantly higher compared to the control (56.9 ± 4.99% vs. 44.6 ± 4.96%; $p<0.0001$). Average progressive motility (PM) of the fresh samples was 67.1 ± 2.26%. The mean post-thaw motility in the PTAT group was significantly higher compared to control (43.3 ± 4.94% vs. 31.9 ± 4.30%; $p<0.0002$).

The average effect size of PTAT-preconditioning on post-thaw motility of equine semen was larger in the autumn season (TM: 12.3 ± 1.74%; PM: 11.4 ± 2.24%) compared to the spring season (TM: 6.59 ± 1.41%; PM: 7.15 ± 1.34%), nevertheless both effects were significant (Table 2 and Table 3).

4.2. Results of a case study

One 19 years old purebred Arabian stallion (stallion No. I.), which was of particular interest for the stud farm, was studied both in the spring and autumn seasons. (Table 1).

The effect of the optimal PTAT treatment of 5 MPa for 10 minutes for this specific stallion was significant in both the spring and autumn breeding seasons for both total and progressive post-thaw motility. Effect size in the spring was 5.78 ± 2.51% (TM) and 6.52 ± 2.27% (PM) (p=0.0442 and p=0.0166, respectively), while in the autumn it was 14.8 ± 1.26% (TM) and 11.2 ± 3.33% (PM) (p=0.0003 and p=0.0285, respectively). (Figure 4)

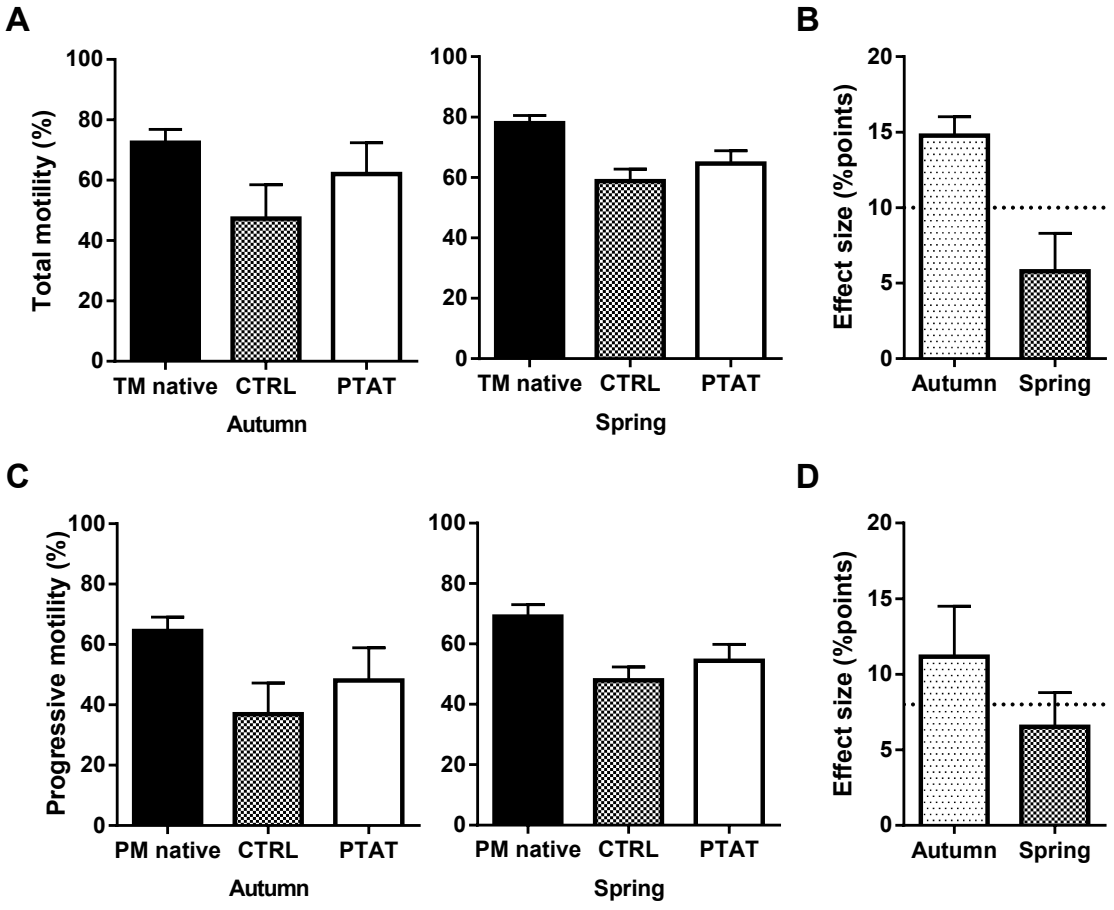

Figure 4: Total and progressive motility of fresh (TM native and PM native) and cryopreserved-thawed semen in the control (CTRL) and preconditioned (PTAT) groups of stallion no. 1 in the spring and autumn breeding seasons (A, C). Effect size in the spring and in the autumn (B (total motility) and D (progressive motility).

5. Discussion

In the present study we report that preconditioning treatment of equine semen can be individualized and inserted into the routine cryopreservation protocol. The treatment has a significant effect on cryosurvival, with a larger effect size in the autumn breeding season compared to the spring. The treatment related post-thaw improvements are significant with regards to both total and progressive motility.

Previous studies have shown that preconditioning treatment has improved semen quality in both bovine and porcine experiments resulting in increased post-thaw motility, greater proportion of cells with normal morphology and increased fertility after cryopreservation when comparing treated and untreated samples (Pribenszky et al., 2007, 2008c, 2011; Horváth et al., 2016). As described earlier Huang et al. (2009) has presented the possible underlying causes for the results on a porcine model: the results revealed that the levels of three proteins differed significantly due to the PTAT treatment. Ubiquinol-cytochrome c reductase complex core protein 1, perilipin, and carbohydrate-binding protein AWN precursor were identified as treatment-response proteins being significantly higher in treated samples after cooling and freezing-thawing compared to the untreated samples. These proteins support the fundamental functions of the spermatozoa and may be the explanation to the improved qualities of treated semen versus untreated semen after cryopreservation (Huang et al., 2009). The insemination of these porcine semen samples resulted in increased pregnancy and farrowing rates and increased litter size (Kou et al., 2008). This finding was later confirmed by an independent randomized controlled trial by Horváth et al. (2016) as mentioned earlier.

Further studies on embryos and oocytes have revealed increased cryosurvival of mouse, bovine, ovine and fish embryos as well as porcine oocytes, where positive effects of the PTAT treatment was measured in increased re-expansion and continued in vitro developmental rate, increased cell number and reduced level of apoptosis (Pribenszky et al., 2008a, 2008b; Ledda et al., 2010; Jiang et al., 2016; Faragó et al., 2017). Jiang et al. (2016) further examined the possible molecular-biological background by showing that the optimized treatment protocol promoted embryo competence through down-regulation of genes related to cell death and apoptosis, and up-regulation of genes related to RNA processing, cellular growth, and proliferation.

The concept of preconditioning can be compared to the famous saying from German philosopher Friedrich Nietzsche (1889); “What doesn’t kill me, makes me stronger”. When the cells are put under harsh conditions, but still within the cell’s tolerance level, the cells will build up a higher tolerance to future stress and have therefore become “stronger”. The combination of the fine-tuned, mild hydrostatic pressure and temperature impact to the cells elicit a cellular stress reaction in a controlled and reversible manner within the cells: while cells sense, assess and counteract a controlled, non-harmful stress factor, their tolerance level, general resistance to stress temporarily increases (eustress – defined originally by Hans Selye (1975); (Pribenszky et al., 2010; Pribenszky and Vajta 2011; Jiang et al., 2016). This beneficial, temporary increase of stress tolerance prepares the cells to withstand forthcoming stressors. Such stressors like the ones associated with the process of cryopreservation, where osmotic, cold, or mechanic stresses are afflicted on the cells. Due to the pre-conditioning, cells may be more prepared to restore normal function after the lapse of the forthcoming stress (cryopreservation and thawing), resulting in higher survival and, more importantly improved function, compared to untreated controls (Jiang et al., 2016).

In agreement with the above findings, equine semen preconditioning with a fine-tuned, specific sublethal stress treatment (PTAT treatment) was found to improve total and progressive motility of the spermatozoa after cryopreservation and thawing. However, equine spermatozoa benefitted from treatments with a lower pressure level than both bovine and porcine spermatozoa. While 30 MPa was mostly used in both bovine and porcine sperm treatment with positive results, the equine sperm showed improved semen qualities at a lower pressure levels like 5-10 MPa and no improvements when 20 MPa pressure was used. While improvement in motility is not directly associated with fertility, these are quality markers that mirror the function of the cells and their ability to fertilize better and support and maintain a normal embryo development. A correlation between pre- and post-freeze motility were observed by Janett et al. (2003b) in Franches-Montagnes stallions, and they concluded that semen with higher initial motility will freeze better and end up with a higher post-thaw motility.

Unlike in other species and cell types, the PTAT treatment of equine semen needs to be individually defined for each donor due to individual stallion variations. However, this

makes cryopreservation using hydrostatic pressure as preconditioning time consuming in the initial phase when a new stallion is introduced into the cryopreservation program. When using the individual optimal PTAT parameters for semen treatment of each stallion before the cryopreservation, robust improvements could be achieved in the individual stallion's semen during the series of the cryopreservation program in both the spring and autumn breeding seasons. A larger effect size was observed in the autumn, which may suggest that preconditioning treatment may have a larger benefit when the circumstances are suboptimal. Seeing as both total motility (TM) and progressive motility (PM) after thawing of the control (CRTL) samples were lower in the autumn compared to the spring, which might indicate that cryopreservation in the autumn is suboptimal compared to the spring. However, a larger case number is needed to draw any conclusions. These results also contrast some of the previous mentioned studies on seasonal effect on semen quality (Janett et al., 2003a, 2003b; Kuhl et al., 2016). Therefore, the exact effect of the season on cryopreservation is still unclear.

PTAT treatment showed benefits in improving frozen semen quality in the Arabian horse, which has wider implications in semen freezing, breeding and genetic conservation programs. Which may be a break-through when it comes to global export and import of semen from top stallions from all corners of the world. This will result in the horse owner and breeder having more choices and can chose the stallion and its genetic material that suit them the best. While this study was conducted exclusively in the Arabian horse breed, there is a possibility that similar results will be found in other horse breeds and can therefore be very significant for the future of the equine industry. However, for assessing the effect size properly, studies with larger case numbers are needed, as well as studies with different breeds in order to assess the applicability also for other horse breeds.

6. Summary

The success of the cryopreservation of stallion semen is hindered by the differences between the horses and the variation within the donors' own performance. Together with quality breeding and the growing need for genetic improvement of the breeds a predictable, stable cryopreservation protocol is demanded by the industry. Attempts are made to individualise cryopreservation protocols for each stud. On the other hand, emerging techniques augmenting the cryopreservation protocols may improve the output. Such a technique is semen preconditioning, performed as part of the semen preparation protocol, before the freezing step. The positive effect of preconditioning treatment with the use of hydrostatic pressure (PTAT treatment – Pressure Triggered Acquisition of Tolerance) has been well documented in bovine and mouse embryos and porcine and bovine semen. In the present report we show that preconditioning treatment of equine semen can be individualized and inserted into the routine cryopreservation protocol.

Semen of six Arabian horses was cryopreserved in the spring and autumn seasons in the routine of the lab, 4 – 16 repetitions were performed. The treatment had a significant effect ($p<0.001$) on cryosurvival, with a larger effect size in the autumn breeding season compared to the spring. The treatment related post-thaw improvement was tangible in both total and progressive motility.

7. Hungarian title and summary

Összefoglaló

A ménsperma mélyhűtésének sikerét rontja az egyes mének közötti és a donorok saját teljesítményén belüli eltérések. A minőségi tenyésztéssel és a fajták genetikai fejlődésének növekvő igényével összefüggésben az iparnak kiszámítható, stabil krioprezervációs protokollra van szüksége. A mélyhűtési programok minősége javítható az egyes mének krioprezervációs protokolljainak individualizálásával valamint új technikák bevezetésével. Ilyen technika a sperma előkezelése, prekondícionálása, amelyet az előkészítési protokoll részeként hajtottunk végre a fagyasztási lépés előtt. A hidrosztatikus nyomás alkalmazásával végzett előzetes kondicionálás (PTAT kezelés – Pressure Triggered Aquisition of Tolerance) pozitív hatását szarvasmarha- és egérembriókban, sertés- és szarvasmarha-spermában jól dokumentált. Jelen kutatásban megmutatjuk, hogy a ló sperma prekondícionálása beilleszthető a rutin krioprezervációs protokollba és jelentősen javítja a ménsperma mélyfagyasztásának mutatóit és kiszámíthatóságát.

Hat arab ló spermáját a tavaszi és őszi szezonban mélyhűtöttük a bábolnai laboratórium protokolljának megfelelően, a labor rutinjába illesztve. 4-16 ismétlést hajtottunk végre ménenként. Az örökítő anyagot ménenként kezelt és kezeletlen csoportokra osztottuk, és a motilitási paramétereket (CASA-val mérve) hasonlítottuk össze a csoportok között. A kezelés szignifikáns hatást fejtett ki ($p < 0{,}001$) a fagyasztást-felolvasztást követő túlélésre, mindkét tenyészszezonban.

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