EFFECT OF DIFFERENT EQUILIBRATION TIMES AND FREEZING RATES ON BULL SPERMATOZOA CRYOTOLERANCE

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ABSTRACT

Sperm cryopreservation is a complex process consisting of several steps, the details of which are not yet universally agreed upon. The objective of this study was to evaluate the effects of different equilibration times and freezing rates on post-thaw resilience and functional integrity. Collected semen from five Holstein bulls (30 samples in total) were split into 4 aliquots to produce frozen straws after 2 different equilibration times (2 h and 4 h) and freezing rates (2-phase and 3-phase). After thawing, the sperm characteristics were measured by flow cytometry. A linear model procedure was used to determine the effects of bull, equilibration time, freezing rate and interaction between them, on sperm variables. The evaluated sperm characteristics included spermatozoa with intact plasma and acrosomal membrane (PMAI), PMAI with high mitochondrial membrane potential (HMMP), spermatozoa with plasma membrane damage (PMD), and spermatozoa with acrosome damage (AD) were analysed immediately, 1 h and 2 h after thawing. In conclusion, the extension of the equilibration time from to 4 h in combination with 2-phase freezing rate led to a significant improvement in all measured parameters of frozen-thawed spermatozoa ($P < 0.01$).

Keywords: equilibration, freezing rate, cryopreservation, thawing, bull, spermatozoa

INTRODUCTION

Semen cryopreservation and the associated use of insemination doses has become an integral part of the dairy cattle breeding process worldwide, in which the use of artificial insemination (AI) provides extensive distribution of genetically superior sires (Ugur *et al.*, 2019). In this matter the AI is considered the most important of the assisted reproductive techniques. The most crucial element of AI success is represented by an insemination doses quality, the production of which is related to the reduction or arrest of the spermatozoa metabolism and thereby prolonging gamete life (Yoshida, 2000; Beran *et al.*, 2013). Worldwide, the predominant system in production of insemination doses is currently based on freezing (Yang *et al.*, 2018). Semen cryopreservation consists of several steps, including equilibration and freezing which influence the quality of thawed sperm

significantly as the spermatozoa cannot endure low temperatures without any harm (Tirpan *et al.*, 2017; Dias *et al.*, 2018). Specifically, cryopreservation leads to severe decline in sperm quality which may be manifested in sperm function lesions, and ultrastructural changes in plasma membrane, acrosomal membrane, or mitochondria. The loss of viability is fairly considerable and usually occurs in at least half of the sperm count (Layek *et al.*, 2016; Khalil *et al.*, 2018). Despite the fact that each step of the cryopreservation protocol has been carefully evaluated, there is still no consensus on which course of these processes is best for semen quality. Therefore, aforementioned steps may be modified to improve both the fertility of bull semen and the optimization of the laboratory production lines (Michel *et al.*, 2016; Fleisch *et al.*, 2017; Dias *et al.*, 2018). The bull ejaculate is most commonly

cooled to 4 to 5 °C and then equilibrated at the same temperature for varying lengths of time (Leite *et al.*, 2010). The equilibration time is however an important factor in sperm post-thaw survival as spermatozoa of many animal species including bear, camel, dog, sheep, goat or pigs improve their post-thaw characteristics when the equilibration time is prolonged (López-Urueña *et al.*, 2014; Ahmad *et al.*, 2015; Belala *et al.*, 2016; Malo *et al.*, 2017; Schäfer *et al.*, 2017; Rajani *et al.*, 2020). Equilibration itself allows sperm to adapt to lower temperatures, facilitates the movement of cryoprotectants across cell membranes (when using penetrating cryoprotectants) and allows water to be transported out of the cell, minimising damage caused by osmotic imbalances and by ice crystal formation during freezing-thawing process (Muiño *et al.*, 2007; Sieme *et al.*, 2016). Similarly, various freezing rates of variable success are being carried out during cryopreservation up to present (Gillis *et al.*, 2020). Nowadays, a rapid freezing in sophisticated programmable freezers is performed immediately after equilibration in order to avoid crystalisation, or sperm morphological damage, and obtain the best post-thaw quality possible (Shah *et al.*, 2016; Dias *et al.*, 2018; Khalil *et al.*, 2018). Previous studies evaluating cryopreservation protocol modifications on post-thaw sperm quality relied on subjective sperm analysis to varying extent. Therefore, this study was conducted to determine objectively the effects of different equilibration times (2 h vs 4 h), freezing rates (2-phase vs 3-phase), and their interactions on post-thaw resilience and functional integrity.

MATERIALS AND METHODS

Animals and Semen Collection

Holstein bulls (n = 5) kept in a private Artificial Insemination Centre (Central Bohemian Region, Czech Republic) were collected for the purpose of this work in 6 successive weeks during spring 2020 (6 ejaculates per bull obtained in total). Semen collection was standardly performed with artificial vagina as a part of the animals' daily routine. The sires were healthy, fertile, sexually mature, 3–5 years old, and used for semen collection. Bulls were stabled under the same optimal feeding, watering and management conditions.

Semen Processing, Thawing and Analysis

The obtained ejaculates were evaluated immediately after the semen collection using an electronic precision scale and the AndroVision® CASA (computer assisted sperm analysis) system (Minitübe GmbH, Tiefenbach, Germany). Only ejaculates with volume ≥ 1.5 mL, sperm concentration ≥700×106 sperm/mL, and motility ≥70% were further processed and cryopreserved. After initial quality control, the samples were diluted to final concentration of 40×106 spermatozoa/mL^{-1} in phospholipid extender AndroMed® (Minitübe GmbH, Tiefenbach, Germany). The diluted semen was then automatically packaged in 0.25 mL French straws (IMV, L'Aigle, France), slowly cooled to a temperature of +4 °C, divided into 2 halves and equilibrated for either 2 or 4 hours in a cooling box. Afterwards, the straws were divided into 2 halves once more, and frozen utilising a two-phase or three-phase freezing rate as recommended by previous study by Doležalová *et al.* (2016) using a programmable DigitCool® freezer (IMV Technologies, L'Aigle, France). The frozen doses were then deposited into liquid nitrogen and stored there for one year till the thawing process. The thawing was performed in a water bath at 38 ± 1 °C for 30 seconds (Rubio-Guillén *et al.*, 2007). After thawing, each sample was transferred into the Eppendorf Tube and kept inside of an INB 400 incubator (Memmert GmbH, Schwabach, Germany) in the dark at 38 °C for the entire duration of a 2 h long incubation. Subsequently, flow cytometric evaluation was performed according to Pytlík *et al.* (2022). Two insemination doses, from each production week, were flowcytometrically analysed twice. Such analyses were accomplished using a NovoCyte digital flow cytometer, model number 3000 (Acea Biosciences, part of Agilent, Santa Clara, California, USA) immediately 0 (< 15 min designated as 0 h), 1 and 2 h after thawing. The assessed sperm subpopulations were as follows: spermatozoa with intact plasma and acrosomal membrane (PMAI), PMAI with high mitochondrial membrane potential (HMMP), spermatozoa with plasma membrane damage (PMD), and spermatozoa with acrosome damage (AD).

Data and Statistical Analyses

The data were analysed using the statistical software SAS 9.3 (SAS Institute Inc., Cary, NC, USA). The data for sperm variables were examined for normal distribution (Shapiro-Wilk test) and for homogeneity of variance (Bartlett's test), and further analysed using the generalised linear model (PROC GLM). The best model was selected based on the Akaike information criterion. The Tukey-Kramer method was used for the evaluation of differences of least squares means. The model equation used for an evaluation was as follows:

$$Y_{ijk} = \mu + A_i + B_j + C_k + (AB)_{ij} + e_{ijklm},$$

where:
Y_{ijk}.....dependent variable (PMAI, HMMP, PMD, AD);
μ........average value of the dependent variable;
A_i.......fixed effect of the bull: i (1, $n = 144$; $i = 2$, $n = 144$; $i = 3$, $n = 144$; $i = 4$, $n = 144$; $i = 5$, $n = 144$);
B_j.......fixed effect of the equilibration time: j (2, $n = 360$; 4, $n = 360$);
C_k......fixed effect of the freezing rate: k (2, n = 360; 3, n = 360);

D_l.......fixed effect of the evaluation time: l (0, n = 240; 1, n = 240; 2, n = 240);

BC_{jk}...interaction between fixed effect of the equilibration time and the freezing rate;

e_{ijklm}...residual error.

Significant differences between groups were evaluated at $P < 0.01$, and $P < 0.05$.

RESULTS

The preservation of spermatozoa characteristics after thawing varied significantly ($P < 0.01$ and $P < 0.05$) as these differences were observed between almost all bulls in every evaluated parameter. The effects of bull individuality, in detail, is presented in Tab. I.

Incubation time had an effect on post-thaw quality parameters ($P < 0.01$ and $P < 0.05$). As can be further seen from the Tab. II., the same time development can be observed for the PMAI and PMD parameters. Both characteristics showed an improvement ($P < 0.05$) in the first hour after thawing compared to results obtained immediately after thawing. The only parameter that followed a decreasing trend throughout the entire incubation period was HMMP ($P < 0.01$).

As evident from Tab. III, the significant interactions between equilibration time and freezing rate were found for all parameters evaluated after thawing. Considering the equilibration conditions, there were differences ($P < 0.01$) in PMAI, PMD and AD. These post-thaw characteristics showed better results when equilibrated for 4 h. Within the freezing rate,

I: *The effect of sire on post-thaw bull spermatozoa characteristics evaluated by flow cytometry*

Level	PMAI %	HMMP %	PMD %	AD %
bull 1	15.25 ± 0.777[A]	45.08 ± 1.097[A]	84.49 ± 0.785[A]	41.30 ± 1.178[A]
bull 2	26.85 ± 0.777[B,a]	41.08 ± 1.097[A,C,a]	72.96 ± 0.785[B,a]	34.99 ± 1.178[B,a]
bull 3	30.09 ± 0.777[B,b]	42.16 ± 1.097[A,C]	69.50 ± 0.785[B,b]	36.82 ± 1.178[A]
bull 4	41.30 ± 0.777[C]	36.25 ± 1.097[B,C,b]	57.97 ± 0.785[C]	29.12 ± 1.178[C]
bull 5	23.12 ± 0.777[D]	47.89 ± 1.097[A,D]	76.49 ± 0.785[B,b]	39.60 ± 1.178[A,b]

Results are presented as least square means ± SEM; PMAI = spermatozoa with intact plasma membrane and acrosome, HMMP = PMAI sperm with high mitochondria membrane potential, PMD = sperm with damaged plasma membrane, AD = spermatozoa with damaged acrosome. Different letters (A, B, C) indicate differences between groups within a column (P < 0.01). Different letters (a, b, c...) indicate differences between groups within a column (P < 0.05).

II: *Comparison of incubation-mediated changes on post-thaw bull spermatozoa subpopulations development*

Evaluation time (h)	PMAI %	HMMP %	PMD %	AD %
0	26.25 ± 0.602[a]	54.03 ± 0.850[A]	73.45 ± 0.608[a]	34.60 ± 0.913[a]
1	28.29 ± 0.602[b]	40.86 ± 0.850[B]	71.22 ± 0.608[b]	37.61 ± 0.913[b]
2	27.43 ± 0.602	32.59 ± 0.850[C]	72.17 ± 0.608	36.90 ± 0.913

Results are presented as least square means ± SEM; PMAI = spermatozoa with intact plasma membrane and acrosome, HMMP = PMAI sperm with high mitochondria membrane potential, PMD = sperm with damaged plasma membrane, AD = spermatozoa with damaged acrosome. Different letters (A, B, C...) indicate differences between groups within a column (P < 0.01). Different letters (a, b, c...) indicate differences between groups within a column (P < 0.05).

III: *The effect of equilibration time and freezing rate on post-thaw bull spermatozoa variables evaluated by a flow cytometer*

Equilibration time (h)	freezing rate	PMAI %	HMMP %	PMD %	AD %
2	2	25.09 ± 0.695[A]	47.41 ± 0.981[A]	74.49 ± 0.703[A]	42.81 ± 1.054[A]
	3	24.69 ± 0.695[Á]	39.16 ± 0.981[B]	74.96 ± 0.703[A]	35.61 ± 1.054[B]
4	2	30.62 ± 0.695[B]	44.15 ± 0.981[A]	68.94 ± 0.703[B]	34.98 ± 1.054[B]
	3	28.89 ± 0.695[B]	39.27 ± 0.981[B]	70.73 ± 0.703[B]	32.08 ± 1.054[B]

Results are presented as least square means ± SEM; PMAI = spermatozoa with intact plasma membrane and acrosome, HMMP = PMAI sperm with high mitochondria membrane potential, PMD = sperm with damaged plasma membrane, AD = spermatozoa with damaged acrosome. Different letters (A, B, C...) indicate differences between groups within a column (P < 0.01).

the HMMP parameter had greater values ($P < 0.01$) when a 2-phase freezing rate was used rather than a 3-phase one.

DISCUSSION

This study was designed to evaluate the effect of equilibration times, and freezing rates on post-thaw quality of bull spermatozoa. The both mentioned stages of cryopreservation are imperative for success of sperm freezing (Ahmad *et al.*, 2015). As expected, bulls responded unequally to cryopreservation protocols. Individual intermale variation in the proportion of evaluated sperm subpopulations after thawing is a well-documented phenomenon (Mohammed and Ahmed, 2017) and as such was observed in our study. This is in line with Ugur *et al.* (2019) who also reported rather low post-thaw viability of sperm and significant differences between sires at the same time. The reason of varying freezability lies in different protein compositions and their expression levels in seminal plasma and sperm of each individual (Ugur *et al.*, 2019). Moreover, a successful cryopreservation is also dependent on specific biochemical and metabolic properties of sperm cells (Loomis and Graham, 2008). Better sperm resistance to cryopreservation therefore resides in genetic variation as discussed by Barbas and Mascarenhas (2009).

It is pertinent to mention that different dynamics of post-thaw variables subjected to 2 h incubation was observed. Our findings of the PMAI parameter time development are in accordance with Anzar *et al.* (2011), yet in disagreement with Fleisch *et al.* (2017) and Ansari *et al.* (2014), who found an 11% and 18% decrease in this variable during 3- and 2-hour long incubation, respectively. For instance, sperm viability did not significantly vary during 3 h equilibration in a study by Muiño *et al.* (2007). A possible explanation of our results could be given by greater instability of membrane structures and by intensified fluorescent probe permeation caused by stress environment conditions immediately after thawing. Regarding the HMMP parameter time course progress, the analogous observation as ours was found in a study of Bucher *et al.* (2019) and Gürler *et al.* (2019), where over 9% and 15% drops were detected during 3-hour long equilibration, respectively. In a study conducted by Anzar *et al.* (2011), on the other hand, the opposite trend was proved as HMMP rose after a 2-hour long incubation period. Varela *et al.* (2020) discussed that samples with lower HMMP manifest extensive level of cryocapacitation, an undesirable trait which prevents normal spermatozoa functioning in the female reproductive tract. Despite contradicting findings in mentioned peer literature, we conclude that HMMP more precisely reflects sample actual functional status and serves as a better indicator of sample quality than PMAI. Plasma membrane

integrity is another key element in spermatozoa proper functioning due to the important role in interactions between internal and external cell environment or regulations of many functions related to fertilisation and resistance to temperature change challenges (Singh *et al.*, 2018). As discussed by Pini *et al.* (2018) cryopreservation causes not only lethal but sublethal damage as well. The latter one includes alterations to sperm proteins, lipids or sugars which may account to increased susceptibility to sperm phagocytosis and clearance from the female reproductive system. Unlike Rastegarnia *et al.* (2014) or Sellem *et al.* (2015) we did not find significant incubation-mediated decrease in our study for PMD, yet the numerical values differed throughout the incubation. Our findings are similar to our previous study by Pytlík *et al.* (2022), where an improvement in PMD was detected in 1 h after thawing. Unfavourable alterations of spermatozoa acrosome, e.g. capacitation-like changes or acrosome reaction, more frequently occur in cryopreserved semen and are ultimately responsible for shorter longevity of spermatozoa in female reproductive tract (Bucher *et al.* 2019, Varela *et al.* 2020). The AD time development findings are in line with those of Rastegarnia *et al.* (2014) and Sellem *et al.* (2015), who reported a 24% and 5% decrease in acrosomal integrity during the 4-hour long course of the incubation.

Many aspects of cryopreservation protocols have to be considered when targeting to obtain maximum fertility, including the equilibration duration. It is necessary to mention that glycerol penetration and its equilibration on both sides of plasma membrane is performed in a rapid manner (2–5 min), therefore the equilibration time is more crucial for sperm membranes adaptation to low temperatures (Leite *et al.*, 2010). The present study showed that there were significant interactions between equilibration and freezing rates for sperm quality after thawing. More beneficial effects in the majority of post-thaw variables were proved for equilibration time rather than for freezing rates. In more detail, there were greater values for post-thaw sperm quality and functional variables when the equilibration time was longer. Similarly to our results, Leite *et al.* (2010) determined 4 h long equilibration as the most appropriate for PMAI, PMD and AD in comparison to non-equilibration or equilibration lasting 2 h. Similarly, Shahverdi *et al.* (2014) found worsened PMD or AD after 2 h equilibration in comparison to 4 h one. Yet, there are other studies like Foot and Kaproth (2002) who found better post-thaw quality after 18 h equilibration than after 4 h. In more detail, our results showed higher figures in PMAI, PMD and AD variables than those published by Leite *et al.* (2010), yet this post-thaw characteristics strongly depend on sample production conditions as different breed, diluents and freezing rates were used in both studies. As mentioned, many studies investigated whether and

how longer equilibration, e.g. overnight or several day long equilibration, affects sperm in vitro post-thaw quality or in vivo fertility. For instance, Michel *et al.* (2016) found no difference in non-return rate at 90 days for insemination doses produced either after 8 h or 18 h long equilibration. Although, the post-thaw quality was in the favour of samples equilibrated for 18 h. Similar conclusion was reached by Fleisch *et al.* (2017) who also did not find an effect on non-return rate in samples equilibrated in the range from 4 h up to 72 h. However the post-thaw quality was ameliorated when equilibration lasted 24 h to 72 h rather than 4 h. Another study, evaluating conception rate, by Murphy *et al.* (2018) demonstrated that increased equilibration time up to 72 h had no significant effect on post-thaw in vitro sperm quality nor the calving rate. These authors suggest that the possibility of longer equilibration provides semen processing centres flexibility in terms of the length of the time during which semen can be stored prior to freezing without detrimental effects on final quality.

As discussed by Dalal *et al.* (2018a) there are two critical temperature ranges wherein damage to male germplasm takes place during freezing. The period of supercooling (0 to -5 °C) and the formation of ice crystals (-6 to -15 °C). Simoultaneously, an optimum freezing rate must be slow enough to allow water to exit the cells to prevent intracellular ice formation, and fast enough to avoid severe cell dehydration. Therefore, the success of cryopreservation is dependent on the course of freezing (Clulow *et al.*, 2008). In the case of freezing rates, unconvincing results were obtained for most of the spermatozoa post-thaw variables. The only exception was HMMP parameter, which was better maintained when the 2-phase freezing rate was applied. The fact that mitochondria did not follow the same pattern as other variables is in line with Leite *et al.* (2010) who also reported that mitochondria were not affected by equilibration time. We hypothesise that mitochondria responded more favourably when there is a combination of a slower freezing pace in the critical temperature range and faster pace during freezing below -10 °C. Our finding is, however, in disagreement with Dalal *et al.* (2018b) who executed large freezing trial and detected the optimum post-thaw semen quality, including greatest HMMP, when faster freezing (-30 °C/min) was performed. Yet, the similar results to our study were obtained by Doležalová *et al.* (2016) who observed greater preservation of mitochondria functions and other spermatozoa features when 2-phase freezing rate was used compared to 3-phase one. Similarly, Dias *et al.* (2018) concluded that slower pace of freezing (-15 °C/min) exhibited better post-thaw quality parameters than faster (-19 °C/min) frozen samples.

The prolonged equilibration can be recommended, however a field fertility study is convenient to be performed in order to confirm this surmise.

CONCLUSION

In conclusion, the extension of the equilibration time from 2 h to 4 h improves membrane integrities and mitochondria functions of cryopreserved semen after thawing. Simultaneously, more suitable values for all measured parameters were reached when 2-phase was executed. Therefore, the optimal values of spermatozoa variables were measured when cryopreserving protocol combined 4 h equilibration and 2-phase freezing rate.

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