ACTA UNIVERSITATIS AGRICULTURAE ET SILVICULTURAE MENDELIANAE BRUNENSIS

Volume 65 84 Number 3, 2017
https://doi.org/10.11118/actaun201765030815

EFFECT OF DIFFERENT THAWING METHODS ON BULL'S SEMEN CHARACTERISTICS

Martina Doležalová[1], Martin Ptáček[1], Luděk Stádník[1], Jaromír Ducháček[1]

[1]Department of Animal Husbandry, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 957, 165 21 Prague 6 – Suchdol, Czech Republic

Abstract

DOLEŽALOVÁ MARTINA, PTÁČEK MARTIN, STÁDNÍK LUDĚK, DUCHÁČEK JAROMÍR. 2017. Effect of Different Thawing Methods on Bull's Semen Characteristics. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis,* 65(3): 815–822.

The aim of the study was to evaluate the influence of different thawing methods on bull's semen characteristics. The semen was collected and processed from 8 bulls (Holstein, n = 4; Czech Fleckvieh, n = 4) kept in private Sire insemination Centre. Four different thawing methods were tested: control thawing methods (temperature = 38.5 °C, time = 30 seconds), slow thawing method (temperature = 30 °C, time = 50 seconds), moderate thawing method (temperature = 50 °C, time = 15 seconds), rapid thawing rate (temperature = 70 °C, time = 3 seconds). The percentage rate of total and progressive motile spermatozoa above head as well as movement characteristics of straight-line velocity (VSL, μm/s) and linearity (LIN, %) were recorded using CASA system immediately after thawing and after 2 hrs. of heat incubation (±38 °C). Subsequently the differences between semen characteristics measured immediately after thawing and after 2 hours of incubation were calculated. The data were analyzed with SAS software. All the evaluated semen traits reached significantly lowest values in total motility (–8 % to –10.5 %, $P < 0.05$), progressive motility (–5.6 % to –10 %, $P < 0.05$) VSL (–3.9 μm/s to –9 μm/s, $P < 0.05$) and LIN (–1.5 % to –4.8 %, $P < 0.05$) in control thawing method immediately after the thawing compared to others. However, these differences were negligible after 2 hrs. of incubation. The highest values of progressive motility and movement characteristics after thawing and after 2 hours incubation were detected using slow thawing method. Moreover, the rapid method of thawing showed the significantly the lowest results in progressive motility (–3.6 % to –6.6 %), VSL (–3.2 to –5.6 μm/s) and LIN (–3 % to –4.6 %) characteristics assessed after 2 hrs. of incubation compared the others ($P < 0.05$). Control method of thawing was most stable during the incubation and showed the significantly lowest decrease of total motility (–7.6 % to –11.8 %, $P < 0.05$), progressive spermatozoa motility above head (–7.5 % to –9.8 %, $P < 0.05$) and VSL characteristics (–2.4 to –9.7 μm/s, $P < 0.05$) during incubation. Therefore, this thawing protocol can be recommended at this study based on the lowest spermatozoa characteristics decline during incubation for using artificial insemination.

Keywords: CASA, progressive motility, total motility, VSL, LIN

INTRODUCTION

Artificial insemination (AI) in dairy cattle is provided with frozen-thawed semen (Andrabi, 2007; Doležalová *et al.*, 2015). Therefore, the quality of produced insemination doses (ID) is directly related to fertility (Pugliesi *et al.*, 2014). The spermatozoa survivability after cryopreservation is influenced by many factors such as type of semen extender (Moore *et al.*, 2006), packing method, the length of equilibration, freezing rate (Rodriguez *et al.*, 1975; Robbins *et al.*, 1976; Doležalová *et al.*, 2016) and finally thawing rate and handling of IDs just before using (Moore *et al.*, 2006).

Spermatozoa having reached elevated thawed temperatures may be damaged by "cold-shock" in cold or overheating in hot ambient climatic conditions (Kaproth *et al.*, 2005). Also the thawing rate is important due the possibility of spermatozoa intracellular ice recrystallization and its negative influence to spermatozoa motility and acrosomal

integrity (Robbins *et al.*, 1976; Mazur, 1984; Dhami *et al.*, 1992; Nur *et al.*, 2003; Seki, Mazur, 2009). Today, the most of commercial AI centers recommend warm-water thaw methods for bovine semen frozen in straws processed at their centers. The thawing procedure features a straw being removed from liquid nitrogen and placed immediately in 33–35 °C water for a 30–40 s before preparing the artificial insemination gun (DeJarnette *et al.*, 2000; Kaproth *et al.*, 2005; DeJarnette, Marshall, 2005).

Although thawing was initially performed at 37 °C (Salamon, Visser, 1973), higher thawing temperatures of 50 °C (Pelaez *et al.*, 2006) and 70 °C (Hernández *et al.*, 2007) were chosen to minimize the exposure time of sperm cells to potential ice crystal and osmotic damage. As a result, thawing duration differed with lower-volume straws (0.25–0.5 ml) which were thawed at 10 to 20 seconds at 50 °C (Aamdal, Andersen, 1968) or 5 to 10 seconds at 70 °C (Hernández *et al.*, 2007). Also other studies have proven that thawing temperatures as high as 60–80 °C could further improve post-thaw motility (Rodriguez *et al.*, 1975; Senger, 1980; Dhami *et al.*, 1992; Nur *et al.*, 2003).

The quality of insemination doses after thawing used to be carried out subjectively, mainly according to estimation of motile spermatozoa ration (Pugliesi *et al.*, 2014). Recently, there has been a growing interest in spermatozoa motility assessment by computer assisted sperm analysis (CASA) to determine spermatozoa movement characteristics more accurately and objectively than by subjective evaluation (Šimoník *et al.*, 2015). The spermatozoa movement characteristics were in positively correlation with in vitro fertilization rate (Kumar *et al.*, 2003).

The aim of presented study was to evaluate the influence of different thawing methods on spermatozoa motility and movement characteristics evaluated by CASA system.

MATERIALS AND METHODS

Bulls and semen processing

The semen was collected from the group of selected breeding bulls (Holstein, n = 4; Czech Fleckvieh, n = 4) at 3–5 years of age. All the bulls were commercially used for ID production and their semen was collected twice a week during the whole year. All the animals were bred under the same management system of private Sire insemination Centre (Central Bohemian Region, 285 m above sea level, average annual rainfall per year = 650 mm, average annual temperature = 9°C). The semen was collected during August 2013 using an artificial vagina and immediately evaluated in laboratory of Sire insemination station according to methodology applied by trained staff. Volume of fresh semen, density of spermatozoa, and percentage rate of progressive motile spermatozoa above head were evaluated. Semen passing the initial conditions for ordinarily commercial purpose (minimal criteria: 0.7 × 10^6 mm^{-3} of semen density and 70 % of progressive motility) was further processed.

Semen was immediately ordinarily diluted with phospholipid diluent AndroMed® (Minitübe GmbH, Tiefenbach, Germany) to a final spermatozoa concentration (10 million motile spermatozoa per dose). Diluted semen was mixed up for at least 5 minutes at room temperature (25 °C). Then was automatically filled in the French straws (0.25 ml, IMV Technologies, L'Aigle, France), spread on the ramps and inserted into the cooling box, cooled at an average speed of 0.2 °C per min to 4–5 °C, and equilibrated for 120 minutes. After equilibration, the straws were frozen using the controlled freezing methodology Direct Freezing in a freezer box Digitcool® (IMV Cryo Bio System, L'Aigle, France). The 3-phase standard freezing curve was used (Muino *et al.*, 2007). The bull's straws were stored in liquid nitrogen (–196 °C) up to their thawing.

Semen thawing

The thawing of straws was performed in preheated water bath. Four different thawing methods based on various water bath temperature and length of thawing were tested:

- Control method of thawing in the water bath heated at 38.5 °C for 30 seconds
- Slow thawing in the water bath heated at 30 °C for 50 seconds
- Moderate thawing in the water bath heated at 50 °C for 15 seconds
- Rapid thawing in the water bath heated at 70 °C for 3 seconds

Evaluation of semen characteristics

The 2 straws per bull per thawing method were used to create the mixed sample for the next evaluation of spermatozoa motility. Each sample was assessed repeatedly, such that overall of 64 observations were monitored during the whole trial. After thawing the volume of straws were positioned into the 500 µl of physiological solution and placed in the dry heater (Thermo-block Falc®, Treviglio, Italy, ±38°C). The percentage rate of total (Total, %) and progressive (Prog, %) motile spermatozoa above head was evaluated and recorded using CASA system (SCA® Production v. 5.3.; MICROPTIC S.L., Barcelona, Spain) with a phase contrast microscope Eclipse E200 (Nikon, Tokyo, Japan) at 200–300× magnification when five fields of view per each straw were evaluated at least (Tuncer *et al.*, 2011). As supplementary spermatozoa movement characteristics were selected straight-line velocity (VSL, µm/s) and linearity (LIN, %). Semen samples were evaluated immediately after thawing (Total0, Prog0, VSL0, LIN0) and after 2 hours of incubation (Total2, Prog2, VSL2, LIN2). Subsequently the difference between semen characteristics immediately after thawing (0 hrs.) and after 2 hours of incubation (Total0-2, Prog0-2, VSL0-2, Lin0-2) were calculated.

I: *Basic characteristics of the data structure*

	N	AM	MIN	MAX	Sd	CV
Total0	64	78.3	50.9	98.5	11.1	14.2
Prog0	64	57.7	32.1	83.1	10.6	18.3
VSL0	64	38.6	28.1	57.0	7.0	18.1
LIN0	64	47.6	32.9	60.2	6.6	13.9
Prog2	64	42.5	18.0	69.6	12.1	28.5
Total2	64	65.2	35.7	94.0	14.7	22.5
VSL2	64	33.3	18.3	43.2	6.0	18.0
LIN2	64	52.6	38.0	68.6	6.5	12.3
Prog2-0	64	15.2	–3.0	43.6	10.5	69.2
Total2-0	64	13.1	–8.6	42.0	11.6	88.4
VSL2-0	64	5.3	–13.2	29.1	9.2	171.8
LIN2-0	64	–5.0	–19.7	8.6	6.8	–137.7

N = number of observing; AM = arithmetic mean; Sd = standard deviation; MIN = minimal value; MAX = maximal value; CV = coefficient of variance (%); Total0, 2 = total spermatozoa motility at time 0, 2 hrs. (%); Prog0, 2 = progressive spermatozoa motility at time 0, 2 hrs. (%); VSL0, 2 = straight line velocity at time 0, 2 hrs. (μm/s); LIN0, 2 = linearity at time 0, 2 hrs. (%);Total2–0 = decrease of total spermatozoa motility from 0 to 2 hrs. of incubation (%); Prog2–0 = decrease of progressive spermatozoa motility from 0 to 2 hrs. of incubation (%); VSL2–0 = decrease of straight line velocity from 0 to 2 hrs. of incubation; (μm/s); LIN2–0 = decrease of linearity from 0 to 2 hrs. of incubation (%)

Statistical analysis

All statistical analyses were conducted using SAS 9.3. (SAS/STAT® 9.3., 2011), MEANS, CORR and GLM procedures. Correlation analysis among variables (Total0, Prog0, VSL0, LIN0, Total2, Prog2, VSL2, LIN2) was performed using Pearson correlation coefficients. The analysis of variance (ANOVA) was used to evaluate influence of fixed effect of bull and thawing method on dependent variables (Total0, Prog0, VSL0, LIN0, Total2, Prog2, VSL2, LIN2, Total0-2, Prog0-2, VSL0-2, Lin0-2). The effects of bull × thawing procedure interaction and repeatability of measurement were also tested during ongoing analysis. However, both these factors were non-significant for all the evaluated traits, and therefore excluded in the final model. The characteristics of actual spermatozoa motility at time 0 and 2 hrs. were presented in the form of tables, while characteristics of spermatozoa persistence (Total0-2, Prog0-2, VSL0-2, Lin0-2) were expressed as figures using of MS Excel software. The model equation adapted to explain the variability in spermatozoa motility and movement characteristics was as followed:

$$y_{ijk} = \mu + BULL_i + THW_j + e_{ijk}$$

y_{ijk} = dependent variable (Total0, Prog0, VSL0, LIN0, Total2, Prog2, VSL2, LIN2, Total0-2, Prog0-2, VSL0-2, Lin0-2)
μ = overall mean value
BULL = fixed effect of i^{th} bull (i = 8 classes, n = 8 observations in each class)
THW = fixed effect of j^{th} thawing method (j = 4 classes, n = 16 observations in each class)
e_{ijk} = residual error
The differences between the variables estimated were tested by the Tukey-Kramer method at the level of significance $P < 0.05$.

RESULTS

Basic statistics and model description

Short overview of basic characteristics of the dataset structure is presented in Tab. I. Model used to explain the variation in bull's semen characteristics was significant for all the evaluated traits. Also factors in the model were significant in the majority, except of effect of thawing method on Total2, Prog2, VSL2 and LIN2 characteristics as documented in Tab. II.

Correlation analysis

Results of correlation analysis performed among dependent variables are presented in Tab. III. Total0 and Prog0 traits were significantly correlated mutually ($r = 0.93$; $P < 0.001$). These both characteristics (Prog0, Total0) were also significantly correlated with Prog2 and Total2 traits ($r = 0.55 – 0.63$; $P < 0.001$), indicating positive relations among basic spermatozoa motility that persisted from time 0 up to 2 hrs. after incubation. This thesis was demonstrated also by positive correlation ($r = 0.92$; $P < 0.001$) between Total2 and Prog2 traits. No or negative relation was observed among spermatozoa movement characteristics at time 0 (VSL0, LN0) and Total0, Prog0, Total2 or Prog2 traits. As interesting VSL2 was significantly correlated with spermatozoa motility at time 0 and after 2 hrs. of incubation, while non-significant correlation was detected in relation with VSL0 or LIN0. Contrary, absolutely opposite relations to VSL2 characteristic were detected in LIN2 parameter.

Influence of thawing procedure on the bull's semen characteristics

The influence of different thawing method on the bull's spermatozoa motility and selected movement characteristics is presented in Tab. IV. Significantly lowest results of Total0 (–8.0 to –10.5 %) and Prog0 (–5.6 to –10.0 %) were detected in control thawing in comparison with all the others used procedures. However, no significant differences were obvious among slow, moderate and rapid thawing methods in these traits (Total0, Prog0). Significantly highest VSL0 and LIN0 were observed in slow thawing, which differed significantly with control thawing (+9.4 µm/s in VSL0, +4.8 % in LIN0) and moderate thawing (+5.5 % µm/s VSL0, +3.3 % in LIN0) methods. No significant differences were observed in Total2 parameter in relation to different methods of thawing; however, numerically highest values (+1.8 to +3.9 %) were detected in slow method. Others semen characteristics evaluated after 2 hrs. (Prog2, VSL2 and LIN2) were also highest in slow method of thawing with significant differences to rapid thawing. More exactly expressed these differences were 6.6 % in Prog2, 5.6 µm/s in VSL2 and 4.6 % in LIN2. The other aim of the study was to evaluate decrease of spermatozoa motility and movement characteristics from time 0 to 2 hrs. after thawing in relation to particular variations of thawing method. The results are presented in Figures 1 to 4. The significantly lowest decrease of total and progressive motility from time 0 to 2 hrs. of incubation was observed in control thawing (Total0-2 = 7.5 %; Prog0-2 = 9.9 %) in comparison with slow (Total0-2 = 15.1 %; Prog0-2 = 17.4 %), moderate (Total0-2 = 19.3 %; Prog0-2 = 19.0 %) and rapid thawing (Total0-2 = 17.6 %; Prog0-2 = 19.7 %) methods. The highest decrease of VSL parameter from 0 to 2 hrs. after thawing was detected in rapid thawing method (VSL0-2 = 11.8 µm/s), which significantly differed to control (+9.7 µm/s) or

II: *Description of the model*

	MODEL		THAWING		BULL	
	R2	**Pr > F**	**F-test**	**P**	**F-test**	**P**
Total0	0.738	P<0.001	9.37	P<0.001	17.81	P<0.001
Prog0	0.667	P < 0.001	7.37	P < 0.001	13.60	P < 0.001
VSL0	0.684	P < 0.001	13.60	P < 0.001	11.56	P < 0.001
LIN0	0.672	P < 0.001	4.23	P < 0.010	15.08	P < 0.001
Total2	0.788	P < 0.001	0.78	P < 0.509	30.50	P < 0.001
Prog2	0.634	P < 0.001	1.52	P < 0.221	14.20	P < 0.001
VSL2	0.634	P < 0.001	2.54	P < 0.067	2.40	P < 0.041
LIN2	0.493	P < 0.001	1.92	P < 0.138	6.67	P < 0.001
Total2-0	0.600	P < 0.001	6.69	P < 0.001	9.43	P < 0.001
Prog2-0	0.475	P < 0.001	4.46	P < 0.008	5.51	P < 0.001
VSL2-0	0.505	P < 0.001	5.40	P < 0.003	6.65	P < 0.001
LIN2-0	0.384	P < 0.001	3.25	P < 0.030	4.06	P < 0.002

Total 0, 2 = total spermatozoa motility at time 0, 2 hrs. (%); Prog0, 2 = progressive spermatozoa motility at time 0, 2 hrs. (%); VSL0, 2 = straight line velocity at time 0, 2 hrs. (µm/s); LIN0, 2 = linearity at time 0, 2 hrs. (%); Total2–0 = decrease of total spermatozoa motility from 0 to 2 hrs. of incubation (%); Prog2–0 = decrease of progressive spermatozoa motility from 0 to 2 hrs. of incubation (%); VSL2–0 = decrease of straight line velocity from 0 to 2 hrs. of incubation; (µm/s); LIN2–0 = decrease of linearity from 0 to 2 hrs. of incubation (%)

III: *Correlation analysis of bull's semen characteristics*

	Total0	**Prog0**	**VSL0**	**LIN0**	**Total2**	**Prog2**	**VSL2**	**LIN2**
Total0	1	0.93***	–0.08n.s.	–0.41**	0.63***	0.61***	0.27*	–0.20n.s.
Prog0		1	0.15n.s.	–0.30*	0.55***	0.58***	0.31*	–0.13n.s.
VSL0			1	0.68***	–0.30*	–0.20n.s.	0.01n.s.	0.26*
LIN0				1	–0.25n.s.	–0.24n.s.	0.01n.s.	0.46***
Total2					1	0.92***	0.37**	–0.12n.s.
Prog2						1	0.57***	–0.01n.s.
VSL2							1	0.66***
LIN2								1

Total0, 2 = total spermatozoa motility at time 0, 2 hrs. (%); Prog0, 2 = progressive spermatozoa motility at time 0, 2 hrs. (%); VSL0, 2 = straight line velocity at time 0, 2 hrs. (µm/s); LIN0, 2 = linearity at time 0, 2 hrs. (%); n.s. = non-significant; * = significant on P < 0.05; ** = significant on P < 0.01; *** = significant on P < 0.001

IV: *Influence of different thawing process on the bull's semen characteristics*

	Total0	Prog0	VSL0	LIN0	Total2	Prog2	VSL2	LIN2
C	69.6^{a}	50.2^{a}	34.7^{a}	46.0^{a}	62.1	40.3^{ab}	32.6^{ab}	53.2^{ab}
S	79.0^{b}	60.2^{b}	44.1^{c}	50.8^{c}	63.9	42.8^{a}	35.0^{a}	54.8^{a}
M	80.1^{b}	58.8^{b}	38.6^{b}	47.5^{ab}	60.8	39.8^{ab}	34.2^{a}	53.7^{ab}
R	77.6^{b}	55.8^{b}	41.1^{bc}	49.5^{cb}	60.0	36.2^{b}	29.4^{b}	50.2^{b}
RMSE	1.56	1.65	1.08	1.04	1.85	2.00	1.39	1.26

Total0, 2 = total spermatozoa motility at time 0, 2 hrs. (%); Prog0, 2 = progressive spermatozoa motility at time 0, 2 hrs. (%); VSL0, 2 = straight line velocity at time 0, 2 hrs. (μm/s); LIN0, 2 = linearity at time 0, 2 hrs. (%); C = thawing in water bath 38.5 °C for 30 s; S = thawing in water bath 30 °C for 50 s; M = thawing in water bath 50 °C for 15 s; R = thawing in water bath 70 °C for 3 s; RMSE = root mean square error; Means within columns with different letters differed significantly (a,b,c = P <0.05)

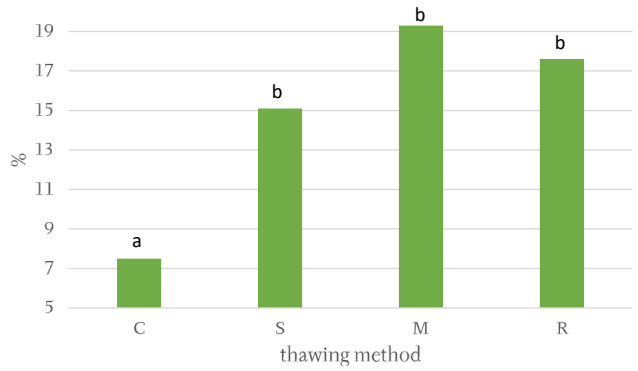
moderate (+7.3 μm/s) thawing methods. These two variations had generally the lowest decrease for the evaluated period of 2 hrs. of incubation. Higher results of LIN characteristic was detected after 2 hrs. of incubation than at time 0 in all the evaluated thawing methods, such that LIN0-2 parameter acquired negative numbers. Therefore, the results presented in Fig. 4 are expressed in absolute values for the better projection. Anyway, the lowest decrease of LIN0-2 during after 2 hrs. of incubation was monitored in rapid method (0.64 %), while the opposite results was detected in control method (7.2 %).

DISCUSSION

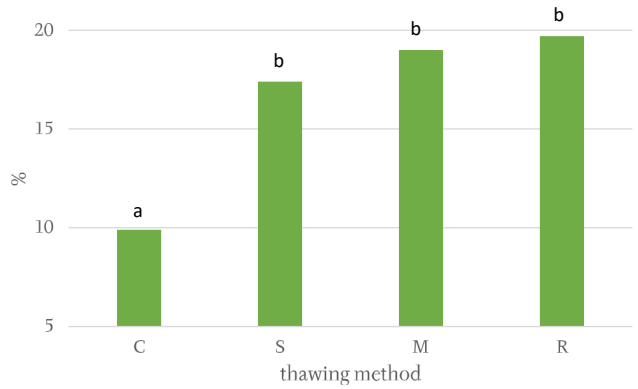
The factors of bull's individuality or thawing method were detected as important in presented study as well as in many previous (Beran *et al.*, 2013; Paldusová *et al.*, 2016; Doležalová *et al.*, 2016). Both these criteria should be therefore tested and proven in specified conditions to optimize methods of IDs processing. Positive effect on spermatozoa motility was described by Rodriguez *et al.* (1975), Senger (1980), Dhami *et al.* (1992) or Nur *et al.* (2003) while using high temperature (reaching 60–80 °C) protocols. Damage of the plasma membrane and cell organelles occurs during slow thawing methods, due to the small, innocuous intracellular ice-crystals that can grow and become recrystallized (Panyaboriban *et al.*, 2016). Therefore, using low thawing temperatures is usually not recommended (DeJarnette *et al.*, 2000; DeJarnette, Marshall, 2005). Contrary, Seidel (1986) reported that spermatozoa damages may occur after high thawing temperature protocols due to reduced spermatozoa efflux of cryoprotective agents out of the spermatozoa and therefore suggested slow thawing protocols as more suitable. Variety of above mentioned authors was underlined by Muino *et al.* (2008) who found no significant effect on spermatozoa motility at time 0 in relation of various thawing rates (control – 35 °C for 40 seconds, moderate – 50 °C for 15 seconds and rapid – 70 °C for 5 seconds). Our results monitored immediately after thawing are ambiguous in this connection, when both too slow thawing method as well as rapid thawing method reached better spermatozoa motility than control method of thawing. The individuality of bull and also the semen processing influenced the results of spermatozoa motility after thawing (Robbins *et al.*, 1976; Moore *et al.*, 2006; Beran *et al.*, 2011; Doležalová *et al.*, 2016). Therefore, these factors should serve as possible explanation of various results presented in this study with those of opposite.

Nevertheless, results of presented study detected after 2 hrs. of incubation are definitely in accordance with Seidel (1986) when rapid thawing using high temperatures decreased spermatozoa motility. The rapid method of thawing straws is not suitable for artificial insemination, because of the lowest values of semen characteristics evaluated after 2 hrs. of incubation. Similar results to ours were described by Rastegarnia *et al.* (2013) who noticed higher proportion of progressive spermatozoa motility and movement characteristics after thawing using higher temperature thawing protocols. However, this relative advantage had disappeared after 2 hrs. incubation.

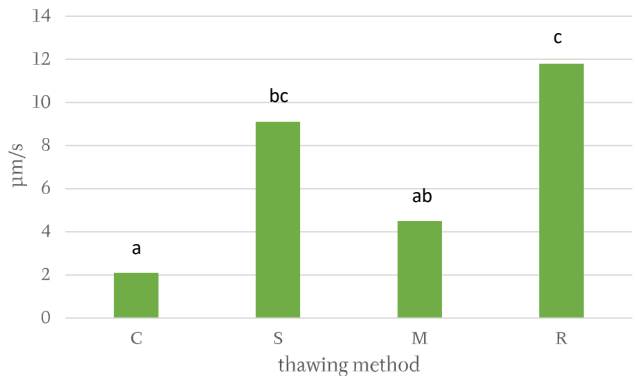
Decrease of sperm motility during incubation was dominantly influenced by thawing method (DeJarnette, Marshall, 2005), which is also in agreement with many previous studies (Linford *et al.*, 1976; Saacke *et al.*, 1980; DeJarnette *et al.*, 2000). Just the lower decline of motility during incubation is also important in particular time periods from the viewpoint of subsequent insemination (Doležalová *et al.*, 2015). Our results clearly indicated that the lowest decline of motility characteristics was demonstrated during control method of thawing, despite the significantly lowest values at time 0 and comparable results after 2 hrs. of incubation. Therefore, this thawing protocol can be recommended at this study for using artificial insemination due the lowest decline of semen characteristics during incubation. These results also corresponded with previously published studies of Narasimha Rao *et al.* (1986) or Larson-Cook *et al.* (2003).



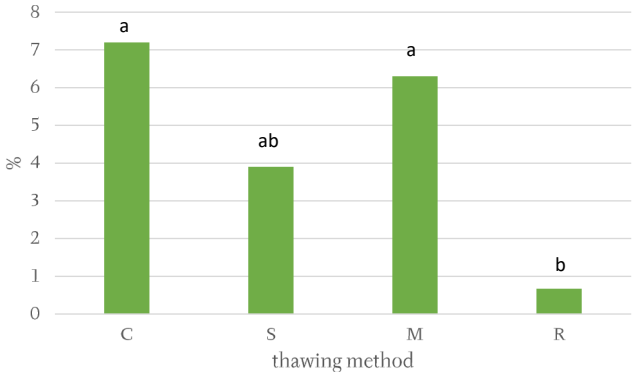
1: *Total spermatozoa motility decrease during incubation based on thawing method*
C = thawing in 38.5 °C water bath for 30 s; S = thawing in 30 °C water bath for 50 s; M = thawing in 50 °C water bath for 15 s; R = thawing in 70 °C water bath for 3 s; Different letters within columns mean significance (a,b = P < 0.05)



2: *Progressive spermatozoa motility decrease during incubation based on thawing method*
C = thawing in 38.5 °C water bath for 30 s; S = thawing in 30 °C water bath for 50 s; M = thawing in 50 °C water bath for 15 s; R = thawing in 70 °C water bath for 3 s; Total0-2= decrease of total spermatozoa motility during 2 hrs. of incubation; Prog0-2= decrease of progressive spermatozoa motility during 2 hrs. of incubation Different letters within columns mean significance (a,b = P < 0.05)



3: *VSL decrease during incubation based on thawing method*
C = thawing in 38.5 °C water bath for 30 s; S = thawing in 30 °C water bath for 50 s; M = thawing in 50 °C water bath for 15 s; R = thawing in 70 °C water bath for 3 s; Different letters within columns mean significance (a,b,c = P < 0.05)



4: *LIN decrease during incubation based on thawing method*
C = thawing in 38.5 °C water bath for 30 s; S = thawing in 30 °C water bath for 50 s; M = thawing in 50 °C water bath for 15 s; R = thawing in 70 °C water bath for 3 s; Different letters within columns mean significance (a,b = $P < 0.05$)

CONCLUSION

Higher progressive motility and spermatozoa movement characteristics immediately after the thawing and after 2 hours of incubation were detected in slow thawing method (30 °C, 50 s). The lowest spermatozoa motility was recorded in rapid thawing after 2 hrs. of incubation. This method is difficult to perform in field conditions and therefore could not be recommended for artificial insemination. Contrary, methods using lower thawing temperatures were more stable during the incubation and usually easier to perform in field conditions. Therefore they were assessed as more appropriate for artificial insemination. However, the modified thawing methods also could be tested for potential application for the specific purposes of in vitro manipulation. Also evaluation of semen characteristics during incubation period longer than 2 hours should be other aim of further research.

Acknowledgments

This study was supported by S grant of MEYS of the Czech Republic and NAZV [No.QJ1210109].

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Contact information

Martina Doležalová: dolezalovamartina@af.czu.cz
Martin Ptáček: ptacekm@af.czu.cz
Luděk Stádník: stadnik@af.czu.cz
Jaromír Ducháček: duchacek@af.czu.cz