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Dedicated to the blessed memory of Gennady V. Eskin

OPTIMAL MODE TO THAW CRYOPRESERVED SPERM OF HOLSTEIN BULL SIRES

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Abstract

As milk yielding increases, the number of animals with defective sexual cycles and quiet manifestations of hunting grows, and therefore there is a need for semen with high quality. Also, at artificial insemination, after dilution of the native semen, packaging, cryopreservation and thawing, the number of spermatozoa per sperm dose decreases. This also necessitates semen with high quality parameters. One of the key stages of the standard processing of cryopreserved bovine semen prior to artificial insemination is the thawing procedure. Therefore, cryopreservation and thawing procedures should be optimized with regard to the proposed modifications of these methods and the changing reproductive abilities of animals. In our study, we compared the effects of previously recommended thawing protocols and those proposed by us on the safety of spermatozoa of Russian Holstein sires and identified the modes that reliably provide a prolonged positive effect on the quality characteristics of spermatozoa and better preservation of their viability after thawing. The semen of 6-7 year-old Holstein bulls was cryopreserved in polypropylene straws (72 doses) with the use of the IMV Technology equipment (France) and in uncoated pellets (90 doses) on the dry ice plates according to GOST State Standard 26030-2015 and the National Technology for Freezing and Handling Semen of Pedigree Bull Sires. Thus, 162 semen doses 0.25 ml each were analyzed. The sperm motility and movement velocity parameters were assessed immediately and after in vitro post-thawed incubation for 5 to 24 hours at 38 °C. The percentage of motile semen was visually assessed with the Olympus microscope CX41 (Japan) at ×150 magnification. Thereafter, in the same samples the motility (%), the number of the highly motile sperm (%), and the sperm velocity (mcm/sec) were automatically measured with the SFA-500 Sperm Analyzer (Biola Company, Ltd, Russia). The dynamic of thawing temperature up to the final value was measured with the Center 304 thermocouple. It has been ascertained that the effects of different procedures used for thawing straws and pellets are quite steady and approximately the same. However, the best results of the male sex cell survival after the long in-vitro incubation period (for 24 hours at the animal body temperature of 38 °C) are in the samples thawed in a water bath at 38 °C for 10 sec when compared to those for the other methods ($p < 0.001$).

Keywords: Holsteins, sperm, thawing procedure, motility, sperm velocity, straws, uncoated pellets

For more than 70-year history of cryopreservation for male germ cells, the method has been widely used both in research programs on biodiversity conservation [1] and for commercial purposes in the creation of sperm banks of different animal species [2] and human [3]. Such a long history of the issue allows researchers to discuss not only the achievements but also the risks of artificial insemination [4, 5]. It is believed that the increase in milk production has a neg-

ative impact on reproduction; in particular, the decrease in the number of calving from the first insemination is noted [6]. The main reason under consideration is the mismatch of energy consumption [7]. As a result, a number of animals with defective sexual cycles and quiet manifestations of hunting, which complicates the determination of the time of insemination, is increasing. In this regard, the need for semen with high qualitative and quantitative characteristics, capable to stay in the female genital tract for a long time (more than 24 hours) without loss of fertilizing ability arises. At the same time, in artificial insemination after dilution of the native semen, packaging, cryopreservation and thawing, the number of spermatozoa decreases, which also significantly increases the requirements for their quality.

The main structural damage to the sperm of bulls and stallions is associated with freezing and subsequent thawing [8-10] and provoked by the phase transition of water into ice and back [11, 12]. The regimes of thawing temperature and speed are crucial for maintaining the integrity of membranes and fertilizing ability of sperm cells [13, 14]. It is known that the formation of intracellular ice damaging the cell includes emergence of crystallization centers and crystal growth. With gradual and slow cooling, the number of crystallization centers is small, but conditions for their growth are favorable. By increasing the cooling rate, the optimum crystal growth temperature can be avoided without damaging cells. Thawing requires the fastest possible temperature rise to prevent secondary growth of ice crystals inside the cells. A particularly vulnerable point in the process of thawing is significant recrystallization of ice (especially inside the cells) at –40 °C. The formation of the ice centers inside cells can lead to ice crystal growth during thawing [15]. To prevent secondary recrystallization, the object to be thawed must pass the zone of ice crystal secondary growth as quickly as possible. Increasing the thawing rate increases the number of viable cells [16].

The simplest and most effective method of thawing cryopreserved sperm is thawing in a water bath (at 37-40 °C according to different protocols) with stirring to prevent local temperature gradients in the water. The higher the temperature of the water bath, the shorter the pause in the rise of temperature in the thawed object after passing –20 °C [17]. Rapid warming-up can increase the survival of cryopreserved cells by reducing the time of ice crystal re-growth and/or the membrane reorganization. In the experiments by F.I. Ostashko [17], in coated semen doses after thawing in running boiling water, the spermatozoa had motility and fertilizing ability, respectively, 7 and 15 % higher than when thawing in the traditional way.

Structural and metabolic disorders in sperms during freezing and thawing are accompanied by damages to the proximal centrioles, mitochondria, and fibrils. The most expressed changes in the ultrastructure of sperm cells identified in acrosomes and mitochondria, slight structural and metabolic changes in the activity of hyaluronidase, acrosin, aspartate aminotransferase, and respiration occur at temperatures of about 4-6 °C [18, 19].

In the routine practice of breeding enterprises, the use of complex tests based on the determination of ultrastructural damage of sperm is difficult [20-24]. For this purpose, the technologies of automated sperm differentiation by the functional state are proposed, in particular, complex computer analysis of sperm with of exposed microscopy of moving spermatozoa (CASA) [25]. The method for measuring the concentration, total motility, number of actively mobile (fertile) spermatozoa and the average speed of their movement by laser analysis of frequency spectrum of optical density fluctuations at spermatozoa movement through an optical channel [26] is well proved. This method is adapted for practical pur-

poses in Russian breeding enterprises [8, 27].

There is still no consensus on the effects of water bath temperature and time of thawing on the preservation of the functional characteristics of thawed spermatozoa. Many papers are devoted to the problem [28-30], but in most of them, information about the optimal conditions of semen dose thawing is quite contradictory, and these temperatures range from 20 to 75 °C [28, 31-33]. In Russia, in accordance with the national technology, the method of thawing in a water bath at 38 °C for 10 s is widely accepted. In the present work, we for the first time compare different modes of thawing of semen with fixing the end temperature and on this basis chose the optimum, making it possible to maintain prolonged viability of the thawed sperm in 24-hour incubation at 38 °C.

The work objective was to compare different deconservation modes (i.e. existing and proposed by us) for sperm cryopreserved in polypropylene straws and uncoated pellets, and the dynamics of sperm qualitative characteristics (motility, the speed of movement, survivability) during subsequent incubation in vitro at 38 °C.

Techniques. Sperm obtained from Holstein bull sires aged 3-7 years was cryopreserved in polypropylene straws (72 doses) on equipment of IMV company (model IS4, France) and uncoated pellets (90 doses) on the plates of dry ice according to GOST 26030-2015 and the National Technology of Freezing and Use of Semen of Breeding Bull Sires.

When thawing, the sealed tip of straws taken from the water bath was cut off, the temperature of the internal content was measured with a thermocouple Center 304 (Center Technology Corp., Taiwan), the melted semen dose (0.23 ml) was extruded, by pressing the piston on polypropylene tube, into penicillin vial with 0.8 ml of diluent OptiXcell (IMV, France) and heated to 30 °C. The following defrost modes were used: 35 °C, 30 s; 38 °C, 10 s (control); 50 °C, 5 s; 70 °C, 3 s (18 doses per option). When thawing the semen frozen in uncoated pellets, they were quickly taken one by one from the tube with sterile tweezers pre-cooled in liquid nitrogen and placed in penicillin vials with 0.8 ml of OptiXcell diluent or with 2.9 % sodium citrate. In the dry thawing option, the pellets were transferred into clean, dry vials.

Immediately after thawing and 5, 20, and 24 h after incubation in vitro at 38 °C, motility (%) was assessed visually using a microscope (CX41 model, Olympus, Japan; ×150 magnification). The motility (%), pool of fast sperm cells (%) and the average speed of sperm cells (μm/s) were measured in the same samples using a SFA-500 device (NPO Biola, Russia) with a laser analyzer of the frequency spectrum of optical density fluctuations caused by the movement of sperm through an optical channel with special characteristics. Temperature of semen thawing was controlled in dynamics with a thermocouple Center 304 (Center Technology Corp., Taiwan). The percentage was calculated of motile and fast-moving sperm, as well as the number and speed of spermatozoa with straight-forward motion (SFM) at different incubation periods.

The data were processed by the variation statistics methods; the tables show the mean values (M) for each series of the experiment and the standard errors of means (±SEM). The significance of differences was assessed by Student's t-criterion. Differences were considered statistically significant at $p < 0.05$.

Results. Thawing of semen in uncoated pellets was carried out under different protocols (Table 1).

Straws with the semen of one batch were simultaneously thawed in water baths in the specified modes; the temperature inside the straws was measured immediately after thawing. In options, the temperature of the semen was

26.0; 25.3; 26.0; 243 °C (average for all modes 25.0±0.4 °C, the differences statistically insignificant). Therefore, regardless of the mode of deconservation, the final temperature of thawed sperm was almost the same.

1. Modes of thawing of uncoated pellets with cryopreserved sperm of Holstein bulls

Diluent buffer	Diluent temperature, °C	Time, s
In a water bath (40 °C)		
OptiXcell diluent (France)	38	10
Sodium citrate (2.9 %)	38	10
Dry thawing		Up to full thawing
Sodium citrate (2.9 %)	4	12-13
OptiXcell diluent (France)	4	12-13
At room temperature (23 °C)		
OptiXcell diluent (France)	23	Up to full thawing
Sodium citrate 2.9 %	23	Up to full thawing
Dry thawing		Up to full thawing
In the refrigerator (4 °C)		
Dry thawing		Up to full thawing

The compared thawing protocols did not impact significantly the number of sperm cells with SFM (fluctuations of 35-38 %), which was confirmed with SFA-500 analyzer (34.4-36.5 % of cells, the differences are insignificant) (Table 2). This is consistent with the data of other authors for semen of Buffalo [28, 31], cattle [29], and zebu [31]. Similar results were obtained for fast-moving sperm (variations from 23.8 to 24.2 %, the differences are insignificant). The highest speed of sperm movement was in samples thawed at 35 °C, and it decreased during deconservation with increasing temperature of water bath (at the maximum temperature the differences were significant, $p < 0.001$). During thawing of cryopreserved Buffalo sperm, the maximum speed was at + 70 °C [28].

2. Motility and the proportion (%) of mobile spermatozoa in semen of Holstein bulls evaluated by two methods immediately after thawing of 72 doses by different modes (OAO "Reproduction Head Center of Agricultural Animals", the Moscow Region, 2018)

Thawing temperature, °C	Microscopy	SFA-500 analyzer		
	motile spermatozoa, %	motile spermatozoa, %		average rate, μm/s
		slow + fast	fast	
0 h incubation in vitro, 38 °C				
35	35.0±0.90	34.4±0.09*	23.9±0.30	100.7±1.2
38 (control)	38.3±0.53	35.1±0.30	23.7±0.20	97.8±1.03
50	36.6±0.53*	36.5±0.76	24.2±0.40	96.3±0.6
70	35.0±0.90**	34.4±0.70	23.8±0.30	91.3±0.16***
5 h incubation in vitro, 38 °C				
35	30.0±0.90	38.4±0.70	27.8±0.20*	97.8±2.30
38 (control)	30.0±0.93	37.8±0.13	28.7±0.40	102.8±1.70
50	30.0±0.93	34.5±0.66***	26.2±0.20***	102.0±1.70
70	28.3±1.40	38.9±0.20***	27.3±0.20**	96.6±1.36**
20 h incubation in vitro, 38 °C				
35	20.0±0.00***	24.0±0.5**	15.1±0.30***	81.6±0.30
38 (control)	23.3±0.53	26.3±0.56	16.7±0.05	81.6±1.30
50	20.0±0.09***	19.8±0.26***	12.8±0.33***	76.9±1.50*
70	18.3±0.50***	19.8±0.60***	12.5±0.43***	79.1±0.30
24 h incubation in vitro, 38 °C				
35	10.0±1.70***	11.4±1.60***	5.8±0.80***	71.0±0.30***
38 (control)	15.0±0.00	18.4±0.4	11.4±0.46	79.3±1.36
50	8.3±0.53***	10.7±0.7***	4.9±0.6***	62.7±2.70***
70	5.6±0.70***	8.2±0.93***	4.1±0.43***	73.1±0.86***

*, **, *** Differences with control are statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

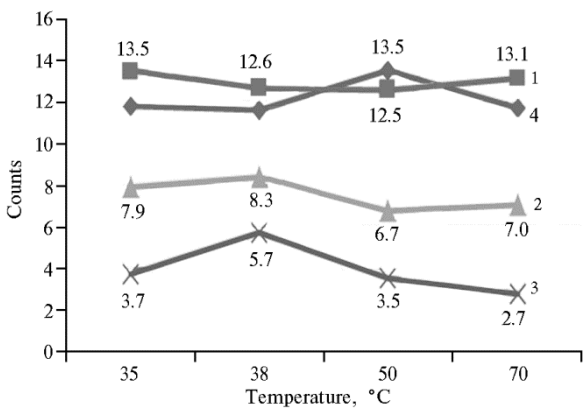
After 5 h incubation in vitro (see Table 2), there was a discrepancy in the results of the motility estimates: visually 28.3-30.0 % of spermatozoa were motile, whereas computer analysis detected more motile gametes (by 4-10 %). In addition, sperm analyzer recorded an increase in motility compared to that immediately after thawing. Similar data were obtained after 2-hour incubation

[29]. Number of such cells was significantly higher (38.9 %) under rapid deconservation (70 °C) and significantly lower when cells were thawed at 50 °C.

Note, the share of fast-moving (fertile) sperm (28.7 %, an average speed of 102.8 μm/s) was significantly higher for the standard thawing technology. The differences between the control and experimental groups were statistically significant in all cases. Probably, this can be explained by the fact that visually sperm cells with SFM are predominantly recorded, whereas a sperm analyzer records all the spermatozoa moving in different trajectories with a speed greater than 25 μm/s. This minimum speed threshold is set to cut off trim cells that do not have their own motility and move with the flow of the fluid [8]. After 20 hours of incubation (see Table 2), the number of spermatozoa with SFM decreased by 10 % on average, with the average speed 20 % lower as compared to that after 5 hours of incubation.

It should be noted that spermatozoa, deconserved by the standard method, preserved more cells with SFM. This trend was observed both in routine microscopy and in the automatic examination (the observed differences are statistically significant). One day after thawing and incubation in vitro at 38 °C (see Table 2), the best preservation (18.1 % of motile sperm) was also characteristic of semen deconserved in a water bath at 38 °C by the standard procedure (the differences with samples in other modes were from 6.7 to 10.0 %, being statistically significant at $p < 0.001$).

The results show that deviations from the conventional method of semen thawing reduce its qualitative characteristics and fertilizing ability. This underscores the need for strict compliance with established regulations. In several publications, it is also reported that the best results are obtained by thawing sperm doses in the range of temperatures from 37 to 40 °C [30, 31]. Strict adherence to the method of sperm thawing can improve the effectiveness of artificial insemination, especially in late ovulation (more than 24 hours after identified estrus; the number of such animals among highly productive individuals is more than 50 %) [27].



Sperm (×10^{6}cell per semen dose) **with a straight-forward movement depending on the temperature of defrosting and different periods of subsequent incubation**: 1 — 5 h, 2 — 20 h, 3 — 24 h, 4 — no incubation (Holstein bulls, OAO Reproduction Head Center of Agricultural Animals, the Moscow region, 2018).

Daily dynamics of cell activity, expressed in absolute values (Fig.), shows that, the number of motile sperms immediately after thawing averaged 33-35 million, and about a third of them are spermatozoa with SFM (when counting sperms with speed below 25 μm/s). After 5 h of incubation in all groups, the number of sperm cells with SFM became 8-12 % higher (except semen thawed at 50 °C). Perhaps, the observed increase is due to the beginning of hyperactivation processes.

It is known that freezing and thawing trigger spontaneous capacitation. It is believed that sperm capacitation is thermally dependent, and the blocking effect is overcome when the sperm returns to a temperature of 37 °C [32]. The key factor here is the partial cryopreservation of the cell membrane, in particular, in the area of the acrosomal cover, resulting in changes in intracellular calcium con-

centration [34]. Even though in cattle sperm motility correlates with fertilizing ability and can be successfully used for its rapid testing, in practice, it is necessary to control the life expectancy of germ cells outside the body.

With increasing incubation period (up to 20 and 24 h), the greatest number of SFM spermatozoa (respectively 8.3 and 5.7 million) remained in samples defrosted in a water bath at 38 °C for 10 s. After 24 h, in samples thawed in a water bath at other temperatures, the number of SFM cells was 2-3 million less (see Fig.). This should be taken into account when inseminating, as in the female genital tract sperm accumulates in a special reservoir of the oviduct, attached to its walls without losing the fertilizing ability during a day, provided its functional integrity is preserved [33].

In experiments with uncoated pellets, the standard conditions of deconservation were changed. According to the approved protocol in Russia, the dose of the semen should be placed in a bottle with 1 ml of 2.9 % sodium citrate, preheated to 38 °C and thawed at 40 °C for 8-10 s to a thin ice rod. We carried out thawing outside the water bath at room temperature of 23 °C, and also used a 40 °C water bath but citrate buffer pre-cooled to 4 °C. Optimal results of pellet thawing were in using a water bath (Table 3). Moreover, the initial indicators of the semen were slightly higher in all variants with chilled citrate. However, after 5 hours of incubation, active sperm was 11.6 % more when defrosting in citrate buffer warmed to 38 °C.

The final thawing temperature determined by thermocouple in the germ cell suspension during thawing in a water bath in both cases was the same and on the average reached about 28 °C with a slight increase in thawing time when using a cooled buffer. Upon defrosting pellets in citrate buffer at room temperature (23 °C), the thawing time increases, the final temperature of the suspension significantly ($p < 0.05$) reduces to 13.9 °C, and the viability of sperm significantly deteriorates compared to that in using water bath (40 °C). Ultimately, this will reduce the effectiveness of artificial insemination.

3. Quality of semen cryopreserved in the uncoated pellets after thawing in a water bath with different diluters and incubation (0 h and 5 h) (Holstein bulls, OAO Reproduction Head Center of Agricultural Animals, the Moscow Region, 2018)

Microscopy		SFA-500 analyzer			Thawing time, s	Semen dose temperature, °C
motile spermatozoa, %		motile spermatozoa, %		average rate, μm/s		
0 ч	5 ч	slow + fast	fast			
Sodium citrate (2.9%)						
Control (1 ml diluent, t = 38 °C, t of defrosting 40 °C)						
36.6±1.60	28.3±1.60	36.0±2.30	21.6±0.90	84.3±2.30	10.0±0.00	28.5±4.20
Group I (1 ml diluent, t = 23 °C, without a water bath)						
33.3±3.30	16.6±4.40*	29.3±1.00*	18.8±1.20	96.6±4.90*	23.3±2.00***	13.9±4.50*
Group II (1 ml diluent, t = 4 °C, t of defrosting 40 °C)						
41.6±1.60*	25.0±2.80	47.3±2.80*	23.1±2.50	73.2±9.90	13.6±1.20	28.3±0.30
OptiXcell synthetic diluent (France)						
Control (1 ml diluent, t = 38 °C, t of defrosting 40 °C)						
31.6±4.40	30.0±5.00	40.5±3.50	23.6±3.10	84.3±6.50	11.3±1.30	28.6±0.80
Group III (1 ml diluent, t = 23 °C, without a water bath)						
35.1±0.10	30.0±2.80	48.8±8.10	28.2±2.30	86.8±5.00	14.6±0.60*	18.3±0.60**
Group IV (1 ml diluent, t = 4 °C, t of defrosting 40 °C)						
36.6±1.60	26.6±3.30	38.0±1.50	22.0±0.20	83.9±3.20	12.0±1.00	29.3±1.30

*, **, *** Differences with control are statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Currently in Russia, breeding enterprises predominantly use imported synthetic diluents for semen. In the current experiment, when replacing the citrate buffer with the OptiXcell synthetic medium, defrosting of uncoated pellets gave ambiguous results that require a further study (see Table 3). As in the citrate buffer variant, immediately after deconservation in a water bath (40 °C), sperm motility was higher in the diluent cooled to 4 °C, but after 5-hour incuba-

tion of active sperm, it was 11.3 % higher in use of initially warm (38 °C) diluent. In this case, the final temperature of the resulting suspension of germ cells and the time of thawing of the sperm did not differ from those for the citrate buffer. Several different patterns emerged at deconservation of uncoated pellets in the synthetic diluent at room temperature, i.e. the thawing was 8.7 s faster, and final temperature of samples increased to 4.4 °C compared to that for citrate buffer. The motility and viability of spermatozoa in the group, where sperm were thawed at room temperature with the OptiXcell synthetic diluent, tended to improve (with not confirmed statistically significant differences). The obtained data indicate the need to study the mechanisms of action of this synthetic diluent during freezing/thawing. It is assumed that the proteins of the seminal plasma bind to the fraction of low density of the egg yolk, which is part of the medium, and this is crucial in the preservation of the cell membrane of sperm [35, 36].

In the experiment with dry thawing (without diluent), a directly proportional relationship was shown between sperm motility and ambient temperature (Table 4). Thus, the best results were obtained at 40 °C (control): 45 % of spermatozoa had SFM immediately after thawing, and 30 % remained viable after 5 h of incubation at 38 °C. With increasing thawing time and decreasing ambient temperature, the proportion of viable spermatozoa decreased: at room temperature after 5 h the survival rate was 21 % compared to 66 % in the first option. With slow deconservation (4 °C), thawing time increased sharply (up to 820 s), which had a detrimental effect on sperm viability: only 6.7 % of the sperm cells retained SFM right after thawing, and only single cells were SFM after 5 h.

4. Quality of semen cryopreserved in uncoated pellets after dry thawing (without diluters) **and incubation** (0 h and 5 h) (Holstein bulls, OAO Reproduction Head Center of Agricultural Animals, the Moscow Region, 2018)

Thawing mode	Motile spermatozoa, %		Thawing time, s	Semen dose temperature, °C
	0 h	5 h		
In a water bath at 40 °C (control)	45.0±2.80	30.0±0.00	56.0±3.05	27.7±8.20
At room temperature 23 °C	23.3±6.00**	5.0±0.00***	222.3±76.00*	12.2±5.14
In the refrigerator at 4 °C	6.7±1.60***	1.7±1.60***	820.0±40.00***	15.2±1.80

*, **, *** Differences with control are statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Table 4 does not contain the data obtained with the SFA-500 analyzer: the characteristics of the device indicate the limits of sperm concentration (5-100 million/ml) for qualitative and quantitative analysis of the sample with an acceptable error; beyond these values, the error increases sharply. In the current experiment, in dry thawing, concentration in a sperm dose increased to 160-190 million/ml, which made the interpretation of the results impossible (additional use of media and buffers to dilute the semen would distort the original effect).

Thus, deconservation of semen packed in straws and uncoated pellets, is applicable worldwide and constantly developing. In the current experiment, the best results with bovine sperm were achieved by thawing for 10 s in a water bath at 38 °C for straws and 40 °C for uncoated pellets. These protocols reliably provide a prolonged positive effect on sperm quality and better preservation of its viability after thawing than other methods ($p < 0.001$). Our results also show that one of the modern modifications of these procedures, namely the use of synthetic yolk-free diluents, requires an in-depth study of the effect of this media on sperm quality of different animal species to clarify the existing protocols of deconservation. The obtained data confirm the well-known fact that even small deviations from the optimal conditions can significantly worsen the properties of sperm and ultimately reduce the effectiveness of artificial insemination. In some cases, the effects of modifications are ambiguous, which indicates a possible complex mechanism of influence of the agents used on the characteristics of

sperm cells and, as a consequence, on artificial insemination.

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