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Effect of Different Thawing Procedures on the Quality and Fertility of the Bull Spermatozoa

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Abstract: The aim of this research was to improve the indicators of motility, survival and fertilizing ability of spermatozoa by optimizing temperature factors and the duration of exposure at unfreezing straws. Straws by volume 0.25 mL were thawed at water bath temperatures at 65, 67 and 70°C for 6-7 sec and at 75°C for 4-6 sec. Impact of exposure time and temperature thawing in the water bath on motility and survival of spermatozoa were studied. Studies indicate that for the procedure of defrost water bath straws in 7 sec for temperature conditions of 65, 67 and 70°C, indicators of progressive motility and absolute survival rate were significantly higher than for the control group an average on 11.4% ($p<0.01$). Optimum exposure time (6-7 sec) and temperature range (65-70°C) defrosting semen doses were defined. Owing obtained the positive result, method of thawing was developed which increases the indicators of motility, survival and fertilizing capacity of bull semen.

Key words: Progressive motility, viability, thawing rate, temperature of defrosting, fertilizing capacity

INTRODUCTION

Frozen semen in straws has become the universally accepted unit of storage and transfer of bovine genetics to cattle procedures which depends on preserve the functional activity of spermatozoa (viability and fertilizing ability) (Bearden *et al*., 2004). High viability and motility of spermatozoa are important factors for successful Artificial Insemination (AI) because a significant correlation between post-thawing sperm viability and subsequent conception rate has been reported (Correa *et al*.,1996). The freezing and thawing of semen inevitably reduces the proportion of motile spermatozoa and causes ultra structural, biochemical and functional damages (Senger, 1980). It has been shown that an increase in post-thaw viability will result in increased fertility of the semen (Rastegarnia *et al*., 2013). Thawing procedure is just as important as the freezing procedure in terms of its impact on the survival of spermatozoa (Nur *et al*., 2003). Defrosting of sperm should be at maximum speed. Increasing the speed of thawing frozen semen increases the number of sperm that restore maximum motility (Smirnov, 1982). The rapid thawing of semen decreases the harmful effects of recrystallization processes and hydration, preventing damage to sperm membrane and cytoplasm. In this case, when passing through the temperature danger zone (-50 to -30 and -30 to 0°C) ice crystals do not have time to be formed and sperm switches directly from the glassy state to the liquid state (Ostashko, 1978; Marshall, 1984; Smirnov, 1982; Bearden *et al*., 2004). Various factors of interaction with thawing procedures which affect the post thawing motility of sperm such as type of extender, concentration of glycerol, method of semen packing, cooling rate, semen handling during cryopreservation procedure (Rodriguez *et al*., 1975; Robbins *et al*., 1976) and experimental conditions such as available facilities, tools and chemicals, vary among countries and areas (Vishwanath and Shannon, 2000; Thibier and Wagner, 2002). Thus, the methods of freezing and thawing frozen spermatozoa should be examined in each country and area (Hayashi and Isobe, 2005). Many researches have been conducted to determine the optimal thawing temperature, duration and increased to know the adequate thawing rate that may give highest percentage of viable spermatozoa after post thawing process (Rodriguez *et al*., 1975; Senger, 1980; Pace *et al*., 1981; Dhami and Sahni, 1993; Correa *et al*., 1996; Nur *et al*., 2003). However, a number of studies have shown that thawing temperatures as high as 60-80°C could further improve post-thaw motility (Senger, 1980; Ahmad, 1984; Narasimha Rao *et al*., 1986; Dhami *et al*., 1996). In some countries, pellets of sperm thawed at +55°C . Some researchers propose to use for thawing frozen semen of bulls higher temperatures from +50 to +75°C or even 100-150°C (Bugrov, 1978; Ostashko, 1978). Many studies have been conducted to assess the

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influence of high thawing temperatures on sperm survival and motility, using different thawing rates for bulls (Rugg *et al*., 1977; Chandler *et al*., 1984; Nur *et al*., 2003; Al-Badry, 2012; Rastegarnia *et al*., 2013), boars, rams and dogs (Eriksson and Rodriguez-Mortinez, 2000; Paulenz *et al*., 2004; Pena and Linde-Forsberg, 2000).

Improving defrost modes of sperm bulls and improve sperm quality indicators are relevant and has important theoretical and practical significance. The aim of this research was to improve the performance of motility, survival and fertilizing ability of spermatozoa by optimizing temperature factors and the duration of exposure at unfreezing straws. This study was consisted of two stages: to determine the optimum thawing procedures in order to know the adequate thawing rate that may give highest percentage motility and viability of spermatozoa after thaw process and to evaluate the relationship between this technique of thawing and fertilizing ability of spermatozoa.

MATERIALS AND METHODS

Studies were performed using cryopreserved of sperm bulls, frozen in French straws by volume 0.25 mL, in the laboratory of the company of "Progress" (Ukraine).

Thawing procedures: Straws by volume 0.25 mL were thawed at water bath temperatures at 65, 67 and 70°C for 6-7 sec and at 75°C for 4-6 sec. Under instructions from AI of cattle, straws by volume at 0.25 mL are recommended to thaw in a water bath at 35°C for 20 sec was used as the control.

Semen evaluation: Immediately after thawing, the content of each straw was emptied in a 2 mL Eppendorf tube at 38°C . The sperm suspension was incubated at 38 °C and was evaluated for post thaw motility indicators and viability of spermatozoa through every hour to cell death. For AI 295 heads of Ukrainian red-white dairy cattle breeding in the farm "RVD-Agro" (Ukraine) were used. Straws were thawed immediately before insemination at 65-70°C for 6-7 sec. Fertilization in control group was conducted after thawing sperm in a water bath at 35°C for 20 sec.

Motility: Indicators of progressive motility and the dynamic characteristics of sperm movement were assessed with a computer-assisted sperm motility analyzer (Sperm Vision) (CASA) and microscope Olympus CX-31. The dynamic characteristics and progressive motility were analyzed immediately after thawing (0 h) and every hour of incubation at 38°C. Then the absolute Survival Rate of Spermatozoa (ASR) was calculated. Among the dynamic characteristics of sperm movement mean Velocity (VAP, μm/sec), straight-line velocity (VSL, μm/sec), the average distance of movement (DAP, μm/sec) and distance in a straight line (DSL, μm/sec) were studied (Mortimer, 2000).

Statistical analyses: Materials of researches were calculated by methods of Mathematical Statistics Means of the Software Package (Statistica).

RESULTS

Motility: Studies indicate that for the procedure of defrost water bath straws in seven sec at temperature conditions of 65°C, indicators of Progressive Motility (PM) were significantly higher than for the control group by 5.4% ($p<0.05$). At the same time, the indicators of PM obtained with 6 sec exposure were lower than for the control group by 2.6% ($p>0.05$) (Table 1).

It was found that by the thaw rate straws in 6 and 7 sec for temperature at 67°C, PM values were significantly higher than for the control group by 5.4 and 6.5% ($p<0.01$), accordingly. The procedure of the thawing straws 6 and 7 sec for the temperature regime of 70°C, PM values were significantly higher than the control group by 5.1 and 10.5%, accordingly ($p<0.01$) (Table 1).

At temperature of 75°C and the procedure of thawing straws 4-6 sec indicators of progressive motility and absolute survival rate were lower and not statistically significant ($p>0.05$).

Parameters of dynamic characteristics movement of spermatozoa are presented in Table 2. It is data obtained

Table 1: Indicators of progressive motility spermatozoa for temperature by thawing 65-70°C, after thawing frozen semen (0 h of incubation) (M±SE, %)

Temperature defrost (°C)	n	Thawing rate in a water bath (sec) (control) 6	7	20
35 (control)	90	-	-	62.7±0.8
65	20	65.3±2.3	68.1±2.1[a]	-
67	60	68.1±1.4[b]	69.2±1.4[c]	-
70	60	67.8±2.2[a]	73.2±2.1[a]	-

[a]$p<0.05$; [b]$p<0.01$; [c]$p<0.001$ levels significantly to control

Table 2: Dynamic motion characteristics frozen-thawed of sperm bulls by temperature 65-70 °C and thawing rate 6-7 sec (M±SE)

Indication	35°C (20 sec) (control)	65 (7 sec)	67 (7 sec)	70 (6 sec)
The average distance of movement (DAP) (μm)	26.3±0.5	26.3±0.8	27.5±0.5	27.1±0.9
Distance in a Straight Line (DSL) (μm)	20.5±0.4	20.3±0.8	21.2±0.5	20.7±0.9
Average Path Velocity (VAP) (μm sec^{-1})	59.2±0.9	59.5±2.0	62.4±1.3[a]	62.0±2.3
Straight-line Velocity (VSL) (μm sec^{-1})	46.3±0.9	45.9±1.9	48.2±1.2	47.2±1.2

average distance of movement was higher than for the control group on 1.2 μm ($p>0.05$) and straight-line velocity of sperm higher on 1.9 μm sec^{-1} ($p>0.05$). At the same time, average path velocity of the sperm was significantly higher for the control on 3.2 μm sec^{-1} ($p<0.05$). Accordingly, at temperature of 65 and 70°C for the thawing rate 7 and 6 sec parameters of dynamic characteristics were not statistically significant ($p>0.05$) (Table 2).

Viability: For the duration of the defrost straws in a water bath for 7 sec at temperature regime of 65°C progressive motility spermatozoa after incubation 3 and 5 h at 38°C was significantly higher than in the control group at 9.7% ($p<0.05$) and on 16.9% ($p<0.01$), respectively (Fig. 1).

Found that at the duration of defrost straws for 7 sec at temperature regime 67°C progressive motility after incubation 1, 3 and 5 h at 38°C was significantly higher than in the control group by an average of 5.6% ($p<0.05$). It should be noted that for the thawing rates of straws seven sec for temperature regime of 70°C, PM was significantly higher than in the control group by an average of 8.3% ($p<0.05$) (Fig. 1). For the control temperature the survival rate is 6 h (lim 5-7 h) and for temperatures 65, 67 and 70°C with thawing rate 7 sec are 8 h (lim 6-8 h) that prolonged survival rate of spermatozoa by 2 h. For temperature regime of 65°C and duration of the defrost straws in a water bath for 6 sec, the Absolute Survival Rate (ASR) after thawing was significantly higher than in the control group on 16.0% ($p<0.05$) and an exposure of 7 sec on 22.4% ($p<0.001$) (Fig. 2).

For duration of defrost straws 6 and 7 sec for temperature regime 67°C, ASR was significantly higher than in the control group by an average of 11.0% ($p<0.01$). Respectively, for the thawing rate straws 6 and 7 sec for temperature regime of 70°C, ASR values were higher than in the control group on 10.7% ($p<0.05$) and on 16.5% ($p<0.001$) (Fig. 2).

Artificial insemination: Based on these results a method has been proposed to improve the quality of thawed semen. The method was used for artificial insemination of cow's cattle livestock. Insemination of cows were conducted by the way of thawing straws in a water bath at the temperature range 65-70°C and the duration of exposure 6-7 sec. The fertilization of cows compared with the control group were higher at 11.6% ($p>0.05$) (Table 3).

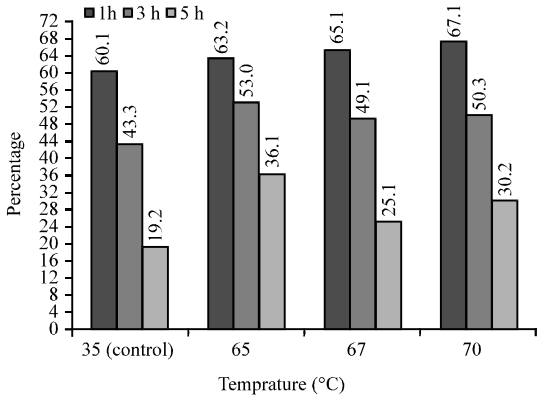

Fig. 1: Effect of thawing temperature on motility of sperm during incubation

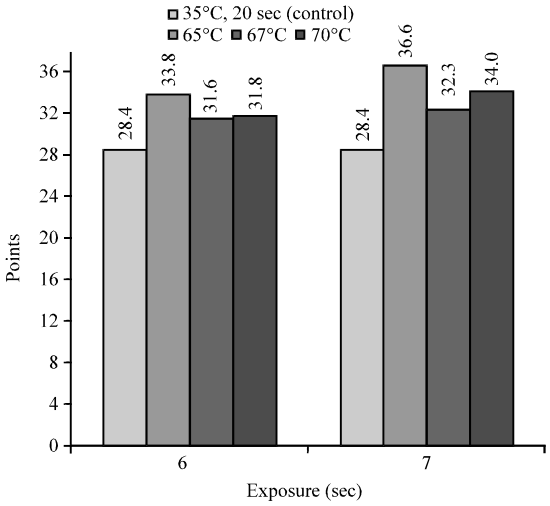

Fig. 2: Absolute survival rates of spermatozoa at temperatures 65-70°C

Table 3: Fertilization of cows by using the proposed method of defrosting straws

The method of defrosting straws	Inseminated cows (heads)	Fertilization after 1st insemination (heads)
Control (35°C, 20 sec)	145	77
Proposed method (65-70°C, 6-7 sec)	150	97

DISCUSSION

In the present study, the progressive motility, dynamic parameters and viability were significantly increased by thawing at 65-70°C for 6-7 sec, compared with the control temperature and thawing rate of 35°C for 20 sec.

The rapid thawing of semen decreases the harmful effects of recrystallization processes and hydration,

preventing damage to sperm membrane and cytoplasm (Watson, 1995; Holt, 2000; Sukhato *et al*., 2001). In this case, when passing through the temperature danger zone ice crystals do not have time to be formed and sperm switches directly from the glassy state to the liquid state (Ostashko, 1978; Smirnov, 1982). Overall, straws by volume 0.25 or 0.5 mL, should be thawed in a water bath at 35-38°C for 20-40 sec (Narasimha Rao *et al*., 1986). A practical thaw for bull spermatozoa is recommended by most AI organizations, is as 35°C water bath for at least 30 sec (Dhami *et al*., 1996; Nur *et al*., 2003; Hayashi and Isobe, 2005). However, a number of studies have shown that thawing temperatures as high as 60-80°C could further improve post-thaw motility (Senger, 1980; Ahmad, 1984; Narasimha Rao *et al*., 1986; Dhami *et al*., 1996). Many studies have been conducted to assess the influence of high thawing temperatures on sperm survival and motility, using different thawing rates for bulls (Rodriguez *et al*., 1975; Robbins *et al*., 1976; Rugg *et al*., 1977; Chandler *et al*., 1984; Nur *et al*., 2003; Al-Badry, 2012; Rastegarnia *et al*., 2013), boars, rams and dogs.

Thawing procedure of semen boars (Eriksson and Rodriguez-Martinez, 2000), rams (Paulenz *et al*., 2004) and dogs (Pena and Linde-Forsberg, 2000) was performed at 70°C for 8 sec that increased parameters motility and viability spermatozoa.

Defrosting of bull's semen was performed at 60°C for 8 sec (Al-Badry, 2012), at 65°C for 7and 5 sec (Robbins *et al*., 1976), at 70°C for 5-6 sec (Nur *et al*., 2003; Muino *et al*., 2008; Rastegarnia *et al*., 2013) and at 75°C for 7 sec (Rodriguez *et al*., 1975; Rugg *et al*., 1977; Chandler *et al*., 1984).

A variety of studies had evaluated a range of different thawing rates for buffalo bull semen frozen in straws. The positive correlation between sperm motility and thawing rate recorded in the present study is in line with Ahmad who generally concluded that the more rapid thawing rates result in better sperm motility and acrosomal integrity (Ahmad, 1984). For cryopreservation of buffalo spermatozoa in Tris-based extender, analyzed Narasimha Rao *et al*. (1986) tested two thawing rates (37°C for 30 sec and 75°C for 9 sec). They concluded that the best value for post-thaw motility was observed for semen thawed at 37°C for 30 sec. The effect of thawing rates (40°C for 60 sec, 60°C for 15 sec and 80°C for 5 sec) on post-thaw motility of buffalo spermatozoa cryopreserved in Trisbased extender. Has shown that thawing at 60°C for 15 sec yielded a higher sperm motility compared to other rates. In another study, Dhami *et al*. (1996) determined the thawing rates for buffalo semen.

The thawing rates investigated were 4°C for 5 min, 40°C for 1 min or 60°C for 15 sec. They concluded that thawing of semen at 60°C for 15 sec yielded high post-thawing spermatozoa recovery and longevity.

For the procedure of thawing straws during 6 and 7 sec for temperature conditions of 65, 67 and 70°C indicators of progressive motility were significantly higher compared with control group. These results cohere with reports by Robbins *et al*. (1976), Rugg *et al*. (1977), Nur *et al*. (2003) and Rastegarnia *et al*. (2013). Dynamic motion characteristics at temperature regime 67°C and exposure time for seven sec were higher compared with the control. Similar results were found in Rastegarnia *et al*. (2013). However, by another temperature defrost (65 and 70°C) of bull sperm observed no differences in dynamic motion characteristics.

For the duration of the defrost straws in a water bath for seven sec at temperature regime of 65, 67 and 70°C progressive motility spermatozoa after incubation 1, 3 and 5 h at 38°C were significantly higher than in the control group. These results were in similar with some results reported by some authors (Muino *et al*., 2008; Al-Badry, 2012; Rastegarnia *et al*., 2013). The absolute survival rate obtained in this study for temperature regime of 65, 67 and 70°C for thawing rate 6 and 7 sec was significantly higher compared to thawing at 35°C for 20 sec. High indicators of motility and viability increase fertility of the spermatozoa bull. Similar results were obtained by Rugg *et al*. (1977), Pace *et al*. (1981), Ahmad (1984), Narasimha Rao *et al*. (1986) and Dhami *et al*. (1996).

CONCLUSION

Studies indicate that for the procedure of defrost water bath straws in seven sec for temperature conditions of 65, 67 and 70°C, indicators of progressive motility and absolute survival rate were significantly higher than for the control group an average on 11.4%. The application of the proposed method of defrosting straws provides improved quality of thawed bull sperm and increases the fertilization of cows after insemination on 11.6%. Researchers recommend thawing straws, by volume in 0.25 mL, in a water bath at 65-70°C for 6-7 sec.

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