Effects of Diluent Component, Freezing Rate, Thawing Time and Thawing Temperature on Acrosome Morphology and Motility of Frozen-thawed Boar Sperm

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ABSTRACT : This study was carried out to obtain informations regarding the effect of N-acetyl-D-glucosamine in the LEY (lactose-egg yolk) diluent according to incubation time in 5 ml maxi-straw and the effects of freezing rate, thawing temperature and thawing time in the LEN (lactose-egg yolk and N-acetyl-D-glucosamine) diluent on acrosome morphology and motility of frozen-thawed boar sperm. The study showed that the LEN diluent was higher post-thaw NAR (normal apical ridge) acrosome than the LEY diluent for 0.5 h incubation at 37°C. However, there were no differences between the LEN and LEY diluents on post-thaw sperm motility according to incubation time. The straws frozen from 5.0 cm (20°C/min) to 17.0 cm (1°C/min) above the liquid nitrogen surface did not show any significant differences on post-thaw sperm motility. However, the straws frozen above 5.0 cm from the liquid nitrogen surface were higher NAR acrosome than those frozen above 17.0 cm. The post-thaw percentages of motile sperm and NAR acrosome were significantly higher ($p<0.05$) for the maxi-straws submerged for 40 or 45 sec in a 52°C water bath than for 30, 35, 50 or 55 sec. The mean sample temperatures of maxi-straws after 40 or 45 sec submersion were 20.7 or 26.4°C. In conclusion, the sample temperature of the thawed semen was very important for post-thaw sperm survival in the LEN diluent of 5 ml maxi-straw. When the temperature of the thawed semen was 20.7°C, the percentages of motile sperm and NAR acrosome were highest. *(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 11 : 1553-1558)*

Key Words : Diluent, Freezing Rate, Thawing Temperature, Boar Sperm

INTRODUCTION

During the last 30 years, several methods for deep-freezing of boar sperm have been presented. For each of the methods, individual research groups have developed their own freezing and thawing diluents (Polge et al., 1970; Crabo and Einarsson, 1971; Graham et al., 1971; Pursel and Johnson, 1971; 1975; Visser and Salamon, 1974; Westendorf et al., 1975; Larsson and Einarsson, 1976; Paquignon and Courot, 1976).

Pursel et al. (1978) reported that incubation of boar sperm for 1 h with as little as 0.1% OEP (Orvus ES Paste) in a diluent devoid of egg yolk had a marked deleterious effect on acrosome morphology and sperm motility. The most popular diluents for boar sperm freezing were glucose and 22.5% egg yolk (Polge et al., 1970); lactose, 25% egg yolk, and sodium triethanolamine lauryl sulphate (OEP; marketed as Equex STM, Nova Chemical Sales, Inc., Scituate, Massachusetts, USA; Westendorf et al., 1975); tris, fructose, ethylene diamine tetraacetic acid (EDTA), citric acid and 15% egg yolk (Visser and Salamon, 1974); tris, glycine, citric acid, glucose and 22.5% egg yolk (Obando et al., 1984); and tes, tris, glucose, OEP and 20% egg yolk (Pursel and Johnson, 1975). Yi et al. (2002) reported that 0.05% soluble N-acetyl-D-glucosamine was the optimum concentration in the first diluent of lactose-egg yolk diluent and a concentration of 0.05% soluble N-acetyl-D-glucosamine significantly enhanced the cryopreservation of boar sperm. Recently, the Hulsenberg straw freezing method (Westendorf et al., 1975) has been most extensively used in the field.

The freezing rate is the most important factor controlling the life or death of the cell during freezing. Mazur (1985) stated that each type of cell had an optimal freezing rate for surviving the freezing process; the rate varied widely depending upon a number of cellular characteristics, the cryoprotectant, the concentration of cryoprotectant, the components of the diluent, and thawing rate.

Park and Pursel (1985) reported the results of an experiment in which 5 ml maxi-straws were frozen at constant freezing rates of 10, 16 and 24°C/min from -5°C to -80°C. The results indicated that post-thaw motilities after 0.5 and 2 h incubation at 37°C were significantly higher for sperm frozen at 16°C/min than for sperm frozen at 10 or 24°C/min. Percentages of NAR (normal apical ridge) acrosomes immediately after thawing and 2 h after incubation at 37°C were significantly higher for sperm frozen at 16°C/min than for sperm cooled at 24°C/min but were not significantly different from frozen at 10°C/min.

Kim et al. (1989) reported that the straws frozen from 5 cm to 25 cm above the liquid nitrogen surface did not show any significant differences on post-thaw sperm

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motility and NAR acrosome.

Thawing in certain solutions was reported to enhance the post-thaw percentage of NAR acrosomes (Crabo et al., 1972; Pursel and Johnson, 1976). Mazur (1985) reported that the warming phase of the freeze-thaw process was just as important to cell survival as the cooling phase.

Westendorf et al. (1975) thawed maxi-straws by submerging them in water varying in temperature from 55 to 90°C. More recently, maxi-straws have been thawed at different combinations of times and temperatures, e.g., 50°C for 50 sec (Perezcanto-Fernandez, 1978; Scheid et al., 1980), 50°C for 45 sec (Aumuller, 1982), 55°C for 55 sec (Froede-Garibay, 1979), 52°C for 52 sec with a 6 ml volume (Schuler et al., 1979) and 52°C for 40 sec with a 5 ml volume (Pursel and Park, 1987).

Our objective in this research was to obtain informations regarding the effect of N-acetyl-D-glucosamine in the LEY (lactose-egg yolk) diluent according to incubation time in 5 ml maxi-straw and the effects of freezing rate, thawing time and thawing temperature in the LEN (lactose-egg yolk and N-acetyl-D-glucosamine) diluent on acrosome morphology and motility of frozen-thawed boar sperm.

MATERIALS AND METHODS

Semen collection

Semen was collected from three Duroc boars twice weekly. The filtered sperm-rich fraction was collected by the glove-hand technique into a 250 ml insulated vacuum bottle. The sperm-rich fractions of ejaculates with greater than 85% motile sperm and NAR acrosome were used.

Frozen semen processing

The sperm-rich fraction (30 to 60 ml) of ejaculate was collected into an insulated vacuum bottle. Semen was slowly cooled to room temperature (20 to 30°C) by 2 h after collection. Semen was transferred into 15 ml tubes, centrifuged at room temperature for 10 min at 800 g, and the supernatant solution was poured off. One volume of concentrated sperm was resuspended with 1 volume of lactose-egg yolk (LEY) diluent or 1 volume of lactose-egg yolk and N-acetyl-D-glucosamine (LEN) diluent (the first diluent to provide 1.0×10^9 sperm/ml) at room temperature. Semen was cooled in a refrigerator to 5°C over a 2 h period and 1 volume of a LEY+6% or 1 volume a LEN+4% glycerol diluent (the second diluent) was added to 1 volume of cooled semen. Straws (Minitub Gmbh, Germany) were immediately filled with 5 ml of semen and steel or glass balls were used to seal the end of the straws. The air bubble was adjusted to the center of each straw and the straws were horizontally placed on aluminum rack and set into a liquid nitrogen tank containing liquid nitrogen (LN). The straws were situated 5 cm above the LN, and kept at that level for 20 min before the straws were transferred into LN storage.

Composition of diluents for boar sperm

The compositions of diluents used for deep-frozen semen in these experiments are summarized in Table 1.

Sperm evaluation

Immediately after each straw was thawed in 52°C water bath for 40 sec, 5 ml of their contents were added to a test tube containing 30 ml of BTS (Beltsville thawing solution) at 22 to 24°C. One ml aliquot of the diluted semen was then added to 1 ml of 1% glutaraldehyde in BTS for the 0.5 h acrosome morphology evaluation and 29 ml were incubated at 37°C. After 0.5 h incubation, 10 μl aliquot were transferred onto glass slides and 18×18 mm cover-slips were applied. The percentage of motile sperm was estimated at 37°C by light microscope at 250×.

The acrosome morphology of 100 sperm per sample at 0.5 h after thawing was evaluated by phase contrast microscopy at 1000×. Acrosomes were differentially categorized into four morphological classes: normal apical ridge (NAR), damaged apical ridge (DAR), missing apical ridge (MAR) and loose acrosomal cap (LAC) as described by Pursel et al. (1972). All samples were coded at thawing and the identity of the treatment was unknown during evaluation.

Temperature measurement during freezing and thawing

One straw from each group was randomly selected to measure straw temperature. The straw was fitted with a type K copper-constantan thermocouple that was passed into the straw. The tip of the thermocouple was held in the center of the straw by a polyethylene collar. Thermocouple was connected to one temperature recorder (μR1000 Recorder Model 436001, Yokogawa Electronic Corporation, Japan) during freezing and thawing. Processes were calculated.

Experimental design

Experiment 1 was conducted to investigate the effect of

Table 1. Composition of diluents for boar sperm freezing

Ingredients	LEN	LEY
First diluent		
Lactose hydrate, g	11.0	11.0
Egg yolk, ml	20.0	25.0
N-acetyl-D-glucosamine, g	0.05	-
Distilled water, ml	100.0	100.0
Second diluent		
Lactose hydrate, g	11.0	11.0
Egg yolk, ml	20.0	25.0
Glycerol, ml	4.0	6.0
Orvus ES Paste, ml	1.0	1.0
Distilled water, ml	100.0	100.0

LEN and LEY diluents on post-thaw sperm motility according to incubation time. Samples were incubated for 0.5, 2, 3 and 4 h at 37°C.

Experiment 2 was also conducted to investigate the effect of LEN and LEY diluents on post-thaw NAR acrosome according to incubation time. Samples were incubated for 0.5, 2, 3 and 4 h at 37°C.

Experiment 3 was designed to determine the effect of aluminum rack distance from liquid nitrogen (LN) surface on post-thaw sperm motility and NAR acrosome in the LEN diluent of 5 ml maxi-straw. Aluminum rack distances were 5, 11 and 17 cm from LN.

Experiment 4 was carried out to investigate the effect of submersion time and sample temperature in 52°C water bath on post-thaw sperm motility and NAR acrosome in the LEN diluent of 5 ml maxi-straw. Submersion times were 30, 35, 40, 50 and 55 sec at 52°C.

Experiment 5 was designed to investigate effect of different thawing temperature and time on post-thaw sperm motility and NAR acrosome in the LEN diluent of 5 ml maxi-straw. Thawing temperatures were 4, 22, 37, 45, 52, 62, 72 ad 84°C and thawing times were 12 min 31 sec, 1min 52 sec, 1 min 3 sec, 43 sec, 40 sec, 35 sec, 30 sec and 15 sec, respectively.

Statistical analysis

Analyses of variance (ANOVA) were carried out using the SAS package (SAS, 1988) in a completely randomized design. Duncan's multiple range test was used to compare mean value of individual treatments when the F-value was significant ($p<0.05$).

RESULTS

Comparison of LEN and LEY diluents on post-thaw sperm motility according to incubation time

As shown in Table 2, there were no differences between the LEN and LEY diluents on post-thaw sperm motility according to incubation time. The sperm motility after 2, 3 and 4 h incubations was decreased compared to that after 0.5 h incubation in the LEN and LEY diluents.

Table 2. Comparison of LEN and LEY diluents on post-thaw sperm motility (%) according to incubation time in 5 ml maxi-straw

Item	Incubation time (h)[1]			
	0.5	2	3	4
LEN	53.3±1.3^{a}	48.7±1.5^{ab}	44.7±2.3^{b}	38.0±2.8^{c}
LEY	49.3±1.4^{a}	42.7±1.4^{b}	40.0±2.5^{b}	37.7±2.8^{b}

[a,b,c] Means with different letters within the same row were significantly different ($p<0.05$).

[1] Mean±SE for 12 replications.

Comparison of LEN and LEY diluents on post-thaw NAR acrosome according to incubation time

The present study showed that the LEN diluent was higher NAR acrosome than the LEY diluent at 0.5 h incubation as shown in Table 3.

However, there were no differences between the LEN and LEY diluents on post-thaw NAR acrosome at 2, 3 and 4 h incubations. The NAR acrosome after 2, 3 and 4 h incubations was dramatically decreased compared to that after 0.5 h incubation in the LEN and LEY diluents.

Effect of aluminium rack distance from liquid nitrogen surface on post-thaw sperm motility and NAR acrosome

As shown in Table 4, the straws frozen from 5.0 cm (20°C/min) to 17.0 cm (1°C /min) above the liquid nitrogen surface did not show any significant differences on post-thaw sperm motility. However, the straws frozen above 5.0 cm from the liquid nitrogen surface were higher NAR acrosome than those frozen above 17.0 cm.

Effect of submersion time and sample temperature in 52°C water bath on post-thaw sperm motility and NAR acrosome

As shown in Table 5, the maxi-straws were thawed by submersion in a 52°C water bath for 30, 35, 40, 45, 50 or 55 sec. The post-thaw percentages of motile sperm and acrosomes with a normal apical ridge (NAR) at the initial microscopic examination were significantly higher ($p<0.05$) for the maxi-straws submerged for 40 or 45 sec than for any other duration of submersion. The mean sample temperatures of maxi-straws after 40 or 45 sec submersion were 20.7 or 26.4°C.

Effect of different thawing temperature and time on post-thaw sperm motility and NAR acrosome

As shown in Table 6, the maxi-straws were thawed to be reached at 19-20°C sample temperature at different combinations of temperatures and times except at 4°C thawing temperature. This results showed that the sample temperatures of maxi-straw during thawing were very important on post-thaw sperm motility and NAR acrosome.

Table 3. Comparison of LEN and LEY diluents on post-thaw NAR acrosome (%) according to incubation time in 5 ml maxi-straw

Item	Incubation time (h)[1]			
	0.5	2	3	4
LEN	63.0±2.1^{ax}	14.5±1.6^{b}	10.7±1.7^{bc}	8.7±1.8^{c}
LEY	48.1±1.8^{ay}	11.5±1.9^{b}	10.3±1.7^{b}	8.2±1.7^{b}

[a,b,c] Means with different letters within the same row were significantly different ($p<0.05$).

[x,y] Means with different letters within the same column were significantly different ($p<0.05$).

[1] Mean±SE for 12 replications.

Table 4. Effect of aluminium rack distance from liquid nitrogen (LN) surface on post-thaw sperm motility and NAR acrosome in the LEN diluent of 5 ml maxi-straw

Rack distance from LN surface (cm)	Rack temperature during freezing (°C)	Freezing rate of maxi-straw from -5 to -80 (℃/min)	Motility[1] (%)	Normal acrosome[1] (%)
5.0	-141	20	51.7±1.1	61.7±2.1[a]
11.0	-95	8	51.0±1.0	56.2±1.5[ab]
17.0	-42	1	49.0±1.6	54.1±2.8[b]

[a,b] Means with different letters within the same column were significantly different ($p<0.05$).

[1] Mean±SE for 12 replications.

Table 5. Effect of submersion time and sample temperature in 52°C water bath on post-thaw sperm motility and NAR acrosome in the LEN diluent of 5 ml maxi-straw

Submersion time (sec)	Sample temperature (°C)	Motility[1] (%)	Normal acrosome[1] (%)
30	8.4±2.7[d]	40.0±2.2[b]	42.6±4.0[c]
35	13.6±2.5[d]	50.0±1.5[a]	47.0±3.2[bc]
40	20.7±1.5[c]	54.0±1.6[a]	61.7±2.1[a]
45	26.4±1.4[b]	53.0±1.2[a]	56.2±2.5[ab]
50	29.4±1.5[b]	43.0±2.0[b]	50.8±4.3[bc]
55	34.7±1.5[a]	37.0±2.2[b]	40.9±2.6[c]

[a,b,c,d] Means with different letters within the same column were significantly different ($p<0.05$).

[1] Mean±SE for 12 replications.

Table 6. Effect of different thawing temperature and time on post-thaw sperm motility and NAR acrosome in the LEN diluent of 5 ml maxi-straws

Thawing temperature, time	Sample temperature (℃)	Motility[1] (%)	Normal acrosome[1] (%)
4°C, 12 min 31 sec	3.9±0.1[b]	21.7±1.7[b]	30.3±1.2[b]
22°C, 1 min 52 sec	19.2±0.0[a]	49.2±2.0[a]	54.8±2.7[a]
37°C, 1min 3 sec	19.8±0.4[a]	50.4±1.6[a]	62.6±2.3[a]
45°C, 43 sec	19.9±0.5[a]	51.7±2.0[a]	63.0±2.5[a]
52°C, 40 sec	21.6±1.4[a]	53.6±1.6[a]	66.1±2.6[a]
62°C, 35 sec	20.3±0.5[a]	48.5±3.0[a]	59.9±2.9[a]
72°C, 30 sec	20.0±0.3[a]	47.8±2.0[a]	61.1±3.7[a]
84°C, 15 sec	19.1±0.3[a]	50.0±0.0[a]	56.3±8.8[a]

[a,b] Means with different letters within the same column were significantly different ($p<0.05$).

[1] Means±SE for 12 replications.

DISCUSSION

The present study showed that the LEN diluent containing 0.05% N-acetyl-D-glucosamine in the first diluent had a beneficial effect on acrosome morphology as compared with the LEY diluent without N-acetyl-D-glucosamine. At present, evidence of the mechanism by which N-acetyl-D-glucosamine protects boar sperm during freezing is lacking. However, Lehninger (1975) reported that many polysaccharides served primarily as structural elements in cell walls and coats, intercellular spaces, and connective tissue, where they gave shape, elasticity, or rigidity to plant and animal tissue as well as protection and support to unicellular organisms.

In early studies, boar sperm were frozen in ampules using low rates of cooling. Under these conditions, sperm motility after thawing was highest when the glycerol concentration exceeded 5%. Since 1970, it is generally accepted that the higher rates of cooling provided by the pellet freezing method (Nagase and Niwa, 1963) in combination with low concentrations of glycerol are advantageous to sperm survival. Schorner (1974) compared pellet and ampule (1°C/min) freezing and reported little difference in post-thaw motility among methods if the diluent contained 5% glycerol. Waide (1975) reported that sperm frozen in thin-walled aluminum packages at rapid cooling rate in liquid nitrogen vapor (+5 to -100°C in 3 min) gave superior post-thaw motility in comparison to sperm cooled at 1-2°C/min from +5 to -20°C and 3°C/min from -20 to -70°C in dry ice-alcohol. The optimal freezing rate for boar sperm has been reported to be 16°C/min with maxi-straw (Pursel and Park, 1985), 30°C/min for 0.5 ml straw (Fiser and Fairfull, 1990) and 50°C/min for 0.25 ml straw (Woelders and Den Besten, 1993).

In this study, we found out that the straws frozen from 5 cm (20°C/min) to 11 cm (8°C/min) above the liquid nitrogen surface did not show any significant differences on post-thaw sperm motility and NAR acrosome in the LEN diluent contained 2% glycerol and 0.025% N-acetyl-D-glucosamine. It has been reported that when boar semen was frozen, the effect of the freezing rate was pronounced in some boars (Eriksson et al., 1997a,b; Medrano et al., 1998). This boar dependence of the freezing rate could explain some of the conflicting results about the importance of freezing rates.

Our results suggest that instead of a fixed freezing rate, boar semen tolerates a range of freezing rates around an optimum (Watson, 1979). This has also been shown for sperm from other species (Robbins et al., 1976; Rota et al., 1998).

Westendorf et al. (1975) reported that the post-thaw percentages of motility and NAR acrosome were higher when thick-walled straws were thawed in 90°C water than when thawed in 55°C water. The temperature of the thawed semen was +20 to +25°C at the time the straw was removed from the bath (Westendorf et al., 1975).

More recently, submersion of straws for specific temperatures and durations has been used for thawing. Perezcanto-Fernandez (1978) compared thawing temperatures of 50°C for 50 sec with 35°C for 60, 90 or 120 sec and saw no advantage for 35°C at any of the durations. Schuler et al. (1979) used a water bath temperature of 52°C and submersion for 52 sec to thaw maxi-straws containing 6 ml of semen.

Amuller (1982) thawed the 5 ml Macrotub straw by submersion in a 50°C water bath for 45 sec with the semen being warmed to 24°C. Pursel and Park (1985) thawed the 5 ml straw by submersion in a 52°C circulating water bath for 28, 34, 40, 46, 52 or 58 sec. The results indicated that the 40 sec submersion with the semen being warmed to 15.2°C resulted in significantly higher post-thaw percentages of motile sperm and NAR acrosome at the initial examination than any of the other durations of submersion.

In this study, we suggest that the progressively lower sperm survival beyond 26.4°C or below 8.4°C was the results of heat shock or cold shock, respectively.

In conclusion, we found out that the temperature of water bath from 22 to 84°C was not important for post-thaw sperm survival in the LEN diluent of 5 ml maxi-straw but the sample temperature of the thawed semen was very important. When the sample temperature of the thawed semen was 20.7°C, the percentages of motile sperm and NAR acrosome were highest.

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