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STUDY ON VIABILITY AND MEMBRANE INTEGRITY OF CRYOPRESERVED SPERMATOZOA OF FRIESIAN BULL UNDER DIFFERENT THAWING TEMPERATURE

Mehrez, A. F.

Animal Production Research Institute, Agriculture Research Center, Ministry of Agriculture, Egypt.

ABSTRACT

The effects of different thawing temperatures (37ºC for 30 second and 20ºC for 60 second) on viability and membrane integrity of cryopreserved spermatozoa with different hypoosmotic conditions (50, 100, 150, 200 and 300 mOsm) and incubation times (0, 15, 30, 45 and 60 min) at the same temperature of thawing using frozen semen were studied on semen from five Friesian bulls. Results showed that immediately after thawing sperm motility (58% vs. 62%), grade of motility (3.3 vs. 3.7) and sperm livability (76 vs. 84%) and abnormality (3.6 vs. 5.2%) improved (P<0.05) in spermatozoa determined immediately after 37ºC than 20ºC thawing temperature. However, percentage of spermatozoa with intact acrosome did not differ significantly (73.6% vs. 79%). Pearson's correlation coefficients between different sperm characteristics of thawed semen revealed a positive correlation between sperm motility percentage and each of, grade of motility and sperm livability, and between sperm livability and grade of motility. Such correlations were significantly (P<0.05) for stronger semen thawed at 37ºC (r=0.92, 0.94 and 0.91, respectively) than those at 20ºC (r=0.41, 0.73 and 0.80, respectively). Correlation coefficient between percentage of sperm motility and abnormality was negative, although it was significant (P<0.05) and strong in semen thawed at 20ºC (r=-0.88) than in those thawed at 37ºC (r=-0.23).

Sperm viability (percentage and grade of motility) showed significant (P<0.05) reduction from 300 up to 100 mOsm in both thawed semen, but the differences between the two thawed semen were not significant at all osmolarity levels. However, percentage of curled spermatozoa significantly (P<0.05) increased by decreasing the osmolarity level from 300 up to 100 mOsm for semen thawed at 37ºC and up to 50 mOsm for those thawed at 20ºc. Yet, the percentage of curling was significantly higher in semen thawed at 37ºC than 20ºC at all osmolarity levels.

On the other hand, sperm viability decreased by increasing incubation time in both thawed semen showing significantly (P<0.05) the minimal values of grade of motility (1.80) at 60 min for 37ºC thawing and at 15 min for 20ºC thawing (1.84). While, motility percentage was reached their minimal values at 45 min in both thawed semen. However, the differences between both thawed semen were not significant. The percentage of curled spermatozoa increased significantly (P<0.05) by advancing incubation time up to 45 min in both thawed semen, but its values were always significantly higher in semen thawed at 37ºC than those thawed at 20ºC.

The correlation coefficient between percentage of curled spermatozoa and each of sperm motility, livability and intact acrosome percentages was significantly positive and stronger in semen thawed at 37ºC than that at 20ºc.

The current study, indicate that thawing at 37ºC for 30 sec results in better sperm quality in terms of high sperm viability and membrane integrity (reactivity to hypoosmotic swelling test, HOS- test).

Keywords: Friesian bulls, cryopreserved spermatozoa, thawing temperature osmotic shock.

INTRODUCTION

Evaluation of frozen semen has been considered as the ascertaining of several particular measured characteristics of processed semen for its contained products, which might reflect the ability of the contained spermatozoa to impregnate females (Saacke, 1983). The rate of thawing markedly affects the viability and livability of spermatozoa that have been frozen (Pickett *et al.*, 1965). Thawing may be faster at the higher temperature than that occurring at the lower temperatures (Pace *et al.*, 1981). Various methods of thawing frozen bovine semen have been attempted (Pace *et al.*, 1981 and Correa *et al.*, 1996). The recommended temperature and duration of thawing may be varied independing on the procedures and solutions used in semen assessment during freezing or according to straw size (Jondet, 1972).

Post-thaw spermatozoa are very susceptible to damage as a consequence of abrupt changes in osmotic conditions induced by rapid dilution (Hammerstedt *et al.*, 1990). Osmotic shock may occur when the extracellular glycerol concentration in post-thawed semen is reduced abruptly by exposure to female reproductive tract fluids during AI (Correa and Zavos, 1995). Because some immotile sperm in hypoosmotic solution have intact plasma membranes and are capable of responding osmotically (Smikle and Turek, 1997), on osmotic swelling test has been used to determine effects of thawing temperature on membrane integrity of spermatozoa.

The aim of this study was to evaluate influence of different thawing temperatures on viability and reactivity of cryopreserved Friesian spermatozoa processed in various osmolarity levels and incubation times to determine the alteration of the sperm cell membrane.

MATERIALS AND METHODS

Frozen semen from five Friesian bulls, packaged in 0.5 ml plastic straws was provided by the International Livestock Management Training Center (ILMTC) Sakha, belonging to the Animal Production Research Institute, Agricultural Research Center, Ministry of Agriculture. The glycerol concentration of the cryopreserved medium was 7% (v/v). The medium used for dilutions and incubation was prepared with fructose (1.25%) and Na-citrate (2.9%) in distilled water to give osmolarity of 300 mOsm using osmometer (Osmett A, Model 5002). The final osmolarity of the tested solutions was modified from 300 up to 50 mOsm via serial dilution by distilled water to obtain four hypoosmotic levels with 50, 100, 150 and 200 mOsm.

Thawing and sperm processing:

One straw from each of five Friesian bulls was allocated to two treatment groups. Frozen semen straws were thawed in a water bath at 37^{o}C for 30 sec or 21^{o}C for 60 sec. Immediately after complete thawing, semen specimens were taken to evaluate grade of motility and percentages of sperm

motility, viability and abnormality as well as percentage of spermatozoa having intact acrosome.

Hypoosmotic swelling test (HOS-test):

Specimen from each semen straw (50μl) was added to one ml of the predetermined hypoosmotic solution into a glass tub and was evaluated immediately (zero time) or at incubation time for 15, 30, 45, 60 minutes at the same temperature of thawing. At each incubation time, 15 μl of well-mixed specimen with each hypoosmotic solution was assessed on a glass slide to make a semen smear, then it was dried and stained with eosin- nigrosin mixture (1:1) stain. All prepared slides were examined using research microscope at x 400 to calculate the percentage of curled spermatozoa in a microscopic field of 200 spermatozoa. Also, at each incubation time, semen specimens were taken to determine individual motility and grade of motility, with different hypoosmotic solutions used.

Sperm evaluation:

Immediately after thawing, percentage of individual motility of spermatozoa was estimated according to Amann and Hammerstedt (1980). The technique used to estimate grade of sperm motility was similar to that described by Zavos *et al.* (1994) as follows: Grade 1, oscillating movement but stationary, Grade 2, slow movement with no fixed direction, Grade 3, slow progressive movement and Grade 4, fast progressive movement. The percentage of live spermatozoa was determined according to Campbell *et al*. (1956). Eosin-nigrosine stain was used to determine sperm abnormaility percentage according to Blom (1983). While, percentage of spermatozoa with intact acrosome was determined using Giemsa stain technique.

At different incubation times with various osmolarity levels, grade and percentage of motile spermatozoa were determine. While, the percentage of spermatozoa with coiled tails was established as described by Zavos (1982).

Statistical analysis:

. The data were analyzed by ANOVA according to Snedecor and Cochran (1980). The least significant difference method (LSD) was used to determine significance among the various means. Pearson's coefficient of correlation was calculated using SAS computer programme (SAS, 1987).

RESULTS AND DISCUSSION

Semen characteristics of thawed semen:

Data obtained in Table 1 show that thawing at 37^{o}C for 30 sec. enabled the recovery of spermatozoa in terms of significantly ($P<0.05$) higher viability (grade of motility and motility percentage), livability and lower ($P<0.001$) abnormality percentages of spermatozoa immediately estimated in semen after thawing. While, percentage of spermatozoa with intact acrosome did not differ significantly. Spermatozoa thawed at 20^{o}C for 60 sec., however, showed inferior results.

Table 1. Effect of thawing temperature on semen characteristics measured immediately after thawing (X±SE).

Trait	Thawing temperature (°C)		Significant
	20°C (60 sec.)	**37°C (30 sec.)**	
Grade of motility (score 1-4)	3.3±0.12	3.7±0.18	*
Gross motility (%)	58.0±0.13	62.0±1.0	*
Sperm livability (%)	76.0±2.6	84.0±2.6	*
Sperm abnormality (%)	5.2±0.72	3.6±0.46	**
Intacted acrsome (%)	73.6±3.2	79.0±2.5	NS

NS: Not significant *** P<0.05** **** P<0.01**

Maintenance of viability and livability of spermatozoa were higher when they were thawed at 37°C for 30 which is in accordance with the recommended thawing procedure for cryopreserved spermatozoa (Pace and Sullivan, 1978 ; Pace *et al.*, 1981 and Correa *et al.*, 1996). Graham *et al.,* 1978) reported that when spermatozoa were thawed optimally, the intracellular ice-crystal formation may be prevented. This may assume the formation of intracellular ice-crystal, when spermatozoa were thawed at 20°C for 60 sec, which may affect intactness of sperm cell membrane, in turn sperm quality of semen. Furthermore, the rate of thawing (Thawing duration) may interact with thawing temperature. Too rapid a thawing rate results in unbalanced rates of egress of glycerol and influx of water, while a very low thawing rate results in recrystallization of micro crystals of intercellular water and subsequent damage to subcellular organelles (Hammerstedt *et al.*, 1990).

Regarding the correlations between different traits of spermatozoa immediately estimated in thawed semen, similar trend of correlation coefficients were obtained in semen thawed at 37°C and 20°C. In semen thawed at 37°C, there was strong and significant (P<0.05) positive correlation between sperm motility percentage and each of grade of motility and live sperm percentage, and between grade of motility and live sperm percentage. Such correlations were lower and insignificant in semen thawed at 20°C (Table 2) This is in agreement with the correlation coefficients reported by some authors, who reported a range of positive correlation (r=0.44, -0.87) between sperm motility and live sperm percentage (Clarke *et al*., 1973 and Garg and Pandit, 1983).

The correlation between sperm abnormality % and each of sperm motility %, grade of motility and live sperm % was almost negative, but it was stronger and significant (P<0.05) between sperm abnormality % and each of percentage of motility and livability of spermatozoa and insignificantly (P<0.05) higher between sperm abnormality percentage and grade of motility in semen thawed at 20°C than in semen thawed at 37 °C (Table2). This may speculate the effect of sperm abnormality on motility of spermatozoa thawed at 20°C.

On the other hand, the correlation of spermatozoa with intact acrosome did not follow a specific trend in both thawed-semen with sperm motality percentage and grade of motility and live sperm %. However, it was

correlated negatively with sperm abnormality %, which was significant (P<0.05) only in semen thawed at 37°C (Table 2).

Table 2: Correlation coefficients among different characteristics of thawed semen (r).

Trait	Thawed semen	Sperm motiliy (%)	Grade of motility	Live sperm (%)	Sperm abnormality (%)
Grade of motility (Score 1-4)	37°C 20°C	0.919* 0.408			
Live sperm, %	37°C 20°C	0.943* 0.7266	0.910* 0.801		
Sperm abnormality, %	37°C 20°C	-0.233 -0.881*	0.088 -0.686	-0.529 -0.872*	
Intact acrosome %	37°C 20°C	0.219 -0.188	0.069 0.461	0.277 0.066	-0.978* -0.136

*** P<0.05**

The obtained stronger correlation coefficients (Table 2) between most of traits in semen thawed at 37°C than 20°C may indicate the higher sperm quality of semen thawed at 37°C.

Response of spermatozoa to HOS-test:

Effect of osmolarity level:

For all osmolarity levels, regardless the incubation time, no significant differences were detected in viability of spermatozoa between semen thawed at 37°C or 20°C. However, susceptibility of spermatozoa to osmolarity levels in term of curled spermatozoa percentage was significantly different as affected by thawing temperature, being higher in semen thawed at 37 C than those at 20°C at all osmolarity levels (Table 3).

As osmolarity level was increased from 50 up to 300 mOsm, sperm viability increased and the reactivity of spermatozoa to HOS-test decreased.

Table 3. Characteristics of cryopreserved semen following thawing and incubation at different osmolarity levels, regradless incubation time. (X±SE)

Trait	Thawing temper-ature	Osmolarity level (mOsm)				
		50	100	150	200	300
Grade of sperm motility (score 1-4)	37°C 20°C	1.59±0.11^{c} 1.54±0.18^{c} NS	1.88±0.14^{c} 1.72±0.21^{c} NS	2.48±0.14^{b}2 .38±0.17^{b} NS	2.68±0.13^{b} 2.64±0.13^{b} NS	3.48±0.11^{a} 3.12±0.16^{a} NS
Sperm motility %	37°C 20°C	9.3±1.0^{d} 6.5±1.0 NS	10.4±1.0^{d} 7.8±1.2^{d} NS	37.8±1.4^{c} 35.2±1.3^{c} NS	55.6±0.9^{b} 53.0±1.4^{b} NS	63.4±1.1^{a} 61.0±1.9^{a} NS
Sperm with curled tail %	37°C 20°C	76.2±1.9^{c} 69.8±1.5^{d} *	71.0±1.6^{c} 52.0±1.9^{c} ***	45.3±1.9^{b} 35.8±2.2^{b} **	36.7±2.3^{a} 30.9±1.8^{a} *	30.8±1.7^{a} 24.2±1.8^{a} *

a, .b ..c and d Means denoted having different superscripts at the same raw are significantly different at P<0.05.

Differences between thawing temperatures (NS ; significant, * P<0.05, P<0.01 and *** P<0.001).**

In both semen thawed at 37 and 20,c maximum sperm viability were recorded significantly ($P<0.05$) with 300 mOsm, while the minimal values were obtained with 100 mOsm osmoalrity level. On the other hand, the trend of change in response of spermatozoa to HOS-test was different, being the highest ($P<0.05$) with 100 mOsm in case of semen thawed at 37 C and with 50 mOsm osmolarity in case of that thawed at 20C. However, the lowest values were recorded significantly in the two semen with 200 mOsm osmolarity. This finding may indicate the highest response of spermatozoa thawed at 37^{o}C to HOS-test than those thawed at 20^{o}C (Table 3).

Bull spermatozoa behave as osmometers (Drevius, 1972) and undergo curling in solution varying widely in osmolarity (Watson *et al.*, 1992). The present results indicated that sperm viability was affected when spermatozoa were exposed to solution over a range of 100-300 mOsm osmolarity, although the differences were almost not significant between both thawed semen. Such trend of change is in agreement with that reported by Bredderman and Foote (1969), who observed that motility of bull spermatozoa decreased gradually as osmolarity was decreased to 94 mOsm. Also, Liu and Foote (1998) reported marked reduction in sperm viability as osmolarity level was decreased up to 100 mOsm.

In accordance with the trend of change in response of spermatozoa to HOS-test, several investigators have documented curling/swelling of membranes in response to low osmolarity in mammalian spermatozoa, being the maximum with 50-60 mOsm osmolarity (Correa and Zavos, 1994 in bull ; Vazquez *et al.*, 1997 in boar and Moussa, 1999 in ram).

It is worthy noting that the highest rate of change in all studied traits were observed with osmolarity level less than 150 mOsm. Cragle and Salisbury (1959) reported insignificant effect of osmolarity level on oxygen uptake by bull spermatozoa within the range from 150 to 392 mOsm. This may support the present change in viability and response of all spermatozoa to HOS-test.

Effect of incubation time:

Sperm viability decreased and response of spermatozoa to HOS-test increased by advancing incubation time from 0 to 60 min. (Table 4). It was observed that grade of motility was more affected by incubation time than sperm motility.percentage Minimal values of motility grade and sperm motility percentage were reached significantly ($P<0.05$) at 45 min in case of semen thawed at 37^{o}C. The corresponding times in semen thawed at 20^{o}C were 15 and 45 min . However, the differences between both thawed semen were not significant at all incubation time (Table 4).

Maximum reactivity of spermatozoa to HOS-test was reached significantly ($P<0.05$) at 30 min for 37^{o}C thawing temperature and at 45 min for 20^{o}C one. However, percentage of curling was always significantly higher in semen thawed at 37^{o}C than at 20^{o}C .The present results are in accordance with those reported for maximum response of spermatozoal at 30 min in bull (Correa and Zavos, 1994) and of 45 min in ram (Moussa, 1999). The response of spermatozoa to HOS-test is depending on cellular water uptake (Osmolarity level) per time unit (incubation time). So, the differences in

response to HOS-test may related to variation in membrane integrity of spermatozoa as affected by thawing temperature (Drevius, 1972).

Table 4. Characteristics cryopreserved semen following thawing and incubation at different incubation times, regradless levels (X±SE).

Trait	Thawing temperature	Incubation time (minute)				
		0	15	30	45	60
Grade of sperm motility (score 1-4)	37°C	2.76±0.25^{a}	2.73±0.16^{a}	2.60±0.15^{a}	2.23±0.16^{ab}	1.80±0.21^{b}
	20°C	2.14±0.19^{a}	2.68±0.15^{b}	2.60±0.20^{b}	2.14±0.18^{b}	1.84±0.15^{b}
		NS	NS	NS	NS	NS
Sperm motility %	37°C	50.6±1.9^{a}	47.7±2.0^{a}	37.4±1.8^{b}	22.7±2.1^{c}	18.2±2.2^{c}
	20°C	48.4±2.2^{a}	45.2±2.2^{a}	352±2.3^{b}	18.5±2.3^{c}	16.2±2.1^{c}
		NS	NS	NS	NS	NS
Sperm with curled tail %	37°C	33.0±1.2^{c}	47.0±1.7^{b}	55.5±1.5^{a}	59.3±1.6^{a}	65.2±2.1^{a}
	20°C	27.7±1.3	32.8±1.5^{c}	44.6±2.0^{b}	51.9±1.8^{a}	55.5±1.5^{a}
		*	***	**	*	**

a, b c and. d Means denoted having different superscripts at the raw are significantly different at P<0.05.
Differences between thawing temperature (NS : Not significant, * P<0.05, P<0.01 and *** P<0.001).**

The present differences between semen thawed at 37°C than 20°C may be attributed to effect of thawing temperature on physical and biochemical properties of sperm cell membrane permeability to water or cryoprotectants as function of the surrounding medium temperature (Hammestedt *et al.*, 1990). Also, Correa *et al*. (1996) found that at 37°C thawing temperature, sperm cell membrane is more fluid than at the lower temperatures, which allows the diffusion of intracellular glycerol during sperm processing.

In supporting the previous results, correlation coefficients (Table 5) between all traits estimated in semen thawed at 37°C and percentage of curling/swelling of spermatozoa at different osmolarities and incubation times were positive stronger and significant, except for sperm abnormality, which had insignificantly (P<0.05) lower and negative correlation with HOS-test (Table 5).

Table 5: Correlation coefficients (r) between thawed semen characteristics and HOS- Test.

Traits	Semen thawed at	
	37°C	20°C
Sperm motility%	0.643**	0.415*
Grad of motility	0.461*	0.320^{NS}
Live sperm %	0.804**	0.613*
Acrosomal status	0.641**	0.303^{NS}
Sperm abnormality	-0.068^{NS}	-0.616*

NS:. Not significant * P< 0.05 **P< 0.01

Similar trend of significant correlation coefficients were reported between HOS-test and each of bull sperm motility (r=0.83, Correa and Zavos, 1995) and livability (r=0.62) and abnormaility (r=-0.07) of ram spermatozoa (Moussa, 1999).

The present results may conclude that sperm viability and sperm membrane fluidity and permeability were improved in semen thawed at 37^{o}C than those thawed at 20^{o}C. Also, HOS-test provides a precis technique for measuring alterations in sperm motility and membrane integrity after freezing and thawing of semen, which could be used as an important indicator of male fertility.

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دراسة على حيوية وسلامة الغشاء الخلوى للحيوانات المنوية المجمدة لطلائق الفريزيان تحت درجات حرارة اسالة مختلفة

علاء الدين فؤاد محرز
معهد بحوث الانتاج الحيوانى - مركز البحوث الزراعية - وزارة الزراعة

يهدف هذا البحث لدراسة تأثير درجات حرارة مختلفة عند اسالة السائل المنوى المجمد على جودة السائل المنوى المسال للاستخدام فى التلقيح الصناعىوذلك بدراسة خصائص السائل المنوى الطبيعيه بعد الاساله مباشرة ثم تحضين السائل المنوى المسال على نفس درجة حرارة الاساله فى محاليل مختلفة الاسموزية (٥٠ ، ١٠٠ ، ١٥٠ ، ٢٠٠ و ٣٠٠ مل أزمول) وعلى اوقات تحضين مختلفة لدراسة تأثيراختبار الأسموزيه على الحيوية (النسبة المئوية للحركة – درجة الحيويه) والنسبة المئوية لالتواء ذيل الحيوانات المنوية. استخدم فى هذه الدراسة السائل المنوى المجمد لخمس طلائق فريزيان تم توزيعها على مجموعتين تم اسالة قصيبات السائل المنوى المجمد لكل طلوقة على درجة ٣٧°م لمدة ٣٠ ثانية (المجموعة الاولى) وعلى ٢٠°م لمدة ٦٠ ثانية (المجموعة الثانية) وقد تم اجراء تقييم السائل المنوى بعد الاسالة مباشرة أو استجابة الحيوانات المنوية لاختبار الاسموزيه .

وقد اظهرت النتائج ما يلى:

(1) كانت جودة السائل المنوى المجمد والمسال على درجة حرارة ٣٧°م لمدة ٣٠ ثانية احسن معنويا من المجموعة الثانية (٢٠° م لمدة ٦٠ ثانية) من حيث درجة الحيوية و النسبة المئوية لكل من الحيوانات المنوية المتحركة والحية والغير شاذة بينما لم تختلف النسبة المئوية للحيوانات المنوية ذات الاكروسوم الطبيعى بعد الاسالة مباشرة.

(2) لم تختلف درجتى حرارة الاسالة معنويا فى تاثيرها على النسبة المئوية للحيوانات المنوية المتحركة او درجة الحيوية ولكن كانت قيمتها اكبر دائما فى السائل المنوى المسال على ٣٧°م عن ٢٠°م بينما كانت الفروق معنوية والنسبة المئوية لألتواء الذيل والتى اكدت الاستجابة الاكبر معنويا لاختبار الاسموزية للحيوانات المنوية المسالة على ٣٧°م عن ٢٠°م.

(3) كان هناك معاملات الارتباط موجبة بين النسبة المئوية لالتواء الذيل للحيوانات المنوية مع كل من النسبة المئوية للحيوانات المنوية المتحركة والحية وذات الاكروسوم الطبيعى وكانت هذه المعاملات اقوى معنويا فى السائل المنوى المسال على ٣٧°م عن ٢٠°م بينما كانت معاملات ا رتباط من النسبة المئوية لالتواء الذيل مع النسبة المئويةللحيوانات المنويه الشاذة سالبة معنويا فى السائل المنوى المسال على ٢٠°م عن ٣٧°م.

وقد اكدت الدراسة على ان اسالة السائل المنوى على درجة حرارة ٣٧°م لمدة ٣٠ ثانية تعطى سائل منوى ذو جودة اعلى من تلك المسالة على ٢٠ °م لمدة ٦٠ ثانية.