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MOTILITY AND ACROSOMAL STATUS OF BULL'S SPERM PRESERVED AT ROOM TEMPERATURE (25°C), BY COOLING (5°) OR BY FREEZING (-196°C)

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SUMMARY

The purpose of this study was to determine the maximum sperm longevity in semen stored at various temperatures. Semen from four healthy bulls (2 Holstein and 2 Guernsey) was collected by using an artificial vagina and incubated at 37°C. After evaluation of the initial semen quality, raw semen was diluted at 37°C in CUE-Tris to a concentration of 60×10^6 cells /ml. and filled into French (0.5 ml.) straws. Thereafter, straws were split into three equal parts. Each part was preserved by one of the storage treatments (at room temperature, by cooling and by freezing). Ten straws from each storage treatment were thawed on the *3rd*, *6th* and *9th* day of storage. Straws were thawed at 40°C for 20 seconds and incubated at 37°C for 3 hours. Evaluation of percent progressive motility and percent intact acrosomes was carried out at 0-time and 3-hour of incubation to investigate the post-thaw decline in both evaluation characteristics.

Semen stored for three or six days at room temperature or by cooling showed higher progressive motility and percent intact acrosomes than that preserved by freezing for the same period. Elongation of the storing period up to 9 days revealed a marked drop in both progressive motility and acrosomal integrity of sperm preserved at room temperature or by cooling than that stored by freezing.

As sperms remain viable for 5-6 days, it is recommended to store semen diluted in CUE-Tris at 25°C or 5°C for the use in artificial insemination within six days of storage. This will reduce costs incurred by freezing in liquid nitrogen.

Keywords: Bovine, semen, extenders, preservation, temperature, motility acrosomes

INTRODUCTION

In some areas of the world, freezing is not widely available or is expensive. Hence, considerations were given to investigate suitable semen diluents and preserving techniques for storage of semen at wide range of temperatures to help in extention of artificial insemination to such areas. In artificial insemination practices, research on preservation of the extended semen concentrated primarily on the maintenance of the fertilizing capacity of spermatozoa for an extended period of time. The livability of the stored spermatozoa depends on the composition of the diluent used and the suitable

kind and amount of antibiotics added to that extender. It has been found that Tris types of organic buffers provide a versatile series of solutions that can be used to formulate desirable semen extenders and they are suitable for ambient temperature storage, refrigerated or frozen semen. In comparing Tris-yolk and CUE extenders for storing semen at 25°C or 5°C for eight days, Foote (1978) found that CUE was best at 25°C and Tris was best at 5°C. Paufler (1967) formulated the CUE-Tris extender and compared between it and Spermasol T and CUE extenders. He found that CUE-Tris was superior to other extenders on the basis of percent 60-90 days non-return data. Recently, the two main laboratory quality parameters used to evaluate the post-thaw fertilizing ability of stored semen are the progressive motility and the acrosomal integrity. Saacke and White (1972) found that post-thaw acrosome retention was more highly correlated with fertility than motility.

The present study was undertaken to investigate the optimum post-thaw sperm viability and acrosomal integrity in semen diluted in CUE-Tris and stored at wide range of temperatures.

MATERIALS AND METHODS

Two ejaculates from each of four healthy bulls (2 Holstein and 2 Guernsey) were collected, using an artificial vagina, pooled and held at 37°C. Sperm cells concentration was measured by diluting 100µl. of raw semen with 7.9ml. of 2.9% sodium citrate solution and using a previously calibrated spectrophotometer. Initial progressive motility was evaluated by diluting 10µl. of raw semen with 2ml. of 5% egg-yolk citrate solution and using a phase contrast microscope (x100) equipped with a heated stage at 37°C. Acrosomal integrity was assessed in samples fixed in 0.2% buffered-glutaraldehyde solution and using a differential interference contrast microscope (DIC) (x1250) equipped with a heated stage (Johnson *et al.*, 1976). Primary and secondary abnormalities were evaluated by using DIC optics (Mitchell *et al.*, 1978).

After evaluation of the initial semin quality, raw semen was split into two equal portions. The first part was centrifuged twice at 750 x g for 30 minutes to separate seminal plasma for further biochemical analysis (El-Hassanein, Unpublished data.). The second portion was diluted at 37°C in CUE-Tris (Foote, 1978) to a concentration of $60x10^6$ cells/ml. Gentamicin, tylosin and lincon spectin were added to the extender at levels of 0.656 mg., 0.084 mg. and 4.8 ml. per milliliter of final extender. Extension process was carried out according to Tuli *et al.* (1991).

After evaluation of post-extention progressive motility and acrosomal integrity, diluted semen was filled into French (0.5 ml.) straws. Immediately afterwards, straws were split into three equal parts for storing at room temperature (25°C) (RT), by cooling to 5°C (CT) or by freezing to -196°C (FT). After equilibration of the third portion of straws at 5°C for about two hours, it was exposed to vapor of liquid nitrogen for 8-9 minutes before plunging it into the liquid nitrogen.

On the 3*rd*, 6*th* and 9*th* day of storage, ten straws from each storage treatment were thawed at 40°C for 20 seconds and incubated at 37°C for three hours (Chandler *et al.*, 1984). Percent progressive motility (PPM) and percent intact acrosomal membrane (PIA) were evaluated immediately post-thaw (0 hour) and after

three hours of incubation to investigate the influence of storage temperature, storage period and post-thaw incubation time on the sperm viability and acrosomal integrity.

Counts of all parameters were examined in two different slides for each sample with 100 spermatozoa per slide and averages were taken. Data of the percentage values were subjected to arcsine transformation. Analysis of variance and Duncan's multiple range test between means of the transformed values were carried out by using the Statgraph program (Version 5).

RESULTS AND DISCUSSION

The different semen characteristics for each bull and the overall average values were presented in Table (1). The mean sperm cells concentration was 828.3 ± 53.3 million.ml^{-1} with an average PPM of 65.0 ± 2.04 and PIA of 90.5 ± 2.72. However, primary abnormalities (refer to spermatozoal head and acrosome) (16.8 ± 1.25%) and secondary abnormalities (associated with cytoplasmic droplets and tail defects) (15.3 ± 2.63%) were relatively higher than those reported for aged bulls by Mitchell *et al.* (1978).

Table 1. Raw seminal quality of bulls used in the present study.

Animal #	Volume[(a)] (ml.)	Concentration (x 10^6 cells/ml.)	% PM	% IA	% PABN	% SABN
I (H)	11.5	979	70	92	14	15
II (H)	15.0	828	65	91	17	8
III (G)	12.5	753	60	83	20	20
IV (G)	12.5	753	65	96	16	18
X ±S.E.	12.9	828.3	65.0	90.5	16.8	15.3
	±0.75	± 53.3	± 2.04	± 2.72	± 1.25	± 2.63

N.B.: (a)- Volume of pooled 1*st* & 2*nd* ejaculates; (H)- Holstein; (G)- Guernsey; PM= Progressive motility; IA= Intact acrosomes; PABN= Primary abnormalities; SABN= Secondary abnormalities.

Dilution of raw semen in CUE-Tris at 37°C caused a reduction in both PPM and PIA by about 16 and 5% from their initial values, respectively (Table 2). The relatively high drop in PPM might be due to the gradual change occurred in the external environment of spermatozoa. Studies have shown that extensive dilution of spermatozoa with simple solutions depressed motility (Mann, 1964), but that the addition of amino acids and macromolecules such as egg albumin or casein minimized this dilution effect. The used concentration of egg-yolk in CUE-Tris extender (10%) might not have been enough to minimize the effect of dilution at 37°C on the spermatozoal PPM. However, the dilution process and the diluent composition led to a maintenance of acrintegrity by about 95% of its initial value.

Three days of storage of semen diluted in CUE-Tris led to a marked decline in PPM by about 48, 59 and 69%, from the post-extention value, for the RT, CT and FT stored spermatozoa, respectively. The difference between the post-thaw PPM values of the three storage temperatures was statistically non-significant (Table 2). However, frozen semen showed the least PPM as compared with RT or CT stored semen. Similarly, Weseli *et al.* (1958) found that frozen semen had a lesser post-thaw PPM than that in the liquid semen (5-7°C). In respect of acrosomal integrity, three days of

storage led to a highly significant difference ($P< 0.01$) between the three storage temperatures. Reduction in PIA in frozen semen reached about 57% from the post-extention value, while it ranged from 16-20% in the liquid semen (RT or CT storage). It is well known that spermatozoa might lose their acrosomes when they die (the false or degenerative acrosomal reaction), and assessment of this type of acrosomal loss is used widely in cryopreservation studies to evaluate the sperm damage. The abrupt disappearance of acrosomal apical ridge at the onset of the false reaction, as it occurs on immotile bovine spermatozoa, provides a sharp irreversible end-point for sperm viability (Saacke and Marshall, 1968). In the present study, frozen semen poorly maintained post-thaw spermatozoal viability and acrosomal integrity as compared with the liquid semen following three days of storage. Almquist and Wiggin (1973) reported improved post-thaw motility, acrosomal integrity and fertility following fast thawing of frozen bovine spermatozoa packaged in plastic straws. However, Brown *et al.* (1982) found that semen straws removed from 35°C water bath after 12 seconds had an internal temperature of approximately -4°C, while straws removed after 1 min had reached 35°C. Rao *et al.* (1986) found significantly improved motility and survival in post-thaw frozen semen by increasing the thawing time from 15 to 30 seconds at 37°C. In the present study, the observed post-thaw spermatozoal damage in frozen semen might be due to a cold shock resulting from a delay in complete thawing of semen. The decline in post-thaw PPM in semen stored for three days at RT or CT seemed to be not due to spermatozoal damage, but might be due to some metabolic problems. It was reported by Foote and Bratton (1950) that the problem of prolonging the motility and fertility of spermatozoa in unrefrigerated extenders appears to be one of achieving sufficient control of spermatozoan metabolism. Metabolic rate tends to be proportional to absolute temperature, so reduced temperature has been the chief means of slowing down chemical reactions and prolonging spermatozoal life (Foote, 1978). The same author added that spermatozoa need protection from autotoxication due to acid products of metabolism, particularly when they are stored without refrigeration. In semen stored at about 5°C and rewarmed to 37°C, the percentage of progressive motile cells was observed to have decreased (Mann, 1964; Salisbury, 1968).

Post-thaw incubation at 37°C for three hours led to a non-significant difference in PPM between the three storage temperatures (Table 2). However, frozen semen showed the least decline in PPM (44%) following three hours of incubation as compared with semen stored either at RT (61%) or CT (56%). In respect of acrosomal integrity, incubation for three hours led to a highly significant difference ($P< 0.01$) between the three storage temperatures (Table 2). Frozen semen showed the highest decline in PIA (24%), following three hours of incubation, as compared with liquid semen (16-19%). It seems that incubated frozen semen maintains spermatozoal viability (motility) better than the incubated liquid semen, however, it poorly maintains acrosomal integrity. These findings were not in agreement with those of Weseli *et al.* (1958) who found that frozen semen (-83°C) showed a more rapid decline in PPM during incubation than did the corresponding liquid semen (5-7°C). It must be taken into consideration that the authors freezed semen at a relatively higher temperature than that in the present study and they used egg yolk-sodium citrate as extender.

Table 2. Mean values (± S.E.) of percent progressive motility and percent intact acrosomes in bull's semen diluted in CUE-Tris and preserved at room temperature (RT), by cooling (CT) and by freezing (FT).

Storing Period (Days)	Storing Type	Percent PM of spermatozoa incubated at 37°C for: 0 hour	3 hours	Percent IA of spermatozoa incubated at 37°C for: 0 hour	3 hours
0-Time	RT	54.8 ± 2.81	23.3 ± 2.66	86.3 ± 2.47	75.0 ± 3.23
3	RT	28.3 ± 6.81 a	11.1 ± 2.42 a	72.5 ± 5.46 a	58.5 ± 4.78 a
	CT	22.5 ± 3.23 a	9.8 ± 2.06 a	69.5 ± 1.21 a	58.5 ± 3.35 a
	FT	16.8 ± 1.89 a	9.5 ± 1.04 a	37.4 ± 5.79 b	28.3 ± 5.21 b
	F-test	n.s.	n.s.	**	**
6	RT	21.3 ± 4.50 a	9.5 ± 2.02 a	47.9 ± 2.29 a	44.3 ± 1.49 a
	CT	18.8 ± 2.56 a	9.3 ± 1.11 a	43.1 ± 5.83 a	36.4 ± 7.53 a
	FT	18.0 ± 2.55 a	10.5 ± 1.55 a	41.6 ± 5.55 a	30.8 ± 3.73 a
	F-test	n.s.	n.s.	n.s.	n.s.
9	RT	5.8 ± 0.85 a	1.5 ± 1.19 a	9.6 ± 2.87 a	3.5 ± 0.74 a
	CT	13.8 ± 1.11 b	3.5 ± 0.65 a	28.6 ± 2.72 b	13.6 ± 0.97 a
	FT	28.8 ± 4.44 c	14.5 ± 3.28 b	42.3 ± 6.61 b	31.0 ± 4.04 b
	F-test	**	**	**	**

N.B.: Means in the same column in each block and followed by the same letter are not significantly different from each other; n.s.= Statistically non significant; **= Highly significant (P<0.01); PM= Progressive motility; IA= Intact acrosomes.

Six days of storage led to a non-significant difference in PPM and PIA between the three storage temperatures (Table 2). The post-thaw decline in PPM ranged from 61-67%, from the post-extention value, for the three storage temperatures. The corresponding decline in PIA ranged from 45-52%. It seemed that CUE-Tris diluter had the same maintenance effect on spermatozoa stored either at RT, CT or FT for six days. Post-thaw incubation for three hours led to a non-significant difference in PPM and PIA for the three storage temperatures (Table 2). However, frozen semen showed the same trend as that found following the post-thaw incubation after three days of storage, i.e. it maintained relatively higher spermatozoal viability than liquid semen but poorly maintained acrosomal integrity.

Nine days of storage led to a highly significant difference (P<0.01) in post-thaw PPM and PIA between the three storage temperatures (Table 2). Frozen semen showed the least decline in PPM (44%) and PIA (51%) as compared with the corresponding liquid semen, while RT storage led to the highest decline in PPM and PIA (89%). It seemed that frozen semen was superior in maintaining post-thaw spermatozoal viability and acrosomal integrity following storage for more than six days. Increasing the storage period up to nine days led to a marked damage for the spermatozoa stored either at RT or CT. In respect of the post-thaw incubation effect, the three storage temperatures showed a highly significant difference (P<0.01) in PPM and PIA. Frozen semen showed the least decline in PPM (51%) and PIA (27%)

following three hours incubation at 37°C as compared to the corresponding values of the liquid semen.

On the other hand, a thorough examination reflected that, irrespective of the storage periods and the incubation times, storage temperatures affected (P<0.05) PPM, but did not affect PIA in semen diluted in CUE-Tris (Table 3). Thawed frozen semen had the highest PPM (23%) and the least PIA (36%) as compared with the liquid semen. It seemed that the composition of the CUE-Tris extender was able to maintain the acrosomal integrity in the stored liquid semen better than in the frozen semen. According to the findings of Saacke and White (1972) and Saacke *et al.* (1980) that acrosomal integrity was more correlated to fertility than the motility, it was suggested that CUE-Tris extender was more suitable for semen preservation at ambient temperature or by refrigeration than for freezing it.

In respect of the effect of the storage period on semen quality, thwas a highly significant difference (P<0.01) between the three storage periods (Table 3). Nine days of storage showed the least PPM (18%) and PIA (27%) than the other two storage periods. This was mainly due to the marked decline in the quality of RT and CT stored semen at the nineth day of storage. Figure (1) reflected that PPM and PIA of spermatozoa diluted in CUE-Tris and stored at RT or CT tended to decrease by increasing the storage period from three to nine days. However, in frozen semen the quality showed a reverse trend, i.e. it increased by increasing the storage period. It is suggested that semen diluted in CUE-Tris must be used within six days of storage at RT or CT to achieve high fertility rates, however, it can be cryopreserved for longer periods for using it in artificial insemination. The highly significant interaction (P<0.01) between the storage temperatures and storage periods (Table 3) indicated that semen quality (PPM and PIA) tended to decrease by increasing the storage period of liquid semen and the reverse was true for the frozen semen, i.e. its quality improved by increasing its storage period.

Irrespective of the storage temperature and storage period, post-thaw incubation at 37°C for three hours led to a highly significant decline (P<0.01) in PPM (37%) and PIA (14%) (Table 3). However, it could be noticed that CUE-Tris extender was able to maintain acrosomal integrity during incubation better than the progressive motility. Figure (1) showed that post-thaw incubated frozen semen maintained higher PPM than liquid semen, however, it poorly maintains acrosomal integrity.

Table 3. Analysis of variance for seminal quality as affected by storing type, storing period and incubation time.

Sources Of Variation		DF	Mean square	
			Progressive Motility	Intact Acrosomes
Corrected Total		71		
Storing Type	(ST)	2	117.43 *	72.99
Storing Period	(SP)	2	211.34 **	2553.32 **
Incubation Time	(INT)	1	1532.21 **	614.82 **
ST x SP		4	284.86 **	895.31 **
ST x INT		2	13.34	14.43
SP x INT		2	8.67	12.29
ST x SP x INT		4	7.54	21.68
Residual		54	20.11	30.76

N.B.: DF= Degrees of freedom; *= P< 0.05 (Significant); **= P< 0.01 (Highly significant)

In conclusion, the present investigation reflected that semen diluted in CUE-Tris and stored for about six days at room temperature (25°C) or by refrigeration (5°C) showed higher seminal quality than that stored by freezing for the same period. However, increasing storage period up to nine days showed a reverse trend, i.e. liquid semen had poorer quality than frozen semen. It is recommended to use semen diluted in CUE-Tris and preserved at 25° C or 5° C in artificial insemination within six days of storage. Cryopreservation of semen diluted in CUE-Tris is recommended for preserving it for longer periods to achieve higher fertility rates. Post-thaw liquid or frozen semen diluted in CUE-Tris must be used in artificial insemination as soon as possible to avoid the marked decline in its quality during the incubation period.

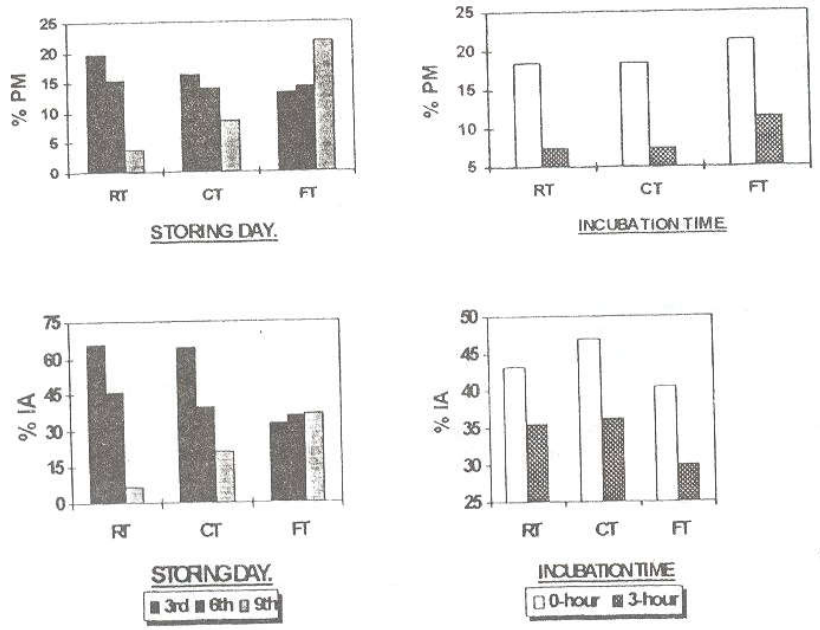

Figure 1. Percent progressive motility (PM) and percent intact acrosomes (IA) of stored spermatozoa as affected by storage temperature, storage period and incubation time. (RT= Room temperature, CT= Cooling temperature, FT= Freezing temperature.)

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تقييم الحركة المتقدمة وصلابة مقدم رأس الحيوانات المنوية فى السائل المنوى للثيران المحفوظ عند درجة حرارة الغرفة (٢٥ °م) أو بالتبريد (٥ °م) أو بالتجميد (-١٩٦ °م).

السيد السيد ابراهيم الحسانين

قسم فسيولوجى الحيوان- مركز بحوث الصحراء- المطرية- القاهرة.

هدفت هذه الدراسة إلى تقدير أقصى فترة طول عمر للحيوانات المنوية وتقييم حيويتها فى السائل المنوى المحفوظ عند درجات حرارة مختلفة . تم جمع السائل المنوى من أربع ثيران بواسطة المهبل الصناعي ، وحضن عند درجة حرارة ٣٧°م . بعد تقييم صفات السائل المنوى ، تم تخفيفه عند نفس درجة الحرارة بالمخفف CUE-Tris حتى تركيز ٦٠ مليون حيوان منوي فى الملليلتر . بعد التخفيف وتقييم صفات السائل المنوي المخفف ، تم تعبئة السائل المنوي المخفف فى أنابيب بلاستك فرنسية سعة ٠,٥ مللى . تم تقسيم الأنابيب المعبأة إلى ثلاثة أقسام متساوية لحفظ كل قسم منها عند إحدى درجات الحرارة التالية: ٢٥°م ، ٥°م ، -١٩٦°م .

بعد الأيام الثالث والسادس والتاسع من الحفظ ، تم غمر عدد عشرة أنابيب من كل مجموعة من حرارات الحفظ فى حمام مائي عند درجة حرارة ٤٠°م لمدة ٢٠ ثانيه (Thawing) ثم تم خلط محتويات العشرة أنابيب من كل مجموعة فى أنبوبة اختبار سعة ٥ملى وتحضينها عند درجة حرارة ٣٧°م لمدة ثلاث ساعات.

تم تقييم نسبة الحركة المتقدمة ونسبة صلابة مقدم رأس الحيوانات المنوية فى العينات المحضنة عند بداية التحضين (صفر ساعة) وبعد ثلاثة ساعات من التحضين ، وذلك لتقدير :-

١- تأثير الحفظ عند درجات الحرارة المختلفة لفترات مختلفة على صفات الحيوانات المنوية.

٢- تأثير التحضين لمدة ثلاث ساعات على صفات السائل المنوي.

أظهرت الدراسة مايلى:-

١- أدى تخفيف السائل المنوي إلى انخفاض نسبة الحركة المتقدمة بحوالي ١٦٪ من قيمتها قبل التخفيف ، كما أدى إلى انخفاض نسبة صلابة مقدم راس الحيوانات المنوية بحوالي ٥٪ .

٢- حفظ السائل المنوي المخفف عند درجة حرارة الغرفة (٢٥°م) أو بالتبريد (٥°م) لمدة ٣-٦ أيام أظهر أعلى صفات خصوبة للحيوانات المنوية عن صفات نظيرتها المحفوظة بالتجميد (-١٩٦°م) لنفس المدة.

٣- أدت زيادة مدة الحفظ لاكثر من ستة أيام إلى انحدار واضح فى صفات السائل المنوي المحفوظ عند درجة حرارة الغرفة أو بالتبريد ، بينما كان التأثير إيجابيا على استعادة الصفات الحيوية للسائل المنوي المجمد بزيادة مدة الحفظ لاكثر من ستة أيام.

مما سبق يتضح أن تخفيف السائل المنوي بالمخفف CUE-Tris وحفظة عند درجة حرارة الغرفة أو بالتبريد يستدعى سرعة استخدامه فى التلقيح الصناعي خلال الستة أيام الأولى من الحفظ . أما بالنسبة للسائل المنوى المجمد، فانه ينصح باستخدامه فى التلقيح الصناعي بعد فترات أطول من الحفظ نظرا للتحسن النسبى فى الصفات الحيوية به مع زيادة مدة الحفظ.