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INNOVATIVE IDEAS FOR RAM SEMEN FREEZING

c. EFFECT OF EXTENDER COMPOSITION, COOLING RATE, AND THAWING METHOD ON RAM SEMEN FREEZABILITY

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ABSTRACT: Two experiments were implemented in this investigation. Semen was collected from four adult Ossimi rams using an artificial vagina in the two experiments (V and VI). Ejaculates were pooled together and gently mixed to study; effect of two cooling rates of the fresh semen were examined i.e., (fast cooling: cooling from 30-32°C to 15°C over 10 minutes versus slow cooling: cooling from 30-32°C to 15°C over 30 minutes), and three thawing rates (T_1, T_2 and T_3) were tested: T_1 involved thawing frozen straws at 42 °C for 35 seconds; T_2 consisted of thawing frozen straws at 100 °C for 4 seconds, followed by 42 °C for 5 seconds; T_3 consisted of thawing frozen straws at 100 °C for 5 seconds, followed by 42 °C for 5 seconds.

In the previous experiment IV, two extenders, A-extender and C-extender, were used. All processing, semen evaluation, and statistical analysis were the same.

The results from experiment V indicated that the fast-cooling rate significantly affected the motility and viability percentages of ram spermatozoa after glycerolation, equilibration, and post–thawing compared to slow cooling. The extender effect on both sperm motility and viability percentages was also significant after glycerolation ($P<0.05$) and equilibration ($P<0.01$), and insignificantly on post–thawing. The final results of post-thawing motility and livability were (66.9 and 65.8%, respectively) for A-extender and (64.4 and 64.3%, respectively) for C-extender.

Experiment VI was run in the same order as Experiment V but used fast cooling. The obtained result from this experiment indicated that the A-extender obtained the best post–thawing motility and viability with (T_2) thawing rate; they were (70.6 and 68.9%), respectively. The C-extender showed the best post–thawing motility and viability percentage with (T3) thawing rate (68.8 and 67.9%), respectively. The effect of interaction between extender and thawing rate on post–thawing motility and viability percentage was significant ($P<0.01$).

It is recommended to use A-extender or C-extender added on one step at 15°C over 10 minutes, a 4.5-hour equilibration period, freezing rate (F_3) and thawing rate (T_2) with A-extender or (T_3) with C-extender to ram semen to improve semen freezability.

Keywords: Ram semen, Freezing, Extender, Cooling rate, Thawing method

INTRODUCTION

Several factors affect ram semen freezability; some important factors are cooling and thawing (Salamon and Maxwell, 2000). Cold-shock sensitivity in ram spermatozoa is higher than in other species (Salamon and Maxwell, 1995). Extender, dilution, cooling rate, freezing rate, and thawing techniques all play a role in the success of ram semen cryopreservation (Pontbriand *et al.*, 1989; Tekin *et al.*, 2006). According to Lebouef *et al.* (2000), rapid cooling of extended semen from 30 to 15°C may not affect sperm survival. However, Watson (2000) reported that the fast cooling from 30 to 10, 5, or 0°C causes injuries in some sperm cells, called "cold shock". Quinn *et al.* (1980) indicated that rapid cooling of diluted ram semen to a temperature above 15°C did not result in additional changes to spermatozoa before freezing and thawing. Similar results were obtained by Fiser and Fairfull (1986), who

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reported that rapid cooling of extended semen from 30°C to 15°C did not affect the survival of ram spermatozoa after freezing and thawing. Meanwhile, cooling from 30°C to either 10 °C, 5 °C, or 0 °C decreased the post–thawing motility of ram sperm.

Thawing frozen semen activates it physiologically; hence, it is essential that thawing is done carefully at an optimal temperature with sufficient time to minimize the loss of semen quality (Borah *et al.,* 2015). Research on thawing protocols has shown that the thawing rate significantly affects the motility and functional traits of frozen-thawed sperm in conventional insemination doses (Rastegarnia *et al.,* 2013). Most available data in the literature indicated that ram semen frozen in straws has been thawed by most investigators at 37 to 42°C. Multiple studies have evaluated the effect of heating rate on sperm function. It has been reported that thawing at high temperatures increases the motility and kinematic characteristics of the sperm (Rastegarnia *et al.*, 2013).

This led us to conduct this study to determine the best cooling rate for the pooled semen before adding the extender and the best thawing method to improve the motility and livability of spermatozoa after thawing.

MATERIALS AND METHODS

Experiment V

Based on the results obtained from the previous experiment IV. This experiment used A and C extenders and freezing rate F_3, which was used in the previous experiment IV, to study the effect of two cooling rates of pooled semen on motility and livability of sperm during pre-freezing and post-thawing processing. Fast cooling rate; the semen was cooled from 30–32°C to 15°C over 10 minutes, versus slow cooling over 30 minutes, as the same cooling rate was used in the previous experiment IV. Then the pooled semen was mixed with the extenders at that temperature and further cooled to 5°C over 1.5 hours. All processing and ram semen evaluations were the same as those reported in the previous experiment IV, and the results obtained were statistically analyzed according to Snedecor and Cochran (1980).

Experiment VI

The results obtained from the previous experiment V indicated that fast cooling was better than slow cooling. This experiment used a fast-cooling rate to study the effect of three suggested thawing rates of frozen semen on post-thawing motility and livability of spermatozoa. The three thawing rates were conducted in a water bath as follows:

At 42°C for 35 seconds (T_1).

At 100°C for 4 seconds, then at 42°C for 5 seconds (T_2).

At 100°C for 5 seconds, then at 42°C for 5 seconds (T_3).

In this experiment, all processing and ram semen evaluation were the same as had been reported in the previous experiment V, and the obtained results were statistically analyzed according to Snedecor and Cochran (1980).

RESULTS AND DISCUSSION

Experiment V

This experiment aimed to examine the effect of the cooling rate on the ram semen from 30–32°C to 15°C, either in 10 minutes (fast cooling) or over 30 minutes (slow cooling), combined with the effect of the extender.

Results obtained in this concept are shown in Table 1. The tabulated data illustrated that cooling rate significantly affected the motility and livability of ram spermatozoa after glycerolation, equilibration, and post–thawing. In this respect, fast cooling recorded greater values of sperm motility and livability at all stages of semen processing, and the respective improvement was much more pronounced after glycerolation of semen processing. The reduction in motility and livability after glycerolation was 2.5 and 4.0%, respectively. After the equilibration period, there were only 4.7 and 4.6%, respectively. This confirms the efficiency of these extenders, which contain a high percentage of glycerol (14.5%), and the new method of adding the extender to semen maintains a very high percentage of sperm motility and livability before freezing.

Table 1: Overall average of motility and livability percentage of spermatozoa as affected by cooling rate

Stage of semen processing	Motility %		Livability %	
	Cooling rate		Cooling rate	
	Fast	Slow	Fast	Slow
Glycerolated	89.4	86.9**	88.6	86.2**
Equilibirated	84.7	82.6*	84.0	81.5*
Post-thawing	65.7	62.5**	65.1	61.5**
Reduction:				
Glycerolated	2.5	5.0	4.0	6.4
Equilibirated	4.7	4.3	4.6	4.8
Post-thawing	19.0	20.1	18.9	20.1
Pre-freezing reduction	7.2	9.3	8.6	11.2
Total	26.4	29.4	27.5	31.2
Recovery rate as % of the initial	71.5	68.0	70.3	66.4

Initial motility : 91.9 % Initial viability: 92.6 %

* Significantly differs (P<0.05).

** Significantly differs (P<0.01).

We also noted that reducing the time required to reach 15°C by using fast cooling from 30 to 10 minutes maintained a high percentage of motility and livability of sperm after adding the extender. This may be due to the sperm maintaining a high degree of vitality and increasing their ability to resist the harmful effects of the extender.

In other words, the fast cooling resulted in greater recovery rates of both sperm motility and livability, which were expressed as percentages of initial values of 71.5 and 70.3%, respectively.

The extender effect on sperm motility and livability was significant (Table 2) after glycerolation equilibration regarding the former two semen traits. In this respect, the C-extender was superior to the A-extender. On the other hand, the post–thawing sperm motility and livability were not significantly affected by the extender. Our results agreed with some investigators; Quinn et al. (1980) indicated that rapid cooling of diluted ram semen to temperatures above 15°C did not result in additional changes of spermatozoa before freezing and thawing. Similar results were obtained by Fiser and Fairfull (1986), who reported that rapid cooling of extended semen from 30°C to 15°C did not affect the survival of ram spermatozoa after freezing and thawing.

During cooling process, the temperature range of 5–15°C is the most critical for cells' damage, because it is more related to changes in plasma membranes' fatty acid composition and lipid class ratios, whilst the uptake of calcium contributes to similar to acrosome reaction, capacitation changes (Drobnis *et al.,* 1993). However, Watson (2000) reported that the fast cooling from 30 to 10, 5, or 0°C causes injuries in some sperm cells, called "cold shock". Our results agreed with this finding, too, since we cooled semen from 30 to 15°C at a fast rate, then from 15 to 5°C over 1.5 h using a slow rate.

Table 2: Interaction effect between extender and cooling rate on motility and livability percentage of spermatozoa

Stage of semen processing	A-Extender			C-Extender		
	Cooling rate			Cooling rate		
	Fast	Slow	Average	Fast	Slow	Average
Sperm motility (%):						
Glycerolated	88.1	86.3	87.2	90.6	87.5	89.05*
Equilibrated	83.1	81.3	82.2	86.3	83.8	85.05**
Post-thawing	66.9	63.1	65.0	64.4	61.9	64.7
sperm livability (%):						
Glycerolated	87.6	85.4	86.5	89.6	87.0	88.3*
Equilibrated	82.5	80.2	81.4	85.4	82.7	84.1**
Post-thawing	65.8	62.1	64.0	64.3	60.9	62.6

Initial motility: 91.9 % Initial viability: 92.6 %

* Significantly differs (P<0.05).

** Significantly differs (P<0.01).

Experiment VI

This experiment was designed to examine the effect of three thawing rates (T_1, T_2, and T3) on ram semen motility and livability in combination with the extender's effect.

Results obtained in this concept are shown in Tables 3 and 4. Thawing rate significantly (P<0.01) affected the percentage of motility and livability of ram spermatozoa. The same was true for either the extender effect or the interaction of extender thawing rate. The effect of thawing rate on sperm motility and livability percentage was significant, being higher for T_1 (42°C for 35 sec) than those of T_2 (100°C for 4 sec, then 42°C for 5 sec) and T_3 (100°C for 4 sec, then 42°C for 5 sec).

The reduction in sperm motility percentage reached the lowest values for T1 (17.8%) versus 20.9 and 19.3% for T2 and T3, respectively. Such differences also reflected a higher recovery rate as a percentage of the initial motility percentage in fresh semen in T1 than T2 and T3 (71.1 vs 67.7 and 69.4%, respectively). It is worth noting that the change trend in the percentage of sperm livability was nearly the same as the sperm motility percentage.

Table 3: Overall average of motility and livability and percentage of ram spermatozoa as affected by thawing method

Stage of semen processing	Motility %			Livability %		
	Thawing rate			Thawing rate		
	T_1	T_2	T_3	T_1	T_2	T_3
Glycerolated	88.2	88.2	88.2	88.0	88.0	88.0
Equilibrated	82.2	82.2	82.2	81.0	81.0	81.0
Post-thawing	64.4 [a]	61.3 [b]	62.9 [b]	63.3 [a]	60.0 [b]	61.9 [b]
Reduction rate (%)						
Pre-freezing	8.4	8.4	8.4	9.7	9.7	9.7
Post-thawing	17.8	20.9	19.3	17.7	21.0	19.1
Total	26.2	29.3	27.7	27.4	30.7	28.8

Initial motility: 90.6 % Initial viability: 90.7 %

a, b, c; values within the same row with different superscripts significantly differ (P<0.05).

Table 4: Interaction effect between extender and thawing rate on percentage of motility and livability of ram spermatozoa

Stage of semen processing	A-extender			C-extender		
	Thawing rate			Thawing rate		
	T_1	T_2	T_3	T_1	T_2	T_3
Sperm motility (%)						
Glycerolated	87.5	87.5	87.5	88.8	88.8	88.8
Equilibrated	81.3	81.3	81.3	83.1	83.1	83.1
Post-thawing	65.6 ^{b}	70.6 ^{a}	56.9 ^{d}	63.1 ^{c}	51.9 ^{e}	68.8 ^{a}
Recovery rate (%)	72.4	77.9	62.8	69.6	57.3	75.9
Sperm livability (%)						
Glycerolated	87.4	87.4	87.4	88.7	88.7	88.7
Equilibrated	80.2	80.2	80.2	81.9	81.9	81.9
Post-thawing	64.4 ^{b}	68.9 ^{a}	55.9 ^{d}	62.6 ^{c}	51.1 ^{e}	67.9 ^{a}
Recovery rate (%)	71.0	75.9	61.6	68.5	56.3	74.8

Initial motility: 90.6 % Initial viability: 90.7 %

a, b, c, d, e values in rows within each trait having different superscripts significantly differ ($P<0.05$).

Based on the present results in Table 4, the thawing rate showed the best results related to motility and livability of spermatozoa in thawed semen, regardless of the type of extender. In this respect, thawing of the frozen ram semen diluted with A–extender at 100°C for 4 seconds, then at 42°C for 5 seconds (T_2) was better than thawing at 42°C for 35 seconds (T_1) or at 100°C for 5 seconds, then at 42°C for 5 seconds (T_3). Meanwhile, thawing of the frozen ram semen diluted with C–extender at 100°C for 5 seconds, then 42°C for 5 seconds (T_3) was better than thawing at 42°C for 35 seconds (T_1) or at 100°C for 4 seconds, then 42°C for 5 seconds (T_2). Post–thawing motility for A-extender was 65.6, 70,6, and 56.9% with T1, T2, and T3, respectively, while they were 63.1, 51.9, and 68.8% with C-extender. Post–thawing livability for A-extender was 64.4, 68,9, and 55.9%, with T1, T2, and T3, respectively, while they were 62.2, 51.1, and 67.7%, with C-extender. Reduction of spermatozoa motility after thawing was 25.0, 20.0, and 33.7% for T1, T2, and T3, respectively, of A-extender and 27.5, 38.7, and 21.8% for T1, T2, and T3, respectively, of C-extender. A–extender gave a high post-thawing recovery rate with T2 being 77.5%, while C–extender gave the best post-thawing recovery rate with T3 being 75.5%, when computed as a percent of the initial. The tabulated data indicate that T_2 is the suitable thawing rate for A-extender, while T3 is suitable for C-extender. This showed that the thawing rate is affected by the composition of the diluent. These findings suggested that not only is thawing temperature an effective factor in post–thaw motility and livability, but also the duration of exposure of the straw to the thawing temperature.

The thawing procedure is just as important as the freezing procedure in terms of its impact on the survival of spermatozoa (Nur *et al.,* 2003). It is known that freezing and thawing procedures drastically decrease ram spermatozoa's viability and motility (Fukui *et al.*, 2010). Medeiros *et al.* (2002), a relatively high proportion (40-60%) of ram spermatozoa preserve their motility after freeze-thawing, but only about 20-30% remain biologically functional. That is the main limitation for using post-thawed semen via the method of cervical insemination (Byrne *et al.*, 2000). Various factors of interaction with thawing procedures affect the post-thawing

motility of sperm, such as the type of extender, concentration of glycerol, method of semen packing, freezing rate, semen handling during, and experimental conditions. (Vishwananth and Shannon, 2000; Thibier and Wagner, 2002). Foote (1999) states that a semen straw is usually thawed in a 37°C water bath for 12–30 seconds. Sperm quality is harmed during freezing and thawing because sperm cells are exposed to two crucial temperature zones (- −5 to −60°C) once during cooling to −196 °C and again while thawing. Fast thawing is required to prevent re-crystallization of intracellular ice (Ntemka *et al.*, 2018). When sperm are thawed quickly, they are exposed to a concentrated solute and cryoprotectant for a short time, and the restoration of intracellular and extracellular equilibrium is faster than when they are thawed slowly. However, many studies have shown that thawing temperatures as high as 60-80°C could improve post-thaw motility (Dhami *et al.*, 1996). Caifeng *et al.* (2021) reported that the highest ram sperm motility and membrane integrity were obtained when the thawing was at 70°C for 5 seconds. Anderson and Aamdel (1972) reported that thawing at 75°C was superior to thawing at 35°C. Johanson *et al.* (1974) found that thawing of ram spermatozoa at 96.5°C was advantageous for sperm motility recovery. Paulenz *et al.* (2004) compared three thawing rates (70°C/5 seconds, 50°C/9 seconds, and 35°C/12 seconds) on lambing rate and concluded the superiority of the 70°C/5 seconds in this concept (77.6%), and the least was the 50°C/9 seconds (66.1%).

Our study agreed with these findings, when using a high temperature for thawing (100°C) led to reducing the period that sperm spend at a temperature between -15 and -5 °C, where ice crystals harmful to sperm are formed. So that thawing at 100°C was much better on sperm motility and livability compared to thawing at 42°C. In addition, thawing at 100°C (the boiling point of water) is easy to perform in farm conditions. On the other hand, Pontbriand *et al.* (1989) found that thawing at 30–40°C or 50–70°C did not affect post-thawing motility of ram spermatozoa. This conflict may be due to the type of extender and the time spent on straw thawing.

The advantages of the study:

The study included innovative ideas for freezing and thawing ram semen.

1. The study succeeded in using new extenders, one of which was Tris extender and the other Tris extender mixed with skim milk. Each one was characterized by containing a high percentage of glycerol, 14.5%, which is very high above 8% without any toxic effect on sperm.
2. The study succeeded in finding a new method for adding the extender to semen by adding the extender to raw semen in one step at a temperature of 15°C. This method was characterized by maintaining a high percentage of sperm motility and livability after a 4.5-hour equilibration period compared to the known methods used. The decrease in sperm motility after the equilibrium period was only 9.3 and 7.5% for the two extenders, respectively, which is a terrific percentage.
3. The study used an easy and simple manual method to control the freezing rate, which is suitable for researchers who cannot use an automatically programmed bio-freezer to do so.
4. The study succeeded in arriving at the new freezing rate and thawing method for freezing semen by thawing in two stages: fast thawing in the beginning at 100°C to prevent re-crystallization of intracellular ice quickly, then a temperature of 42°C to avoid the harmful effects of a significant increase in temperature on sperm. These methods gave better results for sperm motility and viability.
5. The study succeeded in obtaining a high percentage of motility and livability of sperm after thawing, which were 70.6% and 68.9%, respectively, for the Tris extender, and 68.8% and 67.9%, respectively, for the Tris extender mixed with skim milk.
6. The study was able to obtain a high recovery rate of motility and viability of sperm after thawing, reaching 77.9% and 75.9%, respectively, with the Tris extender and

77.1% and 74.8%, with Tris extender mixed with skim milk. We expect this will be accompanied by an improvement in the fertility rate using semen frozen in this method, which allows for its practical use in field conditions.

7. We believe this study will open the way for studying semen freezing with new and innovative ideas not present in previous studies.

In the final analysis, this investigation necessitates the execution of numerous experiments to verify its findings, enhance these findings, and ascertain the fertilization rate that results from using frozen semen in this method, whether through artificial insemination of ewes or in vitro. We believe that these novel concepts can be implemented with an additional species.

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أفكار مبتكرة لتجميد السائل المنوي للأغنام
ج. تأثير معدل التبريد ومعدل الإسالة على قابلية السائل المنوي للأغنام للتجميد.

ممدوح الغنيمي ، حلمي عبد الرحمن ، إبراهيم صديق ، عبد الله نيبر
قسم الإنتاج الحيواني – كلية الزراعة – جامعة المنوفية – جمهورية مصر العربية .

الملخص العربي

اشتملت هذه الدراسة على تجربتين هما:

التجربة الخامسة: استخدمت التجربة مخففين هما مخفف ترس (المخفف أ) ومخفف ترس به لبن فرز ناتج من خلط المخفف أ مع المخفف ب المستخدمان في التجربة السابقة (التجربة الرابعة) بنسبة ٣ أ : ١ ب (المخفف ج) لدراسة تأثير معدل التبريد على قابلية السائل المنوي للكباش للتجميد وفي هذه التجربة تم دراسة معدلين للتبريد هما: تبريد سريع للسائل المنوي قبل التخفيف من درجة ٣٠ – ٣٢ °م إلى ١٥°م على مدى ١٠ دقائق مقارنة بالطريقة البطيئة حيث تم التبريد من درجة ٣٠ – ٣٢ °م إلى درجة ١٥°م على مدى نصف ساعة. واتبعت التجربة نفس الإجراءات المتبعة في التجربة الرابعة مع استخدام معدل التجميد ف٣.

التجربة السادسة: في هذه التجربة تمت مقارنة ثلاث طرق مختلفة لإسالة السائل المنوي المجمد (س١ , س٢ , س٣) وذلك على النحو التالي:

س١: على درجة ٤٢°م / ٣٥ ثانية.
س٢: على درجة ١٠٠°م / ٤ ثانية ثم على درجة ٤٢°م / ٥ ثانية.
س٣: على درجة ١٠٠°م / ٥ ثانية ثم على درجة ٤٢°م / ٥ ثانية.

وقد استخدمت التجربة نفس المخففين المستخدمين في التجربة السابقة. وقد اتبعت التجربة نفس طريقة الإضافة والتبريد وفترة التوازن والتعبأة والتجميد والتقييم المتبعة في التجربة السابقة مع استخدام معدل التبريد السريع. وكانت النتائج المتحصل عليها من هاتين التجربتين كما يلي:

تأثرت النسبة المئوية للحيوانات المنوية المتحركة والحيةً معنوياً سواء بعد إضافة المخفف أو بعد الموازنة وبعد الإسالة بمعدل التبريد للسائل المنوي الخام قبل التجميد (من ٣٠ – ٣٢ °م إلى ١٥ °م) حيث كان معدل التبريد السريع على مدي ١٠ دقائق أفضل من المعدل البطيء على مدي ٣٠ دقيقة.

كان أداء المخفف (أ) أفضل من المخفف (ج) خاصة عند إتباع نظام الإسالة س٢ حيث بلغت النسبة المئوية للحركة بعد الإسالة ٧٠.٦ ٪ ، ٥١.٩ ٪ على الترتيب والعكس في حالة استخدام نظام الإسالة س٣ حيث تفوق المخفف (ج) على المخفف (أ) في النسبة المئوية للحركة بعد الإسالة فكانت ٦٨.٨ ٪ ، ٥٦.٩٪ على الترتيب.

ويمكن التوصية بتجميد السائل المنوي للكباش باستخدام مخفف الترس (أ) أو مخفف الترس المضاف إليه لبن فرز (ج) وإضافته على خطوة واحدة عند درجة ١٥°م على مدى ١٠ دقائق مع فترة موازنة ٤,٥ ساعة واستخدام معدل التجميد ف٣ ومعدل الإسالة س٢ مع المخفف (أ) ومعدل الإسالة س٣ مع المخفف (ج) للحصول على أفضل النتائج.

الكلمات المفتاحية: الحيوانات المنوية، الأغنام، التجميد، المادة المضافة، معدل التبريد، طريقة الذوبان.