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INNOVATIVE IDEAS FOR RAM SEMEN FREEZING

b. EFFECT OF EXTENDER COMPOSITION, EQUILIBRATION PERIOD AND FREEZING RATE ON RAM SEMEN FREEZABILITY

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ABSTRACT: Semen was collected from four adult Ossimi rams using an artificial vagina. Ejaculates were pooled together and gently mixed to study the effects of equilibration period and freezing rate on ram semen freezability in liquid Nitrogen (-196°C). Two extenders were used: Tris-based extender (A-extender) and Tris-based extender containing skim milk (C-extender). Experiment III examined three equilibration periods (3.0, 4.5, and 6 hours). Three Preliminary freezing rates (F_1, F_2, and F_3) were examined in experiment IV. Ram semen freezability was determined based on the percentage of motility and viability of spermatozoa at various stages of the freezing and thawing process.

The results indicated that the equilibration period significantly influenced pre-freezing and post–thawing motility and livability of ram spermatozoa ($P<0.01$). The highest post-thawing sperm motility and livability were found with A-extender or C-extender after a 4.5 h equilibration period. Values in this respect were (56.9 and 56.4%, respectively), for motility and livability with A-extender, while the same traits revealed values of (53.8 and 52.5%, respectively), with C-extender. The lowest values found with 3.0 h were 48.1 and 46.8%, with A-extender and (41.5 and 40.4%, respectively), with C-extender. However, the highest percentage of post-thawing sperm motility and livability after a 4.5 h equilibration period was 54.7 and 55.4 %, respectively.

The freezing rate (F_3) significantly ($P<0.01$) yielded superior post-thawing sperm properties. Meanwhile, F1 yielded inferior values. A-extender was better than C-extender. Post-thawing motility and livability with A-extender were 63.1 and 62.0%, respectively, while with C-extender, the same traits revealed 61.9 and 61.2% values, respectively.

To improve ram semen freezability, it is recommended that an A-extender be added in one step at 15°C with a 4.5-hour equilibration period and a freezing rate (F3).

Keywords: Ram semen, Freezing, Extender, Equilibration, Freezing rate

INTRODUCTION

Several factors, including the equilibration period and freezing rate, affect ram semen freezability (Salamon and Maxwell, 2000).

It was reported that ram semen should not be cryopreserved immediately after ejaculation, and the equilibration of ram semen at 5°C is necessary to secure freezing efficiency (Purdy *et al.*, 2010, and Ntemka *et al.*, 2018). Salamon and Maxwell (1995) reported that traditionally, equilibration has been regarded as the total time spermatozoa remain in contact with glycerol before freezing, during which it penetrates the sperm cell to establish a balanced intracellular and extracellular concentration. Several studies found no significant differences between ram spermatozoa equilibrated for 1 or 3 hours (Watson and Martin, 1975a), 2, 4 or 6 hours (Hinshelwood *et al.*, 1980; Fiser and Batra, 1984), 3 or 5 hours (Kalalharana *et al.*, 1986), and 20 or 150 minutes (Colas, 1975).

Freezing rate for ram semen is an important factor in achieving high-quality sperm after thawing. It is known that freezing and thawing procedures drastically decrease ram

spermatozoa's viability and motility (Fukui *et al.*, 2010). Cells and their surrounding media stay unfrozen when cooled to around −5°C. The exterior media freezes between −5 and −10°C, and the cell contents remain unfrozen. Low temperature, especially sub-zero, imparts physical and chemical changes in the cell membrane, damaging sperm functionality or even causing death. These damages are due to oxidative stress, cold, and osmotic shocks imparted on the sperm during freezing and thawing procedures (Aitkin, 2020).

Freezing rate interacted with glycerol level in the diluent (Fiser and Fairfull, 1984). Additionally, a crucial temperature range (approximately from -5 to -50 °C) has been confirmed as the range when ice crystal formation and spermatozoa dehydration occur (Kumar *et al.*, 2003). Generally, the freezing rate can kill cells either too high or too low. Mazure *et al.* (1972) demonstrated that an optimal cooling rate for cell cryo-survival should exist between high and low rates. Therefore, many studies have tried to improve the quality of ram frozen-thawed semen by modifying the freezing rate and equilibration period. However, there is no agreement among researchers on a specific equilibration period, and freezing rates can be used to ram semen freeze. This has led us to conduct this study to reach an appropriate equilibrium period and method of freezing for the diluents and the technique used to get better results from the motility and livability of sperm after thawing.

MATERIALS AND METHODS

Experiment III

The results of the previous experiment II indicated that A-extender was the best for sperm motility and livability after thawing, while B-extender was the best before freezing. This experiment used A-extender and C-extender, resulting from mixing (3A/1B, v/v respectively). This experiment was designed to study the effect of three equilibrium periods on the motility and viability of sperm during freezing and thawing stages. However, three equilibration periods were examined in this experiment (i.e., 3.0, 4.5, and 6 hours). These periods included 1.5 hours during which the semen was cooled from 15 to 5°C, then 1.5, 3.0, and 4.5 hours at 4–5°C, respectively. This experiment used the same one-step procedures for raw semen at 15°C as the previous experiment II, as well as the same processing and semen evaluation methods.

Experiment IV

Depending on the results of experiment III, this experiment used A and C extenders, 4.5 h as the equilibration period to study the effect of three preliminary freezing rates on ram semen freezability. All processing and semen evaluation were as in the previous experiment, and the obtained results were statistically analyzed according to Snedecor and Cochran (1980). Preliminary freezing rates are shown in the following Table.

Table 1: Preliminary freezing rate and time spent to reach the subzero temperature –16.5 °C (F_1, F_2, and F_3)

Time(minute)	0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5
Temp,°C (F_1)	4.0	- 4.0	- 7.0	- 9.0	-10.5	-12.0	-13.5	-15.0	-16.5	__
Temp,°C (F_2)	4.0	- 1.0	- 7.0	- 9.0	-10.5	-11.0	-11.5	-13.0	-14.5	-16.0
Temp,°C (F_3)	4.0	- 1.0	- 7.0	- 9.0	-10.5	-12.0	-13.5	-15.0	-16.5	__

RESULTS AND DISCUSSION

Experiment III

The effect of three equilibration periods (3.0, 4.5, and 6.0 h) in combination with the extender on pre-freezing and post–thawing percentage of motility and livability of ram spermatozoa was examined in this experiment, and the obtained results are presented in Table 2. The tabulated data indicate that the extender and the equilibration period significantly affected pre-freezing and post–thawing motility and viability

of ram spermatozoa (P<0.01). The interaction effect between extender and equilibration period was only significant on the percentage of pre-freezing (P<0.01) and post–thawing (P< 0.05) sperm motility and livability (Tables 2 and 3).

Table 2: Overall average of motility and livability percentage of ram spermatozoa as affected by equilibration period

Stage of semen processing	Motility %			Livability %		
	Equilibration period, h			Equilibration period, h		
	3.0	4.5	6.0	3.0	4.5	6.0
Pre-freezing	82.9 [a]	80.9 [a]	78.4 [b]	83.8 [a]	82.2 [a]	80.3 [a]
Post-thawing	43.6 [b]	54.7 [a]	52.1 [a]	44.8 [c]	55.4 [a]	52.2 [b]
Changing rate:						
Glycerolated	- 5.3	- 5.3	- 5.3	- 4.9	- 4.9	- 4.9
Equilibration	- 3.1	- 5.1	- 7.6	- 3.1	- 4.7	- 6.6
Post-thawing	- 39.3	- 26.2	- 26.3	- 39.0	- 26.8	- 28.1
Pre-freezing change	- 8.4	- 10.4	- 12.9	- 8.0	- 9.6	- 11.5
Total	- 48.2	- 36.6	- 39.2	- 47.0	- 36.4	- 39.6

Initial motility: 91.3 % Initial livability: 91.8 %

a, b, c; values within the same row with different superscripts significantly differ (P<0.01).

Table 3: Interaction effect between extender and equilibration period on percentage motility of ram spermatozoa

Stage of semen processing	A- extender			C - extender		
	Equilibration period, h			Equilibration period, h		
	3.0	4.5	6.0	3.0	4.5	6.0
Motility %:						
Pre-freezing	82.5 [b]	80.6 [b]	78.1 [c]	85.0 [a]	83.8 [a]	82.5 [b]
Post-thawing	48.1 [c]	56.9 [a]	53.1 [b]	41.5 [d]	53.8 [b]	51.3 [b]
Reduction of motility:						
Glycerolated	5.0	5.0	5.0	3.8	3.8	3.8
Equilibration	3.8	5.7	8.2	2.5	3.7	5.0
Post thawing	34.4	23.7	25.0	43.5	30.0	31.2
Total reduction	43.2	34.4	38.3	49.8	37.5	40.0
Livability %:						
Pre-freezing	81.7 [b]	79.4 [c]	76.6 [d]	83.5 [a]	82.4 [a]	80.2 [c]
Post-thawing	46.8 [c]	56.4 [a]	52.6 [b]	40.4 [d]	52.5 [b]	51.5 [b]
Reduction of livability						
Glycerolated	6.3	6.3	6.3	5.2	5.2	5.2
Equilibration	3.8	6.1	8.9	3.1	4.2	6.4
Post thawing	3.4	23.0	24.0	43.1	29.5	28.7
Total reduction	45.0	35.4	39.2	51.4	38.9	40.3

Initial motility: 91.3 % Initial livability: 91.8 %

a, b, c, d; values within the same row with different superscripts significantly differ (P<0.01).

It has appeared that when the equilibration period increases, the percentage of pre-freezing motility and viability decreases. The post–thawing traits recorded better values with the 4.5 h equilibration period, followed by the 6.0 h equilibration period, while inferior values were obtained by the 3.0 h equilibration period. On the other hand, the C-extender was more convenient than the A-extender for the pre-freezing estimates; meanwhile, the opposite was true for the post–thawing estimates.

The results also indicated that the greatest loss in either sperm motility or viability was detected during freezing and thawing. Increasing the equilibration period to either 4.5 or 6.0 h at that time reduced such loss in sperm motility and viability. On the other hand, A- extender with a 4.5 h equilibration period showed the lowest reduction in motility and livability of spermatozoa after freezing and thawing (23.7 and 23.0 %, respectively). The lowest total reduction in sperm motility and livability were 34.4 and 35.4%, respectively, while the highest recovery rate as a percentage of initial motility and livability were 62.3 and 61.4%, respectively. Our results agreed with those obtained by Anderson and Aamdal (1972), Anderson *et al.* (1973), and First and Fairfull (1984), who reported that an equilibration period of 4.0 h was the best out of 0.0 to 20.0 h equilibration periods.

Although the decrease in sperm motility and vitality with a 3-hour equilibrium period was the least before freezing, the 4.5-hour equilibrium period was the best after thawing. This explains the importance of the duration of the equilibrium period and its impact on the success of semen freezing. Salamon and Maxwell (1995) reported that traditionally, equilibration has been regarded as the total time spermatozoa remain in contact with glycerol before freezing, during which it penetrates the sperm cell to establish a balanced intracellular and extracellular concentration. Equilibration includes the concentration balance of glycerol and other osmotically active diluent components. The equilibration time is important for sperm membranes to adapt to low temperatures (Leite *et al.*, 2010).

The effect of equilibration time on sperm post-thaw motility and survival can be improved by an interaction with an extender (Leite *et al.*, 2010), and the extent of this improvement is dependent on the extender (Fleisch *et al.*, 2017) or cryoprotectant used (Eriani *et al.*, 2017). There is still no agreement on the best equilibration time for semen quality after cryopreservation. Jakub *et al.* (2021) and Lv *et al.* (2019), reported a usual equilibration length for ram sperm in the range of 2–4 h while others reported that the different equilibration times ranging from 1 to 5 hours were employed in rams, with varying post-thaw seminal quality (Galarza *et al;* 2019, and Jha *et al.*, 2019).

Experiment IV

This experiment investigates the effects of three preliminary freezing rates (F_1, F_2, and F3) on post–thawing motility and livability of ram semen. In this experiment, different cooling rates were used. In the preliminary freezing stage, slow cooling rates were used from +4 to -16 or -16.5 °C. When this is done, the cell would be frozen, which made us expect that ultra-rapid freezing rate would be best suited for the next stage, by plunging the straw directly into liquid nitrogen.

The obtained results are shown in Table 4. The tabulated data indicated that the freezing rate insignificantly affected sperm characteristics at pre-thawing stages but significantly affected all the post–thawing sperm characteristics studied ($P<0.01$). In this respect, F_3 was superior, followed by F2, and the inferior values were obtained by F_1. Similar trends were recorded for recovery rate as a percentage of the initial values of motility and livability. The effect of extender on post–thaw sperm motility and livability was also significant ($P<0.01$). A-extender was better than C-extender. Post-thawing motility and livability with A-extender were 63.1 and 62.0%, respectively, while with C-extender were 61.9 and 61.2%, respectively. Recovery rate for motility and livability with A-extender was (69.3 and 66.6%, respectively) and (67.3 and 65.7%, respectively) with C-extender.

Our results agreed with those of Hammadeh *et al.* (2001) and Nur *et al.* (2010), who found that the success of cell cryopreservation is influenced by the rate of freezing and the composition of the solution in which the cells are frozen.

When comparing the preliminary freezing rate, F1, and the freezing rate, F3, we noticed that the difference between them is the cooling speed from +4 to -4°C. This explains the importance of this temperature range on the success of the freezing process, while agreeing with Esteso *et al.* (2018) who reported the better post-thaw sperm quality with a low cooling rate between +5 and −5 °C, followed by a higher cooling rate. Our results agreed with Bag *et al.* (2002), who demonstrated that the initial freezing temperatures significantly affected ram spermatozoa's post–thawing motility. Therefore, semen must cool fast enough to avoid freezing damage and slowly enough to allow for cell dryness without producing intracellular ice. When comparing the preliminary freezing rate of F2 and F3, we notice the difference between them in the accelerating cool from -10.5 to -11°C. This explains the importance of temperature, 11°C, in the success of the freezing process, and this agrees with Salamon and Maxwell (2000), who reported that the temperature of 11°C is a critical degree for ram sperm. Mazure *et al.* (1972) demonstrated that an optimal cooling rate for cell cryo-survival should exist between high and low rates, which also agrees with our results.

Table 4: Effect of extender and freezing rate on percentage of motility, livability, and abnormality of ram spermatozoa at various stages of freezing and thawing

Stage of semen processing	A-Extender			C-Extender		
	Freezing rate			Freezing rate		
	F_1	F_2	F_3	F_1	F_2	F_3
Sperm motility (%):						
Glycerolation (G)	86.9	86.9	86.9	88.1	88.1	88.1
Equilibration (E)	81.2	81.2	81.2	83.1	83.1	83.1
Post-thawing (PT)	56.9 ^{b}	58.1 ^{b}	63.1 ^{a}	53.1 ^{c}	56.2 ^{b}	61.9 ^{a}
Reduction of motility (%):						
Stage 1, (I-G)	5.0	5.0	5.0	3.8	3.8	3.8
Stage 2, (G-E)	5.7	5.7	5.7	5.0	5.0	5.0
Stage 3, (E-PT)	24.3	23.1	18.1	30.0	26.9	21.2
Total reduction	35.0	33.8	28.8	38.8	35.7	30.0
Sperm livability %:						
Glycerolation (G)	85.7	85.7	85.7	87.8	87.8	87.8
Equilibration (E)	80.0	80.0	80.0	82.4	82.4	82.4
Post-thawing (PT)	55.3 ^{b}	55.3 ^{b}	62.0 ^{a}	52.0 ^{c}	54.6 ^{b}	61.2 ^{a}
Reduction of livability%:						
Stage 1, (I-G)	7.4	7.4	7.4	5.3	5.3	5.3
Stage 2, (G-E)	5.7	5.7	5.7	5.4	5.4	5.4
Stage 3, (E-PT)	24.7	24.7	18.0	30.4	27.8	21.2
Total reduction	37.8	37.8	31.1	41.1	38.5	31.9

Initial motility: 91.9 % Initial viability: 93.1 %

a, b, c; values within the same row with different superscripts significantly differ (P<0.01).

The shape of the freezing curve is also important for straw freezing. Del Sorbo *et al.* (1995a) found that it is better to cool the semen according to a parabola–shaped curve instead of

a linear curve of decreasing temperature. This result agreed with the results obtained in the present study. The present finding proved that the freezing rate from 4 to -16 or -16.5 °C significantly affected the revival of the spermatozoa.

Generally, either too high or too low freezing rates are detrimental and can kill cells. Higher freezing rate induces intracellular ice formation, while too low freezing rate may cause excessive dehydration of the cell and the cells membranes are exposed for more extended periods to the hypertonic solutions, with possible deleterious effects such as protein and lipid extraction and the generation of reactive oxygen species (ROS), the acrosome, nucleus, mitochondria, axoneme, and plasma membrane affect by rapid temperature changes as well as the inactivation of acrosomal enzymes and the loss of phospholipids (Dalal *et al.,* 2016; Demir *et al.,* 2018; Murphy *et al.,* 2018). All of these variables have an impact on sperm freezing success.

This experiment's results were concomitant with Filipp *et al.* (2023), who showed that the inhibition of extracellular ice by 1,4-CHD does not improve the results of ram sperm ultra-rapid freezing and the destruction of the plasma membrane. Therefore, they concluded that the intracellular ice plays the role in the sperm cryodamage, at least in the model of ram sperm ultra-rapid freezing. In our experiment, using a slow initial freezing rate and extenders containing a higher concentration of glycerol allowed the sperm cell to lose their intracellular water fast enough, which prevented intracellular ice crystal formation or at least reduced it. Then, after achieving –16.5 °C (sperm cells would freeze), we used ultra-rapid freezing by plunging the strews into liquid nitrogen immediately after reaching –16.5 °C.

CONCLUSION

The results of experiment III indicated that increasing the equilibration period to 4.5 h led to the best results for sperm motility and livability after thawing. However, it also led to increased loss of sperm motility and livability before freezing. The results of experiment IV also indicated that the freezing rate F_3 gave the best results for sperm motility and livability after freezing, but the loss in sperm motility and livability was still high. This led us to conduct other experiments to reduce the loss in sperm motility and livability before and after freezing.

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أفكار مبتكرة لتجميد السائل المنوي للأغنام
ب. تأثير فترة الموازنة ومعدل التجميد 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 الثالثة: تمت لدراسة تأثير ثلاث فترات موازنة (٣ و٤,٥ و٦ ساعات) تبدأ من إضافة المخفف إلى السائل المنوي على قابلية السائل المنوي للكباش للتجميد. استخدمت الدراسة نفس المخففين المسخدمين في التجربة السابق (التجربة الثانية) مع إتباع نفس الإجراءات المتبعة في التجربة السابقة (التجربة الثانية) مع استخدام طريقة الإضافة على خطوة واحدة على درجة حرارة ١٥°م.

التجربة الرابعة: تمت لدراسة تأثير ثلاث معدلات للتجميد على قابلية السائل المنوي للكباش للتجميد.

اتبعت التجربة نفس الإجراءات المتبعة في التجربة السابقة مع استخدام فترة موازنة ٤,٥ ساعة وكانت معدلات التجميد التمهيدي كما في الجدول التالي:

الزمن بالدقيقة	صفر	٠,٥	١,٠	١,٥	٢,٠	٢,٥	٣,٠	٣,٥	٤,٠	٤,٥
درجة الحرارة(ف١)	+٤	-٤	-٧	-٩	-١٠,٥	-١٢	-١٣,٥	-١٥	-١٦,٥	-
درجة الحرارة(ف٢)	+٤	-١	-٧	-٩	-١٠,٥	-١١	-١١,٥	-١٢	-١٤,٥	-١٦
درجة الحرارة(ف٣)	+٤	-١	-٧	-٩	-١٠,٥	-١٢	-١٣,٥	-١٥	-١٦,٥	-

وأوضحت النتائج أن النسبة المئوية للحيوانات المنوية المتحركة والحية تأثرت بفترة الموازنة تأثير معنوي على مستوى ١٪ بعد الإسالة. وكانت فترة الموازنة و4,5 ساعة هي الأفضل بالنسبة للمخففين. كما أوضحت النتائج أن المخفف أ أعطى أعلى نسبة مئوية للحيوانات المنوية المتحركة والحية بعد الإسالة فكانت (٥٤,٧ و٥٥,٤ و١٥,٩١) على الترتيب.

كما أوضحت النتائج أن معدل التجميد للسائل المنوي من درجة ٤ °م إلى – ١٦.٥ °م على مدى ٤ ساعات (معدل التجميد رقم ف٣) أعطى بعد الإسالة سائل منوي كان الأعلى بالنسبة لحركة وحيوية الحيوانات المنوية بينما معدل التجميد رقم ١ أعطى أدنى هذه الخصائص عقب الإسالة.

وأظهر معدل الاسترداد للحركة والحيوية اتجاهاً مماثلاً سواء عند التعبير عنه كنسبة مئوية من القيم الابتدائية أو القيم بعد الموازنة. كما تأثرت قيم النسبة المئوية للحركة والحيوية معنوياً على مستوى ١ ٪ بالمخفف وكان المخفف (أ) أفضل من المخفف (ب) بينما لم يوجد تداخل معنوي لتأثير المخفف مع معدل التجميد على أي من خصائص السائل المنوي بعد الإسالة.

ويمكن التوصية بتجميد السائل المنوي للكباش باستخدام مخفف الترس وإضافته على خطوة واحدة عند درجة ١٥°م مع فترة موازنة ٤,٥ ساعة مع استخدام معدل التجميد التمهيدي (ف٣) للحصول على أفضل النتائج.