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Cryopreservation of Ram Spermatozoa in the Absence of Glycerol as Affected by the Dilution Method and the Presence of High Molecular Weight Compounds

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THE effects of two concentrations of Aquacide I, Aquacide II, dextran (2.5 KDa), dextran (15-20KDa), glycogin, hydroxyethyl starch, and polyvinylpyrrolidone and warm or cold dilution on ram spermatozoa preserved in the absence of glycerol were studied. Dextran (2.5KDa) at 5-10% (v/v) and hydroxyethyl starch at 5-10% (v/v) had better cryoprotecting abilities to unfrozen and frozen-thawed ram spermatozoa in the absence of glycerol as compared with the other compounds or the control. Cold dilution was significantly ($P < 0.05$) superior to warm dilution and maintained a higher percentage of motile cells post-thawing.

Keywords: Cryopreservation, ram semen, high molecular weight compounds, dilution method.

Some high molecular weight compounds have proved to be beneficial to cryosurvival of several cell types (Doebbler, 1966; Ashwood *et al.*, 1972a, b; Pribor and Pribor, 1973; Damjanovic and Thomas, 1974; Lionetti *et. al.*, 1976; McGann, 1978). Nonpenetrating cryoprotectants such as dextran and polyvinylpyrrolidone (PVP) act as external shields for the cell membranes (Karow and Webb, 1965) or may enable the cell membranes to leak reversibly with osmotic stress (Meryman, 1971). These agents function more effectively at high cooling rates (Rowe, 1966; Rapatz and Luyet, 1968). Combinations of a penetrating cryoprotective agent (glycerol) with nonpenetrating cryoprotective sugars, dextran and hydroxyethyl starch (HES) have been used to cryopreserve spermatozoa from many species including the ram (Fiser *et al.*, 1982., Graham *et al.*, Schmehl *et al.*, 1986). It has been shown that dilution method, osmotic pressure, egg yolk level, type of sugar and rates of freezing and

thawing affects the cryopreservation of ram spermatozoa in the absence of glycerol (Abdelhakeam, 1988; Abdelhakeam *et al.*, 1988a,b) However, there are no reports on the effect of polymeric compounds on the preservation of cold diluted ram spermatozoa in the absence of glycerol.

The objectives of this study were to test the effects of dilution method and non-penetrating high molecular weight compounds on the survival of unfrozen and frozen ram spermatozoa in the absence of glycerol.

Material and Methods

Extenders Preparations:

Stock solutions of seven polymeric compounds were prepared in TEST (Tes titrated with Tris) extender, at 375 mOsm osmotic pressure (OP), pH 7.0 and 10% (v/v) of maltose monohydrate solution at 375 mOsm (Abdelhakeam, *et al.*, 1988b). Measured amounts of each compound were added to 100 ml of TEST-Maltose as follows: 4 gm glycogen (GLG), 4 gm dextran 15-20 KDa (DX15), 4 gm dextran 2.5 KDa (DX2.5), 4 gm hydroxyethyl starch (HES), 4 gm polyvinylpyrrolidone (PVP), o.5 gm Aquacide I (AQI) or 0.25 gm Aquacide II (AQII). The final extenders consisted of 30% (v/v) yolk, 10% (v/v) maltose monohydrate solution, and either 5% or 10% (v/v) of each stock solution, with the remaining percentage composed of TEST solution. The final extenders had an OP of 375 mOsm and 7.0 pH. The extenders were centrifuged at 10, 000 g for 10 min and the supernatants were decanted for use. TEST-yolk-maltose buffer without additives was used as the control.

Semen Collection

Semen was collected from 4 to 5 mature rams using an artificial vagina and pooled directly in the same collecting tube. One to three ejculates were collected from each ram (9-11 ejaculates/one day of collection).

Semen Processing

Semen was mixed thouroughly before dilution 1:4 (v/v) semen to extender. Two dilution methods (DM), according to: Time (hr) at 5°C after collection and before dilution + Time (hr) at 5°C after dilution and before freezing, were used either warm dilution at 30 - 37°C soon after collection (O+4) or cold dilution at 5°C after 3 hr(3+1) (Abdelhakeam et al., 1988a, b). All treatments were represented in each semen collection. The semen was cooled slowly to 5°C over 2 hr. After 4 hr total

cooling time semen was packaged into 0.5cc French straws and frozen horizontally at 7 cm above liquid nitrogen (LN_2) level for 10 min, then plunged directly into LN_2. All semen treatments were represented in each freezing batch and stored for 24 hr before analysis.

Thawing and Semen Assay

Percentages of motile spermatozoa were estimated for unfrozen samples just prior to freezing (4 hr at 5°C) and after 24 hr storage at 5°C. Two straws from coded samples were thawed in a water bath at 37-39°C for 30 sec. The percentages of motile spermatozoa were estimated and mean values determined. Semen samples were analyzed immediately after thawing (O hr), and 4 hr at 22 - 24°C (room temperature). All motility estimates were taken at 20 power with a closed circuit black and white television camera attached to the microscope. A homogenized sample of semen was placed on a warm (37°C) glass slide covered with cover slip. The study was repeated six times (different days of collection).

Statistical Analysis

All extenders and dilution methods were analyzed statistically as a factorial design: 6 (replicates) X 15 (extenders) X 2 (dilution methods) using general linear model (GLM, Goodnight, (1979); a least-squares procedure). Mean differences were determined by Duncan's multiple range test (Sall, 1979).

Results and Discussion

The analysis of variance for percentage of motile unfrozen ram spermatozoa at 4 hr (pre-freezing) and 24 hr storage at 5°C showed highly significant differences ($P < 0.01$) between extenders. There was no significant difference between dilution methods on percentage of motile cells at 4 hr storage at 5°C (pre-freezing) ram spermatozoa (Tables 1, 3 and Fig. 1). However, after 24 hr storage at 5°C, the difference was significant ($P < 0.05$). The warm dilution method at 37°C maintained a higher motility (57.2%; $P < 0.05$) compared to the cold dilution method at 5°C (54.3%). Extenders containing 5 and 10% HES (# 12, # 13) or 5% of either dextran (#6 and #8) maintained higher percentages of motile ram spermatozoa pre-freezing (81.0, 80.6% for HES; 79.4, and 80.2% for dextran extenders, respectively) compared with the control (#1; 79.0%). HES extenders maintained the highest percentage of motile unfrozen ram spermatozoa stored for 24 hr at 5°C (#12; 65.0 and #13; 64.4%) compared to the dextran extenders (#6; 60.2 and #8; 63.5%) and the control extender

TABLE 1. Effect of High Molecular Weight (HMWM) Compounds and Dilution Methods (DM) on Percentage of Motile Unfrozen (4 hr and 24 hr at 5°C) Ram Spermatozoa in Absence of Glycerol.

HMWM *	Unfrozen			
	4 hr after dilution **		24 hr after dilution ***	
	Warm Dilution	Cold Dilution	Warm Dilution	Cold Dilution
1- control	78.3 ± 2.9 a - f	79.6 ± 3.0 a - d	62.1 ± 2.9 a - d	63.8 ± 3.1 ab
2- 5% AQI	76.7 ± 1.9 c - h	76.7 ± 2.0 c - h	56.7 ± 1.7 c - g	44.2 ± 3.0 ij
3- 10% AQI	76.7 ± 2.6 c - h	72.5 ± 2.1 hi	55.4 ± 2.3 d - g	41.7 ± 1.7 ij
4- 5% AQII	78.8 ± 2.6 a - f	78.3 ± 2.7 a - f	55.0 ± 2.9 fg	48.3 ± 2.8 hi
5- 10% AQII	75.0 ± 4.0 d - h	74.6 ± 3.3 e - i	45.0 ± 3.4 i	38.3 ± 1.1 j
6- 5% Dx15	79.6 ± 2.4 a - d	79.2 ± 2.1 a - e	60.8 ± 3.0 a - e	59.6 ± 2.6 a - f
7- 10% Dx15	77.9 ± 2.5 b - g	77.1 ± 2.2 c - h	53.3 ± 3.1 f - h	51.7 ± 3.3 gh
8- 5% Dx2.5	79.2 ± 3.1 a - e	81.3 ± 2.6 a - c	62..9 ± 1.9 a - c	64.2 ± 1.4 ab
9- 10% Dx2.5	75.4 ± 2.5 d - h	79.6 ± 2.7 a - d	57.5 ± 2.9 b - g	62.9 ± 1.7 a - c
10- 5% GLG	77.5 ± 2.9 b - g	74.2 ± 3.5 f - i	59.6 ± 3.3 a - f	52.5 ± 2.1 gh
11- 10% GLG	74.6 ± 2.1 e - i	74.6 ± 2.1 e - i	55.8 ± 2.8 d - g	47.5 ± 2.8 hi
12- 5% HES	79.2 ± 1.4 a - e	82.9 ± 2.0 a	63.8 ± 2.3 ab	66.3 ± 1.3 a
13- 10% HES	79.2 ± 2.0 a - e	82.1 ± 2.0 ab	62.9 ± 3.0 a - c	65.8 ± 1.5 a
14- 5% PVP	75.8 ± 2.9 d - h	79.2 ± 2.5 a - e	59.6 ± 2.5 a - f	60.0 ± 2.5 a - f
15- 10% PVP	70.4 ± 4.6 i	73.3 ± 2.8 g - i	47.5 ± 3.1 hi	47.5 ± 2.5 hi

* (1) control TEST - yolk - maltose without additives; (2) 5% (v/v) Aquacide I; (3) 10% (v/v) Aquacide I; (4) 5% (v/v) Aquacide II;(5) 10% (v/v) Aquacide II; (6) 5% (v/v) dextran 15-20 KDa; (7) 10% (v/v) dextran 15-20 KDa; (8) 5% (v/v) dextran 2.5 KDa; (9) 10% (v/v)dextran 2.5 K Da; (10) 5% (v/v) glycogen; (11) 10% (v/v) glycogen; (12) 5% (v/v) hydroxyethyl starch; (13) 10% (v/v) hydroxyethyl starch; (14) 5% (v/v) polyvinylpyrrolidone; (15) 10% (v/v) polyvinylpyrrolidone.

** a-i Means followed by the same letter vertically or horizontally are not significantly different (P > 0.05).

*** a-j Means followed by the same letter vertically or horizontaly are not significantly different (P > 0.05).

TABLE 2. Effect of Different High Molecular Weight (HMWM) Compounds and Dilution Methods (DM) on Percentage of Motile Frozen-Thawed (Ohr and 4hr post-thawing) Ram Spermatozoa in Absence of Glycerol.

HMWM *	Frozen			
	0 hr post-thawing **		4 hr post-thawing **	
	Warm Dilution	Cold Dilution	Warm Dilution	Cold Dilution
1- control	22.3 ± 2.2 i - l	39.4 ± 2.6 a - c	15.2 ± 2.0 g - i	34.0 ± 2.3 a
2- 5% AQI	19.2 ± 1.9 k-m	30.2 ± 2.5 e - g	11.2 ± 2.1 h - m	23.0 ± 3.1 ef
3- 10% AQI	14.4 ± 1.5 mn	26.5 ± 2.4 g - i	8.4 ± 1.7 k - n	16.3 ± 2.9 gh
4- 5% AQII	14.2 ± 2.4 mn	29.8 ± 3.0 fg	9.2 ± 1.3 j - n	21.2 ± 3.7 ef
5- 10% AQII	7.4 ± 1.0 Op	21.3 ± 2.1 i - l	4.3 ± 0.5 no	10.5 ± 1.9 i - m
6- 5% Dx15	17.5 ± 2.3 lm	35.5 ± 2.7 cd	10.0 ± 1.7 i - m	24.4 ± 2.6 de
7- 10% Dx15	11.9 ± 1.2 no	28.1 ± 1.9 f - h	7.0 ± 1.1 m - o	18.8 ± 2.4 fg
8- 5% Dx2.5	20.4 ± 2.1 j - l	41.3 ± 3.0 ab	12.9 ± 2.0 h - l	32.9 ± 2.5 ab
9- 10% Dx2.5	23.6 ± 1.4 h - k	42.3 ± 1.6 a	14.0 ± 1.3 g - j	33.2 ± 1.5 a
10- 5% GLG	23.2 ± 1.6 h - k	35.0 ± 1.9 c - e	14.8 ± 1.3 g - i	25.7 ± 1.2 c - e
11- 10% GLG	22.1 ± 1.7 i - l	31.9 ± 2.6 d - f	12.0 ± 1.6 h - m	23.8 ± 1.8 de
12- 5% HES	24.8 ± 1.4 h - j	40.2 ± 1.4 a - c	14.0 ± 1.8 g - j	28.4 ± 1.2 b - d
13- 10% HES	21.3 ± 1.7 i - l	39.4 ± 1.7 a - c	13.5 ± 1.5 h - k	29.4 ± 1.4 a - c
14- 5% PVP	17.7 ± 1.2 lm	36.9 ± 1.6 bc	7.9 ± 0.8 l - n	24.4 ± 2.0 de
15- 10% PVP	5.8 ± 1.1 P	17.3 ± 3.0 lm	3.1 ± 0.5 o	8.6 ± 1.9 k - n

* (1) control TEST - yolk - maltose without additives; (2) 5% (v/v) Aquacide I; (3) 10% (v/v) Aquacide I; (4) 5% (v/v) Aquacide II;(5) 10% (v/v) Aquacide II; (6) 5% (v/v) dextran 15-20 KDa; (7) 10% (v/v) dextran 15-20 KDa; (8) 5% (v/v) dextran 2.5 KDa; (9) 10% (v/v) glycogen; (12) 5% (v/v) hydroxyethyl starch; (13) 10% (v/v) hydroxyethyl starch; (14) 5% (v/v) polyvinylpyrrolidone; (15) 10% (v/v) polyvinylpyrrolidone.

** a-p Means followed by the same letter vertically or horizontally are not significantly different (P > 0.05).

*** a-o Means followed by the same letter verticaliy or horizontaly are not significantly different (P > 0.05).

(#1; 62.9%), however these differences were not significant. Lowest motility of unfrozen ram spermatozoa was obtained with extenders #3 (10% Aquacide I), #5 (10% Aquacide II), #10 (5% glycogen), #11 (10% glycogen) and #15 (PVP), (Fig. 2).

The results revealed that the addition of certain types or concentrations of high molecular weight compounds to the extender could improve the preservation of ram spermatozoa in the absence of glycerol. Unfrozen ram spermatozoa maintained high percentage of motility for 4 hr (prefreezing) and 24 hr storage at 5°C with the addition of 5 to 10% (v/v)HES or dextran 2.5 KDa compared with the control and the other compounds tested. schmehl *et al.*, (1986) reported that all polymeric compounds studied (17 compounds including dextrans) except HES significantly reduced percentage of motile spermatozoa in fresh semen. However, in our study, dextran 2.5 KDa also maintained the motility of unfrozen ram spermatozoa. This difference could be due to different experimental conditions, the extender used, dilution method and rate, absence of glycerol, the type and concentration of the compounds.

The analysis of variance for the post-thawing percentage of motile spermatozoa at 0 hr and 4 hr showed highly significant differences ($P < 0.01$) between extenders, and also, between DM (Table 3). Also, there was a highly significant ($P < 0.01$) interaction between extenders and DM. Table (3) showed that, although the highest post-thaw motilities were obtained with extenders containing 10% dextran 2.5 KDa (#9; 33%), 5% HES (#12; 32.5), 5% dextran 2.5 KDa (#8; 30.9%) and 10% HES (#13; 30.4%) compared with 30.9% for the control (#1), the differences were not significant. The same extenders and the control maintained the highest percentages of motile spermatozoa at 4 hr post-thawing compared to the rest of extenders used in this study (Tables 2, 3 and Fig. 2). The control extender yielded the highest motility (24.6%), and extenders containing dextran 2.5 KDa (#8, #9) maintained slightly higher percentages of motile spermatozoa (23.0, 23.6%, respectively) than HES extenders (#12, 21.2% and #13, 21.5%; (Table 3). Cold dilution method at 5°C significantly ($P < 0.05$) improved post-thaw motility at 0 hr (33.0%) or 4 hr (23.6%) with all extenders tested in this study (Table 3and Fig. 1) compared with warm dilution (17.7, 10.5% for 0 and 4 hr respectively). In our study, concentrations of 5% or 10% (v/v) of both HES and dextran maintain higher motility of unfrozen and frozenthawed ram spermatozoa compared with the same concentrations of the other solutions. Dextran and HES with glycerol have been used to increase the cryosurvival of ram spermatozoa (Fiser *et al.*, 1982). Schmehl *et al.*, (1986) have shown that

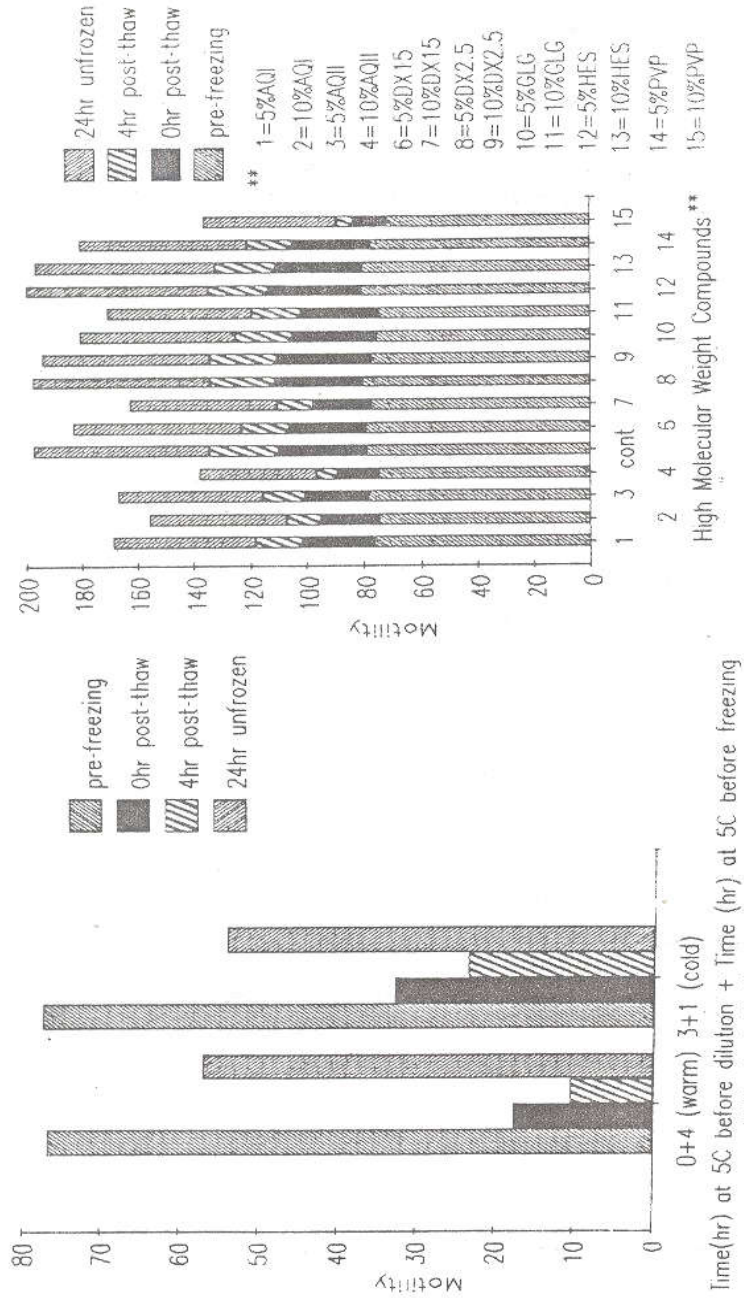

Fig. (1). Motility (%) of unfrozen and frozen-thawed ram spermatozoa as affected by the dilution method regardless of the presence of different high molecular weight compounds in the medium.

Fig. (2). Motility (%) of unfrozen and frozen-thawed ram spermatozoa as affected by the presence of different high molecular weight compounds in the medium regardless of the dilution method.

TABLE 3. Overall Effects of High Molecular Weight (HMWM) Compounds and Dilution Methods on Percentage of Motile Unfrozen and Frozen Ram Spermatozoa in Absence of Glycerol.

High Molecular* Weight Compounds :	Unfrozen **				Frozen ***			
	Time after dilution				Time after thawing			
	4 hr		24 hr		0 hr		4 hr	
	at 5°C				at 24°C			
1- control	79.0	abc	62.9	ab	30.9	abc	24.6	a
2- 5% AQI	76.7	cde	50.4	ef	24.7	fg	17.1	cd
3- 10% AQI	74.6	ef	48.5	ef	20.4	h	12.4	e
4- 5% AQII	78.5	abcd	51.7	def	22.0	gh	15.2	de
5- 10% AQII	74.8	ef	41.7	g	14.3	i	7.4	f
6- 5% Dx15	79.4	abc	60.2	bc	26.5	ef	17.2	cd
7- 10% Dx15	77.5	bcde	52.5	de	20.0	h	12.9	e
8- 5% Dx2.5	80.2	ab	63.5	ab	30.9	abc	23.0	ab
9- 10% Dx2.5	77.5	bcde	60.2	bc	33.0	a	23.6	ab
10- 5% GlG	75.8	de	56.0	cd	29.1	bcde	20.2	bc
11- 10% GLG	74.6	ef	51.7	def	27.0	def	17.9	cd
12- 5% HES	81.0	a	65.0	a	32.5	ab	21.2	ab
13- 10% HES	80.6	ab	64.4	ab	30.4	abcd	21.5	ab
14- 5% PVP	77.5	bcde	59.8	bc	27.3	cdef	16.3	d
15- 10% PVP	71.9	f	47.5	f	11.6	i	5.8	f
Dilution Methods***								
Warm (0 + 4)	76.9	a	57.2	a	17.7	b	10.5	b
Cold (3 + 1)	77.7	a	54.3	b	33.0	a	23.6	a

* (1) control TEST - yolk - maltose without additives; (2) 5% (v/v) Aquacide I; (3) 10% (v/v) Aquacide I; (4) 5% (v/v) Aquacide II;(5) 10% (v/v) Aquacide II; (6) 5% (v/v) dextran 15-20 KDa; (7) 10% (v/v) dextran 15-20 KDa; (8) 5% (v/v) dextran 2.5 KDa; (9) 10% (v/v)glycogen; (12) 5% (v/v) hydroxyethyl starch; (13) 10% (v/v) hydroxyethl starch; (14) 5% (v/v) polyvinylpyrrolidone; (15) 10% (v/v) polyvinylpyrrolidone.

** Means followed by the same letter vertically are not significantly different (P > 0.05).

*** Means followed by the same letter vertically are not significantly different (P > 0.05).

**** Warm dilution method at 37°C soon after collection; Cold dilution method at 5°C 3 hr after collection. (0hr or 3hr at 5°C after collection and before dilution + 4hr or 1hr at 5°C after dilution and before freezing).

dextran and HES provided good post-thaw recovery of motile ram spermatozoa preserved in the presence of glycerol. They stated that the hydrolyzed dextran with lowest molecular weight (0.8 - 1.6 KDa) provided more protection than higher molecular weight dextran (15-20, 70, 200 - 300 KDa) when used for cryopreservation of ram spermatozoa in the presence of glycerol. Same finding was found in our study, lower molecular weight dextran (2.5 KDa) improved the percentage of motile spermatozoa compared to the high molecular weight dextran (15 - 20 KDa).

Aquacide I, Aquacide II, glycogen, dextran 15 - 20 KDa and polyvinylpyrrolidone (PVP) gave little protection to ram spermatozoa in the absence of glycerol. However, Salamon *et al.,* (1973) suggested that these compounds, used in conjunction with other cryoprotective agents, may provide additional protection. Aquacide I is a sodium salt of carboxy-methyl cellulose 70 KDa, and Aquacide II is a sodium salt of carboxy-methyl cellulose, 500 KDa. both of these salts provided little protection to ram spermatozoa preserved in the absence of glycerol as compared with the control (Table 3). It has been suggested that, neither the basic chemical structure nor the molecular weight alone determine the protective properties of polymeric materials but possibly a combination of the two factors (Schmehl *et al.,*(1986) in addition to the type or concentration of the polymeric material present in the media.

The interactions between DM and extenders indicated that the percentage of motile ram spermatozoa preserved in the absence of glycerol may be affected by the type of extender and by the method of dilution. Cold dilution of ram spermatozoa at 5°C provided higher post-thaw protection to ram spermatozoa cryopreserved in the absence of glycerol compared to the warm dilution at 30 - 37°C regardless of the presence or absence of polymeric compounds. These results are in agreement with our previous studies on freezing ram semen in absence of glycerol (Abdelhakeam, 1988; Abdelhakeam *et al.,* 1988a, b).

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تأثير طريقة التخفيف ووجود المركبات عالية الوزن الجزئى على حفظ الحيوانات المنوية للكباش فى غياب الجليسرول

عبد الهادى عبد الحكيم عبد النبى

قسم الأنتاج الحيوانى – كلية الزراعة – جامعة المنيا – مصر.

فى هذا البحث درس تأثير كل من أكواسيد ١ ، أكواسيد ٢، دكشتران (٢،٥ كيلو دالتون) ، دكستران (١٥ – ٢٠كيلو دالتون) ، جليكوجين ، هيدوكسى ايثيل النشا ، ومركب بولى فينيل بيروليدون كلابتركيز ٥٪ أو ١٠٪ (حجم / حجم) من مخفف الكونترول وكذلك كل من طريقة التخفيف الدافئة (٣٠ – ٣٧ م) والباردة (٥ م) على حيوية الحيوانات المنوية المحفوظة سائلة على درجة ٥ م لمدة ٤ ، ٢٤ ساعة وكذلك المحفوظة بالتجميد بعد تسييحها مباشرة إو بعد ٤ ساعة من تسييحها وذلك فى غياب الجليسيرول.

أظهرت النتائج أن أضافة أى من مادتى دكستران (٢،٥ كيلو دالتون) أو هيدوكسى ايثيل النشا بنسبة ٥ أو ١٠٪ (حجم حجم) حافظت على حيويه الحيوانات المنوية المحفوظة سواء فى صورة سائلة على درجة ٥ م أو مجمدة وذلك بالمقارنة ببقية المركبات الأخرى ذات الوزن الجزئى العالى والكونترول.

طريقة التخفيف الباردةللسائل المنوى على درجة ٥ م بعد ٣ ساعات من التجميع نتج عنها حيوية عالية المعنوية بعد التجميد والتسييح بالمقارنة بطريقة التخفيف الدافئ على درجة ٣٠ – ٣٧ م مباشرة بعد التجميع.