J.Animal and Poultry Prod., Mansoura Univ., Vol.7(10): 383- 391,2016

Impact of Conventional and Non-Conventional Extenders on Rams Semen Quality During Storage at 5ºC

Khalifa, E. I[1]. and W. A. Khalil[2]

[1-]Animal Production Research Institute (APRI), Department of Sheep and Goat Research, Ministry of Agriculture, Dokki, Giza, Egypt.

[2-] Animal Production Department, Faculty of Agriculture, Mansoura University.

Correspondence author: xyezz@yahoo.com

ABSTRACT

The aim of the present study was to define the effect of different extender components (as conventional and non-conventional extenders) on rams' semen quality during storage at 5ºC for up to 6 days. Fertility rates of the extended ram spermatozoa stored at 5ºC for up one day were also studied. Four Rahmani rams were used to collect pooled ejaculates by artificial vagina. Semen was collected, evaluated and extended with five extenders included conventional and non- conventional extender ingredients. The conventional extenders were sodium citrate (E1), Tris (E2) and skimmed milk powder (E3) - based glucose- egg yolk. While, non-conventional extenders were salt of sodium chloride 0.9% (E4) and saline solution 0.9% intravenous infusion (E5) based glucose-egg yolk. The final extension rate was 1semen : 6 extender. The extended semen with different extenders media (E1, E2, E3, E4 and E5) were cooled to 5ºC and stored at this temperature for 6 days. After storage time (0, 1, 2, 3, 4, 5 and 6 days), the percentage of sperm motility (SPM), recovery sperm motility (RSM) and positive osmotic resistant (POR) were recorded. Also, fertility rate was carried out with liquid semen stored at 5ºC for up to one day using the best conventional and non-conventional extenders. Thus, twenty-two ewes were used to investigate fertility rate. Ewes were divided into two groups (11/group), the1st and 2nd groups were artificially inseminated with conventional (E2) or non-conventional (E5) extenders which obtained better semen quality than other extenders, respectively. The results indicated that, the cooling extended ram semen with each of E1, E2 or E5 extenders was significantly ($P<0.05$) higher the percentage of SPM, RSM and POR than those extended with E3 and E4 extenders during storage at 5ºC for up to 6 days. However, the cooling ram semen with E2 and E5 showed non-significantly parameters during cooled at 5ºC till 6 days. The statistical analysis of the data revealed a significant positive correlation between extender types and SPM, RSM and POR of ram spermatozoa during storage at 5ºC until 6 days. Surprisingly, fertility rate of ewes artificially inseminated with ram semen preserved at 5ºC for one day of storage performed to assess higher conception rate (84.62%) with E5 (as non-conventional extender) than conception rate (78.57%) with E2 (as conventional extender). Moreover, the superior litter size recorded 1.00 with ram semen extended with E5 compared to 0.86 with E2 extenders. Also, sex ratio of born lambs displayed female lambs 66.67 and 33.33% of male lambs with semen extended with E2 extender. Ram semen extended with E5 achieved female lambs 69.23% and male lambs 30.77%. It is therefore concluded that saline solution (0.9%) intravenous infusion (E5) based glucose-egg yolk intended to keep goodness fertility rate and litter size after AI of cooled semen at 5ºC.

Keywords: *Ram semen, conventional and non-conventional extenders, motility, osmotic resistance, fertility rate.*

INTRODUCTION

Keeping extension semen at cooled temperature condition was reported to be a cheaper alternative than liquid nitrogen which helps to increase using artificial insemination (AI). Another benefit is subsided bacterial growth at 5ºC, which would improve the quality of diluted semen. In addition, the success of AI in livestock species depends on maintenance of viability, motility and fertility of spermatozoa. It is reported that, extender types used to diluted semen is importance, played substantial role in the survival rate of frozen and non-frozen spermatozoa and effective factors on successful storage of spermatozoa. In this context, (Bohlooli *et al*., 2012) indicated that there is a difference between extenders regarding their abilities to viability, progressive motility and membrane integrity during preservation of ram spermatozoa. Furthermore, (Soltanpour *et al*., 2014) stated that a good extender must have equal osmotic pressure to the seminal plasma, buffering capacity, protect sperm from cold shock, nutrient for sperm metabolism, controlling microbial contamination, protect sperm against freezing-thawing damages and preserve sperm viability without more decline in fertility. Semen has been usually diluted with conventional extenders such as Tris plus egg yolk, glucose phosphate solution, egg yolk-citrate solution, homogenized whole milk, fresh and dried skim milk, lactose solution and commercial diluents (Kulaksiz *et al*., 2011). Hence, (Lopez *et al*., 1999) observed that there were no differences between sodium citrate, Tris or milk-based extenders when ovine liquid semen was stored at 5°C during a short period (2 days). Moreover, (Gündoğan, 2009) reported better sperm motility and integrity of sperm membrane in a Tris-based extender than in sodium citrate and skimmed milk extenders. In addition, (Gundogan *et al*., 2011) concluded that the extender types such as Tris, sodium citrate and milk had no effect on spermatozoa parameters during stored at 4ºC. However, (Rakha *et al*., 2013) reported that Tris-based extender showed better sperm viability of ram semen than sodium citrate or milk based during liquid storage. In addition, sodium citrate extender with disaccharides (sucrose or lactose) and polysaccharides (raffinose-citrate-yolk) can be used for successful insemination of ewes and enhancing pregnancy rate after artificial insemination (Stefanov *et al*., 2015). Besides, (Albiaty *et al*., 2016) concluded that progressive motility was 63.87, 61.25% and 39.12% after storage at 5°C for 3 days of ram semen diluted with Tris, sodium citrate and milk extenders, respectively. Moreover, decrease in cooled sperm characteristics was observed of the extended ram semen with milk, Tris and sodium extenders which may be

related to extender types (Acharya *et al*., 2016). Usually, testing the efficiency of different extenders for semen storage, in *in vitro* assays of motility, membrane integrity and acrosome status have been widely proven to be reliable (Govindasamy *et al*., 2016).

Therefore, the present study aimed to define the effect of different extender components (as conventional and non-conventional extenders) on rams' semen quality during storage at 5ºC for up to 6 days. Fertility rates of the extended ram spermatozoa stored at 5ºC for up one day were also studied.

MATERIALS AND METHODS

Experimental study was conducted at El-Serw Experimental Research Station belonging to Animal Production Research Institute (APRI), Ministry of Agriculture, Egypt. The experimental work was carried out from December, 2015 till June, 2016.

Experimental animals:

Four Rahmani rams at 3.0 - 3.5 years of age and 70 -75 kg body live weight were used in the present study. The rams were healthy condition and clinically free from external and internal parasites with the sound history of fertility in herd. Palpation of the external genetalia (testes) showed that they were typically normal and moved free in scrotum. Rams were received rations according (NRC, 2007). All rams were received clean fresh water freely and housed in a yard which provide with common feeding through and a concrete floor provide with common sheltered water trough and they could more freely in enclosed area.

Semen collection and evaluation:

Prior to semen collection, the instruments of semen collection were cleaned using distilled water and dried with sterile paper towel. Then, semen collection was performed using an artificial vagina twice a week for four weeks in existence an estrous ewe for mating. The ram semen samples having at least 70% progressive sperm motility, 17% sperm abnormalities and $2.5x10^9$/ml sperm cells concentration were pooled and used in the experimental work.

Preparation of semen extenders:

The present study was planned to evaluate the extended cooled rams' semen quality as percentage of progressive sperm motility (SPM), recovery of sperm motility (RSM) and positive osmotic resistant (POR) using five extenders as conventional and non-conventional extenders during storage at 5°C for up to 6 days. The conventional ingredients of extenders were sodium citrate (E1), Tris (E2), skimmed milk powder (E3) based glucose- egg yolk. While, non-conventional extenders were salt of sodium chloride (0.9%) (E4) and saline solution (0.9%) intravenous infusion (E5) based glucose-egg yolk. The ingredients of these extenders were as shown in Table (1). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Table 1. The chemical composition of semen extenders used in experimental traits.

Ingredient	Grams/ 100ml of distilled water				
	E1	E2	E3	E4	E5
Sodium citrate (gm)	2.37	-	-	-	-
Tris (gm)	-	3.28	-	-	-
Sodium chloride salt (gm)	-	-	-	0.90	-
Skimmed milk powder (g)	-	-	10.00	-	-
Citric acid(gm)	0.06	1.70	-	-	-
glucose (gm)	0.80	0.80	0.80	0.80	0.80
Egg yolk (ml)	10.00	10.00	10.00	10.00	10.00
*Antibiotic (ml)	1.00	1.00	1.00	1.00	1.00
Saline solution (ml)	-	-	-	-	100.00
Distilled water (ml)	100.00	100.00	100.00	100.00	-

***Each ml contains: Procaine penicillin 200mg and Dihydrostreptomycin sulphate 250mg.**

The conventional extenders included: E1: Sodium citrate extender, E2: Tris extender (hydroxymethyl aminometahne) and E3: Skimmed milk extender prepared as ingredients (skimmed milk powder, glucose) dissolved in 100mL of distilled water, heated to 95 °C for 10 min, then cooled at room temperature before addition of 10% egg yolk and antibiotic completed to 100ml distilled water. However, non-conventional extenders included: E4: Sodium chloride (as salt) extender and E5: Saline solution (0.9%) intravenous infusion.

1. Experimental procedures:

Semen extension:

Semen was collected and evaluated for each ram then, pooled and extended with different extenders media E1, E2, E3, E4 and E5 are illustrated later in (Table1). Semen extension was carried out by adding the appropriate volume of extender to semen slowly. The final extension rate was 1 semen : 6 extender, then, five extended semen (in Falcon tube) was kept below the level of water in water both at 37°C at all times to avoid fluctuations in the temperature of extended semen.

Chilling of semen:

The tubes containing extended semen were placed in a 500ml beaker containing water at room temperature with a thermometer in order to facilitate periodic checking of temperature during the cooling periods. Then, thermometer was put in Falcon tube contained 7 ml of dilution (consisted of 10 ml egg –yolk completed to 100 ml distilled water). Another test tubes containing extended semen only were placed in beaker to maintain the extended temperature similar to that of semen (all the test tubes were covered with dark plastic sheath). The beaker was placed in refrigerator and gradually cooled till their temperature reached to 5°C during a period of 1.5-2.0 hours. The extended cooled semen in different of extenders was kept at 5°C for up to 6 days. After storage time (0, 1, 2, 3, 4, 5 and 6 days), the percentage of sperm progressive motility (SPM), recovery of sperm motility (RSM) and positive osmotic resistant (POR) using five extenders were recorded.

Sperm motility (SPM):

With regarded to extended semen, the SPM was determined using 100 µl / extender type taken and poured individually in test tube which incubated previously in water bath at 37°C after storage period. The drop of incubated extended semen was covered by warmed cover slip and immediately examined using high power (× 400 magnifications).

Recovery sperm motility (RSM):

The recovery of sperm motility (%) was calculated using the following formulae as:

$$\text{Recovery rate (RSM)} = \frac{\text{Sperm motility at 1, 2, 3, 4, 5 and 6 days}}{\text{Sperm motility at 0 day}} \times 100$$

Positive osmotic resistance (POR):

The POR (%) was evaluated using an aliquot (50 µl / extender type) of extended semen added to 1 ml of hypo-osmotic solution (consisted of 0.735 g sodium citrate, 1.351 g fructose and distilled water up to 100 ml to give osmotic pressure at 190 mOsm) and mixed thoroughly. After 30 minutes of incubation at 37°C, one drop of this mixture was placed on a slide and covered with a cover slip then, examined under a phase contrast microscope (× 400 magnifications). A positive osmotic resistant response to the test was evident by coiling of the sperm tail. At least 200 spermatozoa per slide were counted to evaluate totally percentage of coiled tail of spermatozoa in each sample.

2. Fertility rates:

Extended with E2 (as conventional extender) or E5 (as non- conventional extender) having the best semen quality after stored at 5°C for one day used to calculate conception rates of ewes. A total numbers of 22 Rahmani mature ewes (3-4 years old) were divided into two groups (n=11/ each). The 1st and 2nd groups of ewes were artificially inseminated with E2 or E5, respectively. The detection of ewes in manifested estrous was performed in the morning by tester ram. The estrous ewes were cervically inseminated by two doses (within 12 hours as interval period the 1st dose at identify estrous) of extended semen (1 ml / dose) which stored at one day and warmed at 37°C for 60 seconds before insemination. The insemination dose of spermatozoa concentration was adjusted at $\geq 200 \times 10^6$ spermatozoa / ml and placed on os-cervix using open speculum. The pregnancy was diagnosed after ewe passed two oestrus cycles without return to heating again. If ewe returns to heat again after 14-19 days, it will be inseminated again using previous technique. After breeding season, fertility rate was recorded as: conception rate during two services [number of ewes conceived (through 1st and 2nd services) / number of ewes inseminated (through 1st and 2nd services)].

Lambing rate and litter size:

Lambing rate was calculated as single birth (number of ewes lambing single/ number of ewes lambed) and twins birth (number of ewes lambing twins/ number of ewes lambed). Then, Litter size was calculated as number of total lambs born / number of ewes lambing (after 1st and 2nd services).

Sex ratio:

The sex of born lambs was calculated from the following formulae as:

$$\text{Sex ratio (male: female)} = \frac{\text{No. of born lambs in particular sex}}{\text{Total No. of lambs born}} \times 100$$

Statistical analysis:

One way analysis of variance using (SPSS, 2013) program version 22 Inc, was done for the obtained data of chilling semen qualities after transformation of percentages to their corresponding arcsin values according to (Snedecor and Cochran, 1989). Analysis of variance between the extenders and comparison of means at 5% significance level using Duncan's multiple range test (DMRT) was carried out within SPSS program. Also, the conception rate results were analyzed using Chi-Square test. Correlation coefficients between extender types and mean sperm characteristics parameters (represented in SPM, RSM and POR) were calculated using the Pearson's coefficients of SPSS programmes.

RESULTS AND DISCUSSION

Sperm motility (SPM):

The effect of different extenders (E1, E2, E3, E4 and E5) on SPM (%) of the cooled ram spermatozoa during storage at 5ºC for up to 6 days are presented in (Figure1). Diagram in Figure (1) indicated that, SPM (%) was better insignificantly with E1, E2 and E5 than E3 and E4 extenders it reached to 86.25, 86.88, 86.88, 84.38 and 83.75 % at zero time, respectively. Regarding the effect of progress cooled time, the SPM % decreased significantly ($P<0.05$) with the advancement of storage days, it reached to 76.88, 81.87, 75.62, 73.12 and 81.25% at day one of storage with E1, E2, E3, E4 and E5, respectively. Generally, the SPM (%) in all extenders was higher than 50% at 2 days of storage; whereas semen diluted with E2 and E5 was over 70%. This study demonstrated that the different extender types and storage time were significantly ($P<0.05$) associated with the deterioration in SPM (%). These results indicated that sodium citrate sustained its viability better to preserve of ram sperm motility and membrane integrity than milk or Tris-based extenders during storage at 5˚C for up to 2 days (Lopez *et al*., 1999). While, (Paulenz *et al*., 2002) found that a clear advantage of Tris based extender instead of either sodium citrate or milk based extender to maintain sperm motility and integrity of acrosomal membrane during storage of ram semen. On the contrary, (Kulaksiz *et al*., 2012) concluded that skimmed milk extender was better than sodium citrate and Tris extender during liquid state at 4˚C or frozen state at -196˚C. On the other hand, (Albiaty *et al*., 2016) indicated that extended ram semen with Tris and sodium citrate-based extenders were significantly superior than milk based extender. Similarly, Tris based extender (E2) and saline solution (E5) improved SPM (%) compared to ram semen extended with those of E1, E3 and E4 extenders during storage at 5˚C for up to 6 days. Likewise, activity of SPM (%) with E5 extender may be attributed to higher Na^+ concentration than K^+ and Ca^{2+} in ram seminal plasma. This information is consistent with findings of (Asadpour, 2012) who reported that Na^+ concentration in seminal plasma of different sheep breeds as Baluchi × Moghani, Ghezel × Merino and Merino× Moghani was 39.00, 42.00 and 37.66 mg / dL compared to K^+ 12.45, 12.70 and 11.10 mg / dL and Ca^{2+} 10.30, 11.20 and 10.30 mg / dL in same breeds, respectively. Hence, sodium (Na^+) level may be higher in E5 extender could result in Na^+ content in extender, which led to improve sperm parameters than E1, E3 and E4 extenders.

Despite, E4 contained Na^+ cation comes from sodium chloride, but saline solution in E5 extender had adjusted during industry that led to goodness chilling preservation. In addition, the highest value of Na^+ cation presented in saline solution (E5 extender) plus other cations such as, K^+, Ca^{++} and Mg^{++} in the seminal plasma may play essential roles in the sperm activity and establish the osmotic balance (Hamad *et al*., 2014). On the other hand, (Zeny, 2016) indicated that Na^+ generally plays an important role in the activation sperm motility which may be an indication of sperm plasma membrane integrity and Na^+ cation exhibited favorable effects on the seminal quality, antioxidant balance and positive impact on all spermatozoa vitality characteristics.

Continuity of sperm cooling storage at 5ºC from 0 to 6 days, the SPM % parameters decreased markedly in all extender types. It was recorded 30.62, 35.63, 20.60, 15.61 and 35.00% at day 6 of cooled with E1, E2, E3, E4 and E5, respectively. Hence, reducing of SPM in cooling extenders may be associated with waste products of sperm metabolism (as lactic acid). (Soltanpour and Moghaddam, 2014) revealed that a higher accumulation of lactic acid at the length of storage time increased spermatozoal damage which shown through the less spermatozoal motility and durability. Storage at 5ºC does not completely arrest spermatozoa metabolism, therefore, assemblage of toxic production included a toxic enzyme namely membrane –bound aromatic amino acid oxidase (AAAO) released from dead spermatozoa (Aitken *et al*., 2014). On the other hand, free radical might be involved in damaged sperm membrane (Stefanov *et al*., 2015) and reactive oxygen species (ROS) (Acharya *et al*., 2016). In addition, effect of peroxidation comes from polyunsaturated fatty acids in sperm cytoplasm membrane lead to lost cytoplasm membrane, decrease sperm motility and inhibit of fructolysis and respiration (Albiaty *et al*., 2016).

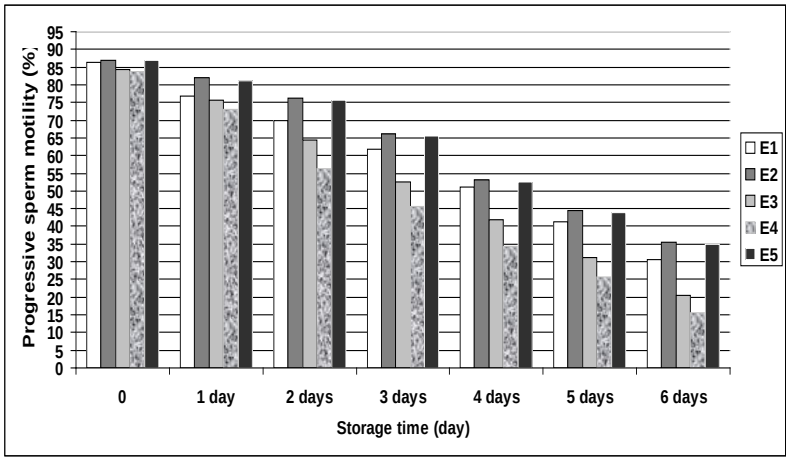

Figure 1. Effect of conventional extenders (E1, E2 and E3) and non-conventional extenders (E4 and E5) on ram sperm motility (SPM) during storage at 5°C for up to 6 days.

Recovery sperm motility (RSM):

Data presented in (Table 2) revealed that the RSM% during storage at 5°C for up to 6 days was significantly (P<0.05) lower with E3 and E4 extenders than E1, E2 and E5 extenders. These results might be attributed to better production of sodium citrate, Tris and saline solution to spermatozoa against cold shock compared to other components of extender during refrigeration at 5°C. Similar trend was reported by (Albiaty *et al*., 2016) with sodium citrate and Tris extenders and (Zeny, 2016) with saline solution. The advancement of storage time at 5°C for up to 6 days decreased significantly (P<0.05) the RSM% among all extenders. These findings may be due to the increase of sperm metabolic activity, consequently increase of AAAO enzyme (Aitken *et al*., 2014), lactic acid (Soltanpour and Moghaddam, 2014) and free radical as ROS (Acharya *et al*., 2016).

Table 2. Mean of RSM % with different extenders during storage at 5°C for up to 6 days.

Storage time (days)	Different extender types					Overall mean
	E1	E2	E3	E4	E5	
1	89.13±3.75	94.24±3.89	89.60±1.49	87.32±1.73	93.50±1.45	90.76±1.89 ^{a}
2	81.12±1.87	87.75±1.99	76.27±1.95	67.19±2.45	87.01±1.02	79.87±1.42^{b}
3	71.69±2.39	76.22±1.39	62.15±2.81	54.50±2.87	75.49±1.56	68.01±1.66^{c}
4	59.35±2.06	61.07±1.67	49.59±2.76	41.08±2.68	60.33±1.95	54.29±1.58^{d}
5	47.75±2.11	50.98±2.15	37.03±2.84	30.61±2.09	50.20±2.55	43.31±1.63^{e}
6	35.38±2.04	40.89±2.46	24.38±3.18	18.66±2.09	40.07±3.54	31.88±1.82^{f}
Means	64.07±2.35^{b}	68.53±2.59^{a}	56.50±2.52^{c}	49.89±2.33^{d}	67.77±2.02^{a}	61.35

a, b, c and d values with different superscripts within same row are significantly different (P<0.05).
A, B, C, D, E and F values with different superscripts within same column are significantly different (P<0.05).

Positive osmotic resistance (POR):

It could be noticed that from (Figure 2) at any storage time, the highest (P<0.05) values of POR (%) was observed with E2 or E5 extenders followed by E1 and E3 extenders. On the other hand, the worst values was observed using E4 extender. The hypo-osmotic swelling test (HOST) has proved to be a good tool for evaluating the membrane integrity of sperm of various domestic animals (Nalley and Arifiantini, 2013). The current results recorded that primary ram spermatozoa characteristic of POR (%) at zero time was non-significant (92.38, 93.38, 91.12, 90.62 and 93.25%) with E1, E2, E3, E4 and E5 extenders, respectively. However, total mean of POR (%) of ram sperm quality storage at 5°C for up to one day was > 80.00 % then, POR (%) of ram spermatozoa with E1, E2, E3, E4 and E5 was reached to 81.00, 85.50, 81.50, 79.38 and 85.12%, respectively. Similarly, (Asadpour, 2012) concluded that the cations Na^+ and K^+ generally establish the osmotic balance and seminal plasma osmolality ultimately plays an important role in the activation sperm cell also potassium ions (K^+) are also intracellular cations and the concentrations in the seminal fluid may be an indication of sperm plasma membrane integrity. The highest values of POR (%) with E2 and E5 extender may be due to the combinations of all beneficial effects of Tris components (E2) which provided the most buffering capacity which penetrated rapidly into the sperm cells membrane and avail as an intercellular buffer (El-Sheshtawy *et al*., 2016). Also, saline solution (E5) extender has been an integral part of dilutor for preservation as they have capability to alter osmolarity of the diluent and have a definite protective effect (Asadpour, 2012). The individual days of storage time at 5°C for up to 6 days were decreased significantly (P<0.05) the POR% of ram spermatozoa counts. Hence, at day 6, the POR (%) of spermatozoa was significantly (P<0.05) reduced among E1, E2, E3, E4 and E5 extenders, it was recorded of 30.38, 36.75, 27.12, 21.62 and 36.88%, respectively. This results is comparable with those of several authors who suggested that progression of cooled time decreased significantly (P<0.05) the POR (%) of spermatozoa may be attributed to AAAO enzyme (Aitken *et al*., 2014), lactic acid levels (Soltanpour and Moghaddam, 2014) and free radical concentration (Acharya *et al*., 2016). At all events (Soylu *et al*., 2007) revealed that supplementation of ram semen with an osmotic pressure at 400 mOsm improved storage ability of spermatozoa without dysfunction in extenders.

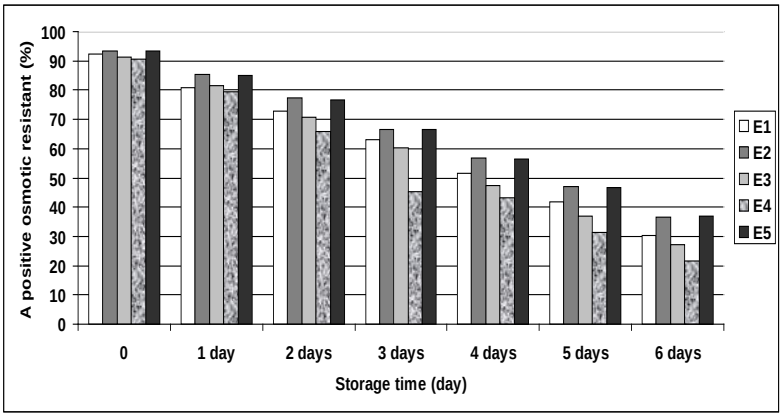

Figure 2. Effect of conventional (E1, E2 and E3) and non-conventional (E4 and E5) extenders on positive osmotic resistance (POR) during storage at 5°C for up to 6 days.

Correlation coefficients among different semen extenders and sperm motility (SPM), recovery sperm motility (RSM) and positive osmotic resistance (POR):

Data presented in (Table 3) indicated correlation coefficient among different extenders of ram semen quality as SPM (%), RSM (%) and POR (%) during storage at 5°C for up to 6 days consecutively. The results of the present investigation showed highly significant (P<0.05) correlation between different extender types and sperm parameters during different storage periods. These results are in agreement with those of (Azizunnesa *et al*., 2014) who reported that the strongest correlation was calculated between spermatozoa characteristics and different types of semen extenders. Besides, the results of present study indicated a significant correlation among sperm motility and hypo-osmotic swelling test (HOST). Similar trend was reported by (Bohlooli *et al*., 2012) revealed a highly significant (P<0.01) correlation in plasma membrane integrity is related to sperm motility.

Table 3. Correlation coefficient between semen extender types and sperm motility (SPM), recovery sperm motility (RSM) and positive osmotic resistance (POR).

Item	Extender	SPM	RSM	POR
Extender	1	0.728*	0.719*	0.877*
SPM		1	1.000**	0.983**
RSM			1	0.985**
POR				1

**** (P<0.05).***
***** (P<0.01).***

Fertility rates:

Fertility rates of ram semen extended with E2 or E5 extenders are presented in (Table 4). Fertility rates of E2 or E5 extenders were 78.57 and 84.62% after two

services, respectively. The highest fertility rates of ewes artificially inseminated with E5 extender may be attributed to rich of Na^+ cation in dilution which associated with high percentages of motile sperm and such semen was considered to be greater quality. (Asadpour, 2012) suggested that Na^+cation generally establish the osmotic balance and seminal plasma osmolality ultimately plays an important role in the activation of sperm cell for the prediction of ram fertility. Hence, a positive and significant correlation as observed between Na^+ cation and sperm motility (Akpa *et al*., 2013). Indeed, existence of Na^+ and K^+ (presented in seminal plasma) activated adenosine triphosphate (ATP) in a variety of tissues included sperm cell then, declined of ATP activity result in less sperm movement and capacitation (Zhou *et al*., 2015). The same later authors explained that binding of Na^+ triggers a crossover conformational change of transmembrane segments (TS) that contain the region responsible for ion transport in cytoplasmic sperm tail. (Salem *et al*., 2013) also found that NaCl is one of the major molecules present in cervical mucus that support the arborization (a fine branching structure at the end of a nerve fiber) of embryo. Moreover, Na^+ cation takes place in the female genital tract to penetrate the oocyte associated with Ca^{2+} and progesterone. (Correia *et al*., 2015) confirmed that progesterone caused a rapid sperm plasma membrane depolarization, increased by Na^+ influx which result in positively in acrosome reaction and penetrate the oocyte activity. In addition, (Correia *et al*., 2015) also suggested that Ca^{2+} and Na^+ fluxes might occur through separate progesterone activation pathways. However, Ca^{2+} and Na^+ appeared to compete for the same entry pathway (Ca^{2+} increase were higher in the absence of extracellular Na^+; depolarization was greater in the absence of extracellular Ca^{2+}). Actually, a gradual decline in motility and fertility was occurred when semen stored at 5°C this decline may be attributed to the action of reactive oxygen species (ROS) generated by the cellular components of semen. Thus, sperm membrane are rich in polyunsaturated fatty acids (PUFAs); this predominance of PUFAs renders sperm highly susceptible to lipid peroxidase (LPO), immediate LPO in membranes of sperm destroys the structure of the lipid matrix, as a result of the invasion by ROS (Agarwal and Sekhon, 2010). The reactive oxygen species (ROS) attacks lead to the impairment of sperm function (sperm motility, functional membrane integrity and fertility) then; use of antioxidants show beneficial effects on reduced free radical (Hong *et al*., 2010). Moreover, (Zeny, 2016) showed that Na^+ cation has an antioxidant balance may be associated with high fertility. Hence, the addition of antioxidants to the extended semen diluents at the period of storage could improved sperm motility, acrosomal integrity, reduce the degree of cellular damage and increase the viability and fertilizating capacity of sperm *in vitro* (Soltanpour *et al*., 2014). On the other hand, (Slanina *et al*., 2015)stated that the majority of extenders with physiological solutions (NaCl 0.9% w/v Intravenous Infusion) convenient for survival of spermatozoa and activated fertility which identical with the seminal plasma of turkey. In addition, sodium chloride based extender solutions have low risk of hypo- or hyperosmotic damage to sperm. Likewise, (Tirpan *et al*., 2016) reported that the highest values of sperm motility, sperm viability and egg fertility were observed when semen of sea bream fish diluted with sodium chloride (NaCl 1.0%). Furthermore, (Vílchez *et al*., 2016) indicated that the presence of NaCl (sodium Na^+ is the principal cation of the extracellular fluid and plays a large part in electrolyte disturbances and chloride Cl^- has an integral role in buffering action) in the seminal plasma of fish (or in the extender medium) is necessary for the preservation of sperm motility.

Table 4. Fertility rates of ewes artificially inseminated with ram semen extended with conventional (E2) or non-conventional (E5) extenders after one day of storage at 5°C.

Item	Conventional extender (E2)	Non-conventional extender (E5)
No. of ewes inseminated at 1st service	11.00	11.00
No. of ewes conceived at 1st service	8.00	9.00
No. of ewes reiterated insemination at 2nd services	3.00	2.00
No. of ewes conceived at 2nd services	3.00	2.00
Total No. of ewes conceived at 1st and 2nd services	11.00	11.00
Total No. of ewes inseminated at 1st and 2nd services	14.00	13.00
Conception rate after 1st and 2nd services (%)	78.57	84.62

Lambing rate and litter size:

Data presented in (Table 5) revealed that the ram semen extended with E5 (saline solution 0.9%) gave higher total lambing rate than ram semen extended with E2 (Tris) after ewes were inseminated with spermatozoa diluted and stored at 5°C for up to one day. Therefore, insemination of ewes with E5 extender could be activated sperm motility to pass through the female tract speedily which may reach to the oocyte and penetrated it rapidly. In this respect, (Torres Flores *et al*., 2008) reported that in the absence of external calcium allows sodium permeation in a high conductance mode and generate intracellular sodium loading thus, sodium influx, and the plasma membrane potential mainly depends on the electrogenic Na+/K+-ATPase, activated by the increase in Na^+ that hyperpolarizes the sperm cells. This ATP decrease when the media contains low in sodium (Na^+), it is interesting that a recovery in sperm motility occurs when Na^+ is still high (Torres-Flores *et al*., 2011). In addition, (Vílchez *et al*., 2016) revealed that the presence of the ion Na^+ in the seminal plasma (or in the extender medium) is necessary for the preservation of sperm motility; Na^+ plays a role in maintaining an appropriate sperm cell and caused an

activity of the spermatozoa. Accordingly, several authors observed positive correlations between litter size and sperm motility. These results are in agreement with the findings of (David *et al*., 2015) who recorded that the greatest relation observed between sperm motility and litter size. The positive role of Na^+ for enhances sperm motility then, insemination of saline solution (contains E5) plus a high natural of Na^+ in genital tract mucus could activate sperm motility and fertility compared to E2 extenders. In this context, (Salem *et al*., 2013) found that Na^+ concentration was higher mineral than P^+, K^+, Cl^+ and Zn^{2+} concentration during oestrus cycle phase as pro-estrus, estrus and post-estrus were 207.76, 222.76 and 243.13 mm/L, 7.85, 8.50 and 9.89 mm/L, 12.41, 10.88 and 11.60 mm/L, 112.56, 121.57 and 111.86 mm/L and 3.67, 2.94 and 2.72 mm/L, respectively.

Table 5. Lambing rates and litter size of ewes artificially inseminated with ram semen extended with conventional (E2) or non-conventional (E5) extenders after one day of storage at 5°C.

Item	Conventional extender (E2)	Non-conventional extender (E5)
Total number of born lambs (ewe group)	12	13
Total No. of ewes inseminated 1st and 2nd services	14	13
No. of ewes lambing single	10	9
Single rate (%)	90.91	81.82
No. of ewes lambing twins	1	2
Twins rate (%)	9.09	18.18
Litter size	0.86	1.00

Sex ratio:

In relation to sexing rate of lambing, the present results observed that ram semen extended with E5 could be recorded higher sex ratio than those ram spermatozoa extended with E2 stored at 5°C for up to one day (Table 6). Actually, sex ratio depending on both X and Y chromosome-bearing sperm which have different morphologically and structurally. The X chromosome-bearing sperm are 2 or 3 folds greater in size than Y chromosome-bearing sperm. The X-bearing sperm has 2.8% more deoxyribonucleic acid (DNA) than the Y-bearing sperm. Also, X-bearing sperm swims more slowly and has a longer life span than Y-bearing sperm. In this context, (Johnson, 2000) reported that percentage of deoxyribonucleic acid (DNA) was higher in X spermatozoa by 4.2% than Y spermatozoa. Indeed, there are some factors such as ATP and pH association with sperm motility to control sexing. The genital pH acidity (increasing ion H+) and negative X- sperm charge may activate X-bearing sperm to reach fertilizing side speeder than Y- bearing sperm which gave female lambs. These results are defined by (Kaneko *et al*., 1984) who found that motility of X-bearing sperm faster than Y-bearing sperm in acidic medium because of X-bearing sperm have a higher net negative charge on the sperm cell surface than Y-bearing sperm. Also the last authors showed that female sperm has a longer life span in acidic environment than the Y sperm though decreased motility. In addition, the pH level in the vagina and cervix can have an effect on gender of lambs X sperm was more robust and slower than Y sperm and resilient to acidity in the vagina. Despite, X sperm may be slower but, it may be able to withstand an acidic environment that weed out the Y-chromosome sperm. Alkalinity would tend to favor the alacrity of male sperm or at least even the pH odds. This could be a reflection of various factors that may influence sex ratio including acidic and alkaline media. This finding is in agreement with (Khalifa *et al*., 2009) who attained that extender with acidic media (at pH 6.6) activated X sperm to give 66.67 % female lambs and 33.33% male lambs however, alkaline extender media (at pH 7.3) realized 81.25 % male lambs and 18.75% of female lambs. Subsequently, acidity of E5 extender contained saline solution (0.9%) could be activated X-bearing sperm to produce female lambs.

Table 6. Sexing lambs of ewes artificially inseminated with ram semen extended with conventional (E2) or non-conventional (E5) extenders after one day of storage at 5°C.

Item	Conventional extender (E2)	Non-conventional extender (E5)
Total lambs born	12	13
No. of female lambs	8	9
Female ratio (%)	66.67	69.23
No. of male lambs	4	4
Male ratio (%)	33.33	30.77

CONCLUSION

In conclusion, saline solution (0.9%) intravenous infusion as non-conventional extender is better in maintaining good ram semen quality, fertility rate, litter size and sex ratio toward to female lambs than other trial extenders. Therefore, it could be recommended to extended ram semen with saline solution (0.9%) during storage at 5°C to improve ram semen quality and fertility rate when used for artificial insemination in ewes. In addition, saline solution (0.9%) cheaper and more a practical standpoint than conventional extender to storage ram semen.

Acknowledgments

The authors are grateful to Prof. Dr. El-Harairy, M. A. Senior of Animal Physiology, Department of Production Faculty of Agriculture, Mansoura University and Prof. Dr. Zeidan, A. E. B. Senior of Animal Physiology (AI) Animal Production Research Institute (APRI) for their generosity to reviewing this manuscript.

REFERENCES

Acharya, M., Burke, J., Smyth, E., Davis, A., Lester, T., Rorie, R., 2016. 107 Effect of semen extender and storage temperature on ram sperm motility over time. Journal of Animal Science 94, 53-53.

Agarwal, A., Sekhon, L.H., 2010. The role of antioxidant therapy in the treatment of male infertility. Human Fertility 13, 217-225.

Aitken, R., Finnie, J., Muscio, L., Whiting, S., Connaughton, H., Kuczera, L., Rothkirch, T., De Iuliis, G., 2014. Potential importance of transition metals in the induction of DNA damage by sperm preparation media. Human Reproduction 29, 2136-2147.

Akpa, G., Ambali, A., Suleiman, I., 2013. Relationships between semen cation concentrations, semen characteristics, testicular measurements and body conformation traits in Red Sokoto goat. Nature and Science 11, 94-99.

Albiaty, N.M.H., Alobaidi, H.J.K., Kareem, A.F., Al-Hakim, A.M., Alnaeb, A.Y., Alkhazraji, A.A.H., 2016. Effect of extenders and preservation periods in some semen characteristics of awassi rams. World Journal of Pharmaceutical Research 5, 234-243.

Asadpour, R., 2012. Relationship between mineral composition of seminal plasma and semen quality in various ram breeds. Acta Scientiae Veterinariae 40, 1027-1034.

Azizunnesa, Z.B., Bari, F., Alam, M., 2014. Effects of Proportion of Egg Yolk and Preservation Time on Chilled Semen from Indigenous Rams. GSTF International Journal of Veterinary Science 1, 18-26.

Bohlooli, S., Cedden, F., PishJang, J., Razzaghzadeh, S., Bozoğlu, Ş., 2012. The effectof different extenders on post-thaw sperm viability, motility and membrane integrity in cryopreserved semen of zandiram. J. Basic. Appl. Sci. Res., 2, 1120-1123.

Correia, J., Michelangeli, F., Publicover, S., 2015. Regulation and roles of Ca2+ stores in human sperm. Reproduction 150, R65-R76.

David, I., Kohnke, P., Lagriffoul, G., Praud, O., Plouarboué, F., Degond, P., Druart, X., 2015. Mass sperm motility is associated with fertility in sheep. Animal reproduction science 161, 75-81.

El-Sheshtawy, R.I., Gamal, A., El-Nattat, W.S., 2016. Effects of pomegranate juice in Tris-based extender on cattle semen quality after chilling and cryopreservation. Asian Pacific Journal of Reproduction 5, 335-339.

Govindasamy, K., Ponraj, P., Thulasiraman, S., Andonissamy, J., Naskar, S., Das, A., Hasin, D., Baishya, S.K., 2016. Efficacy of different extenders on sperm characteristics and fertility in crossbred pigs of north-eastern India. Veterinarski Arhiv 86 515-528.

Gündoğan, M., 2009. Short term preservation of ram semen with different extenders. Kafkas Univ. Vet. Fak. Derg 15, 429-435.

Gundogan, M., Avdateka, F., Yani, D., 2011. Effect of extenders on motility, morphology and osmotic resistance parameters or ram sperm during liquid storage. Revue de MédecineVétérinaire 62, 546-551.

Hamad, A.-W.R., Al-Daghistani, H.I., Shquirat, W.D., Abdel-Dayem, M., Al-Swaifi, M., 2014. Sodium, Potassium, Calcium and Copper Levels in Seminal Plasma are Associated with Sperm Quality in Fertile and Infertile Men. Biochemistry & Pharmacology: Open Access 3, 1-7.

Hong, Z., Hailing, L., Hui, M., Guijie, Z., Leyan, Y., Dubing, Y., 2010. Effect of Vitamin E supplement in diet on antioxidant ability of testis in Boer goat. Animal reproduction science 117, 90-94.

Johnson, L., 2000. Sexing mammalian sperm for production of offspring: the state-of-the-art. Animal reproduction science 60, 93-107.

Kaneko, S., Oshio, S., Kobayashi, T., Iizuka, R., Mohri, H., 1984. Human X-and Y-bearing sperm differ in cell surface sialic acid content. Biochemical and biophysical research communications 124, 950-955.

Khalifa, E.I., Ahmed, M.E.M.E., Abdel-Salaam, I., El-Zelaky, O.A., Abdel-Gawad, A.M., 2009. Effect of semen extender pH value on Rahmani ram characteristics and on altering sex ratio of offspring. Agriculture Research Journal Suez Canal University 9, 49-52.

Kulaksiz, R., Bucak, M.N., Akcay, E., Ssakın, F., Daşkin, A., Ateşşahın, A., 2011. The effects of different extenders and myo-Inositol on post-thaw quality of ram semen. Kafkas Universitesi Veteriner Fakultesi Dergisi 17, 217-222.

Kulaksiz, R., Çebi, Ç., AKÇAY, E., 2012. The effect of different extenders on the motility and morphology of ram sperm frozen or stored at 4 C. Turkish Journal of Veterinary and Animal Sciences 36, 177-182.

Lopez, A., Söderquist, L., Rodriguez-Martinez, H., 1999. Sperm viability in ram semen diluted and stored in three different extenders. Acta Veterinaria Scandinavica 40, 1-9.

Nalley, W., Arifiantini, R., 2013. THE HYPO-OSMOTIC SWELLING TEST IN FRESH GARUT RAM SPERMATOZOA. Journal of the Indonesian Tropical Animal Agriculture 38, 212-216.

NRC, 2007. Nutrient requirements of small ruminants: Sheep, goats, cervids, and new world camelids, National Academies Press, Washington, D.C., U.S.A.

Paulenz, H., Söderquist, L., Pérez-Pé, R., Berg, K.A., 2002. Effect of different extenders and storage temperatures on sperm viability of liquid ram semen. Theriogenology 57, 823-836.

Rakha, B.A., Hussain, I., Akhter, S., Ullah, N., Andrabi, S.M., Ansari, M.S., 2013. Evaluation of Tris–citric acid, skim milk and sodium citrate extenders for liquid storage of Punjab Urial (Ovis vignei punjabiensis) spermatozoa. Reproductive biology 13, 238-242.

Salem, A., Nasrat, A.E., Gamal, M., 2013. Estrous activity detection device in mammals. Frontiers in Bioscience E 5, 798-808.

Slanina, T., Petrovičová, L., Miškeje, M., Kňížat, L., Mirda, J., Lukáč, N., Trandžík, J., Petrovičová, I., Massányi, P., 2015. The effect of diluent, temperature and age on turkey spermatozoa motility in vitro. Journal of Applied Animal Research 43, 131-136.

Snedecor, G.W., Cochran, W.G., 1989. Statistical methods. Iowa State University Press: Ames, IA.

Soltanpour, F., Moghaddam, G., 2014. Effect of diluents on storage of ram semen. Journal of Agri-Food and Applied Sciences 2, 179-183.

Soltanpour, F., Moghaddam, G., Asadpour, R., Rafat, S.A., 2014. Effect of Antioxidant combinations on sperm quality of cross breed rams during liquid storage. International Journal of Advanced Biological and Biomedical Research 2, 732-740.

Soylu, M.K., Nur, Z., Ustuner, B., Dogan, I., Sagirkaya, H., Gunay, U., Ak, K., 2007. Effects of various cryoprotective agents and extender osmolality on post-thawed ram semen. Bulletin-Veterinary Institute In Pulawy 51, 241.

SPSS, 2013. Statistical package for social sciences, IBM®SPSS Statistics Data Editor 22.0 License Authorization Wizard, Chicago, USA.

Stefanov, R.G., Anev, G., Abadjieva, D.V., 2015. Effect of different extenders and storage periods on motility and fertility of ram sperm. Macedonian Veterinary Review 38, 85-89.

Tirpan, M.B., ÖZGÖRAY, E.D., Alemdar, H., AKÇAY, E., 2016. Cryopreservation of seabream (Sparus aurata) semen and fertility. Turkish Journal of Veterinary and Animal Sciences 40, 200-206.

Torres-Flores, V., Picazo-Juárez, G., Hernández-Rueda, Y., Darszon, A., González-Martínez, M.T., 2011. Sodium influx induced by external calcium chelation decreases human sperm motility. Human reproduction 26, 2626-2635.

Torres Flores, V., GarcíaSánchez, N.L., González Martínez, M.T., 2008. Intracellular sodium increase induced by external calcium removal in human sperm. Journal of andrology 29, 63-69.

Vílchez, M.C., Morini, M., Peñaranda, D.S., Gallego, V., Asturiano, J.F., Pérez, L., 2016. Sodium affects the sperm motility in the European eel. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 198, 51-58.

Zeny, Z.A., 2016. Effect of use vitamin E and Zn on seminal plasma enzymes and their effect on trace minerals of shami male Goat. Kufa Journal for Agricultural Science 8, 257 - 265.

Zhou, J., Chen, L., Li, J., Li, H., Hong, Z., Xie, M., Chen, S., Yao, B., 2015. The Semen pH Affects Sperm Motility and Capacitation. PloS one 10, 1-15.

تأثير مخففات السائل المنوى التقليدية وغير التقليدية على جودة السائل المنوى للكباش أثناء التخزين على درجة حرارة ٥م°

عزالدين إبراهيم خليفة [١] و وائل أحمد خليل [٢]

[١]- معهد بحوث الإنتاج الحيوانى – قسم بحوث الأغنام والماعز- وزارة الزراعة - الدقى – الجيزة - مصر

[٢]- قسم إنتاج الحيوان - كلية الزراعة - جامعة المنصورة - مصر

إستخدم فى هذه الدراسة أربع كباش رحمانى لتجميع قذفات السائل المنوى بواسطة المهبل الصناعى. تم تجميع وتقييم السائل المنوى وأستخدم خمس مخففات إشتملت على مخففات تقليدية وغير تقليدية. المخففات التقليدية كانت مخفف سترات الصوديوم (م١) مخفف الترس (م٢) مخفف اللبن (م٣). بينما المخففات غير التقليدية كانت مخفف ملح كلوريد الصوديوم ٩و.% (م٤) و محلول الملحى الفسيولوجى (٩و٠%) المستخدم فى الحقن الوريدى (م٥) وكان معدل التخفيف ١ سائل منوى : ٦ مخفف وتم تخزين السائل المنوى المخفف بالمخففات السابقة على درجة٥م° لمدة ٦ أيام متتالية. بعد التخزين على ٠، ١، ٢، ٣،٤، ٥، ٦ أيام فحصت الحركة التقدمية، ومعدل الرجوع للحركة التقدمية، المقاومة الموجبة للإسموزية. وتم تقييم معدل الخصوبة للسائل المنوى المخفف بالمخفف التقليدى وغير التقليدى الذى أعطى أفضل صفات للسائل المنوى المخزن على ٥ م° لمدة يوم واحد. أستخدم ٢٢ نعجة متشابهة فى الأداء التناسلى والإنتاجى وقسمت النعاح إلى مجموعتين ١١نعجة / مجموعة ولقحت المجموعة الأولى والمجموعة الثانية بالمخفف التقليدى (م٢) والمخفف غير التقليدى (م٥) على التوالى. والنتائج أوضحت أن المخففات م١، م٢، م٥ أظهرت فروق معنوي عالية (٠٥و٠%) فى الحركة التقدمية، الرجوع للحركة التقدمية، المقاومة الموجبة للإسموزية مقارنة بالمخففات م٣، م٤ عند التخزين على ٥ م° لمدة ٦ أيام متتالية. بينما المخففات م٢، م٥ لم تظهر أى فروق معنوية فى الحركة التقدمية، الرجوع للحركة التقدمية، المقاومة الموجبة للإسموزية اثناء التخزين على ٥ م° لمدة ٦ أيام متتالية. أظهر التحليل الإحصائى للنتائج إرتباط معنوى موجب بين أنواع المخففات والحركة التقدمية، الرجوع للحركة التقدمية، المقاومة الموجبة للإسموزية لمنوى الكباش اثناء التخزين على ٥ م° لمدة ٦ أيام متتالية. وأيضا كان معدل الخصوبة أعلى مع المخفف غير التقليدى م٥ (٦٢و٨٤%) من معدل الخصوبة للمخفف التقليدى م٢ (٥٧و٧٨%) بعد التخزين على ٥ م° لمدة يوم واحد. وكان حجم البطن متفوق مع المخفف م٥ (٠٠و١) مقارنة مع المخفف م٢ (٨٦و٠). وكانت النسبة الجنسية للحملان الإناث المولودة ٧٦و٦٦% والذكور ٣٣و٣٠% مع السائل المنوى المخفف مع (م٢) بينما السائل المنوى المخفف مع (م٥) حقق نسبة جنسية للأناث المولودة بلغت ٢٣و٦٩% والذكور ٧٧و٣٠% . ومن هنا يمكن التوصية بأن مخفف المحلول الملحى الفسيولوجى ٩و٠% المستخدم فى الحقن الوريدى مناسب لتخزين السائل المنوى و أفضل لحفظ معدل الخصوبة وحجم البطن بعد التلقيح الصناعى بالسائل المنوى المبرد على ٥ م°.