CRYOPRESERVATION OF RABBITS SPERMATOZOA: EFFECT OF WARMING PROCEDURES, BREED AND POST-THAWING INCUBATION TIME

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SUMMARY

The present work was conducted to determine the effects of three warming procedures (37℃/15s, 50℃/12s or 70℃/10s) and post-thawing incubation time (0 to 2 hrs) on in vitro assessment of sperm cryosurvival (motility) and acrosome status (intact, modified and completely detached) of New Zealand White (NZW) and Baladi Black (BB) rabbits. Semen was collected from mature bucks (6 in each breed) three times a week (two ejaculates per male/time). Only ejaculates with >70% motility were considered, pooled and frozen in liquid nitrogen.

Post-thaw sperm motility and intact acrosome percentages were significantly (P<0.001, 0.01 or 0.05) affected by different thawing procedures used, rabbit breeds, post-thaw incubation time and their interactions. There was a tendency to post-thawing motility and intact acrosome improvements when using moderate (50℃/12s) thawing procedure (27.44 and 39.44%) compared to rapid (70℃/10s, 21.50 and 33.67%) and slower (37℃/15s, 17.33 and 28.33%) warming procedures. The highest percentage of post-thaw motility and intact acrosome (36.00 and 44.50%) were recorded when using moderate thawing procedure immediately after thawing, followed by 29.17 and 38.83% with the same warming procedure after one hour incubation, then 28.50 and 36.83% with thaw rate at 70℃/10s without incubation time. The higher percentage of structural abnormalities was observed with BB rabbits when using slow or rapid thawing procedures compared to NZW rabbits. It can be concluded that moderate warming procedure (50℃/12s) provided significantly more cryosurvival spermatozoa compared with the other thaw rates, especially in semen of the NZW rabbit bucks.

Keywords: Cryosurvival rabbit spermatozoa, warming procedures

INTRODUCTION

The cryopreservation of spermatozoa has allowed specific opportunities for the conservation of genetic resources through sperm banks, the guarantee of a constant commercial supply of semen, and collaboration in breed improvement programs (Holt, 1997). Most of domestic species (including the rabbit), semen cryopreservation is not yet under control (Salvetti *et al.*, 2006). Successful preservation of spermatozoa irregardless of species is dependent on a series of steps aimed to reducing damage to the cell and securing adequate longevity *in vitro* and *in vivo*.

Many factors influencing semen quality through cryopreservation process are known include the technique of semen collection, the optimal dilution to obtain the

final concentration of spermatozoa (Okano *et al.,* 2004), type of buffer and the addition of freezing cryoprotectant agents (Rijsselaere *et al.,* 2002), the combination of extender and cooling rate during the freezing procedure (Pena and Linde-Forsberg, 2000), the thawing technique (Rota *et al.,* 1998), as well as the use of a thawing medium and equilibration time (Linde-Forsberg *et al.,* 1999) and possibly, also the removal of cryoprotectant after thawing.

Various studies carried out on freezing and thawing procedures for animal semen used different temperatures (35 - 75 °C) and times (5 - 60 s) for the thawing of straws (Velasco-Santamaria *et al*., 2006, Paulenz *et al*., 2007 and Muino *et al*., 2008). Most procedures used to thaw spermatozoa from rabbit have been modified from those considered to be standard for domestic ungulates and the optimal thawing procedure for rabbit spermatozoa frozen in straws remains to be established. Therefore, the aim of this study was to *in vitro* assess spermatozoal longevity (cryosurvival) of freez-thawed rabbits spermatozoa as affected by warming procedures and rabbit breeds during different thawing-incubation time.

MATERIALS AND METHODS

Animals: Twelve mature New Zealand White (NZW) and Baladi Black (BB) rabbit bucks (6 in each breed) with an average body weight of 3.26±0.24 and 2.97±0.18 kg, respectively were used. They were housed in flat deck cages in rooms with controlled light (16:8 h L:N). Room temperature ranged from 25 to 28°C. Animals received a commercial diet and water *ad libitum*.

Semen collection, processing and assessment of spermatozoa quality: Semen was collected three times a week (two ejaculates per male from each rabbits breed were collected, with a minimum of 30 min between ejaculate collections) with an artificial vagina. Only ejaculates exhibiting a white colour were used in the experiment, and if gel was present, it was removed. The volume was measured in a graduated conical tube. Sperm motility and morphological evaluations were performed in Tris diluent (0.25 M Tris, 88 mM citric acid anhydrous, and 47 mM D (+) glucose, according to **(**Viudes de Castro *et al*., 1999). Only ejaculates with >70% motility were considered. Sperm-cell concentration (x10^6/ml) of the original suspensions was determined using a hematocytometer. Motility and acrosomal status for cryopreserved sperm were made in the same way as for fresh sperm. The status of the acrosome of the normal sperm (intact, modified or completely detached) was evaluated as described by Tamuli and Watson (1994).

Freezing protocol: Following the initial assessment, the pooled fresh semen collected from each rabbit breeds were extended at 37ºC with the dilution extender A (One part of fresh semen plus two parts of extender A). This dilution extender (Tris-Citric acid-Glucose) has the following composition: 0.25 M of Tris [hydroxymethyl] aminomethane, 88 mM of anhydrous citric acid, and 47 mM of D (+) glucose, and distilled water to 100 ml (Moce *et al.,* 2003). The tubes of diluted semen were placed in water bath of 37ºC and gradually cooled to 5ºC in refrigerator. Then, the semen was diluted at 5ºC with freezing extender (B) which composed of extender A and cryoprotective agent (40 mM sucrose combined with 1.5 M dimethylsulfoxide, DMSO). The diluted semen was mixed with the freezing extender as one part semen + two parts extender A + two parts extender B (1+4 or 1:5 ratio). The pH level was adjusted to 6.8 at final for the sperm survival. The extended semen was packed in 0.5 ml plastic straws, (IMV_ Technologies, L'Aigle, France), and sealed with polyvinyl-

alcohol sealing powder and kept at 5°C for 90 min as equilibration period. The straws were placed horizontally in a rack approximately 5 cm above the liquid nitrogen surface for 10 min, before plunging into liquid nitrogen container (-196ºC).

Warming procedures: The semen frozen straws in each rabbit breeds were divided into three groups and subjected to the one of the thaw procedures (slow: done at 37ºC water bath for 15 second; moderate: done at 50ºC water bath for 12 s and rapid: done at 70ºC water bath for 10 s) then removed from the bath and dried.

Assessment of frozen-thawed spermatozoa longevity: The frozen-thawed semen samples were incubated at 37°C for 2 h in the freezing medium. The percentages of post-thawing sperm motility and acrosomal status (intact, modified and completely detached) were assessed immediately (without incubation time, 0 hrs) and after one and two hours post-incubation.

Scanning electronic microscopy of rabbit spermatozoa: Sperm morphology was assessed by scanning electron microscopy. After rinsing with 0.1 M Hepes buffer with 0.05 M NaCl (250-260 mOs), spermatozoa were fixed for 1 hr in 1.5% glutaraldehyde and 1.5% paraformaldehyde in the same buffer. Then, they were washed by 3 changes of Hepes buffer and placed on small glass cover slips (12 x 12 mm) coated with 0.1% poly-L-lysine solution. Post fixation was done with 1% osmium tetroxide in Hepes-NaCl buffer for 1 hr. Samples were then, dehydrated in a series of increasing concentrations of ethanol, critical point dried, sputter-coated with gold and examined with a Zeiss DSM-982 scanning electron microscope at an accelerating voltage of 30 kV.

Statistical analysis: The experimental trail was replicated seven times (each include 6 straws for each treatment). Data were analyzed using Least Squares Analysis of Variance using the GLM program of SAS (1998) according to the following fixed model:

$$Y_{ijklm} = \mu + R_i + A_j + B_k + C_l + AB_{jk} + AC_{il} + BC_{kl} + ABC_{jkl} + e_{ijklm}$$

Where Y_{ijklm} is the observation; μ is the overall mean; R_i is the random effect of the replicate, i = 1, 2,….7, A_j is the fixed effect of jth breed effect, j = NZW and BB, B_k is the fixed effect of kth warming procedures effect, k = slow, moderate and rapid, C_l is the fixed effect of lth post-thaw incubation time effect, l = 0, 1 and 2 hrs, AB_{jk}, AC_{jl}, BC_{kl} and ABC_{jkl} = the fixed effect of interactions and e_{ijklm} is the random error. The differences among means were tested using Duncan's New Multiple Range Test (Duncan, 1955).

RESULTS AND DISCUSSION

1. Effect of warming procedures on post-thawing sperm quality:

The percentage of post-thaw sperm motility and intact acrosome of rabbit spermatozoa were significantly (P<0.001) affected by different warming procedures used (Table 1). The results are in agreement with those reported by Bruce (2005) who found that the thawing procedure is of critical importance when using cryopreserved semen, which influence the post-thaw survival of a number of cells.

Warming semen with moderate warming procedure (50ºC/12s) had higher post-thaw sperm motility and intact acrosome percentages (27.44 and 39.44%), followed by those thawing with rapid thawing (21.50 and 33.67%), then slower thawing rate (17.33 and 28.33%). Likewise, Eriksson and Rodriguez-Martinez (2000) found that increase thawing rate (50 or 70ºC compared to 35ºC) improved motility and NAR, which considered as an important parameter to monitor for evaluating the potential

fertility of post-thaw semen. Also, Nur *et al*. (2003) found that thawing temperatures higher than 35ºC improved post-thaw sperm motility, acrosomal integrity or sperm morphology. In addition, Moce *et al*. (2003) reported that thawing at 50ºC gave better results (higher motility and NAR) than thawing at higher temperature (70ºC). However, Chen and Foote (1994) reported that the best rabbit sperm motility after thawing was obtained when the thawing temperature was 25ºC for 1 min. Generally, thawing stimulates metabolism and motion of sperm with intact plasma and acrosomal membranes (Bedford *et al*., 1999).

Table 1. Post-thawing motility and acrosome status of rabbit spermatozoa as affected by thawing procedures, rabbit breeds and post-thawing incubation time

Items	Post-thawing motility (%)	Acrosome status (%)		
		Intact	Modified	Completely detached
Thawing procedures (ºC/second):				
37 ºC/15 s	17.33[c]±0.54	28.33[c]±0.37	32.11±0.78	39.56[a]±0.66
50 ºC/12 s	27.44[a]±0.54	39.44[a]±0.37	29.78±0.78	31.33[c]±0.66
70 ºC/10 s	21.50[b]±0.54	33.67[b]±0.37	29.89±0.78	36.44[b]±0.66
Significance	***	***	NS	***
Rabbit breeds:				
NZW	25.11[a]±0.44	36.67[a]±0.30	34.11[a]±0.64	29.22[b]±0.54
BB	19.07[b]±0.44	30.96[b]±0.30	27.07[b]±0.64	43.33[a]±0.54
Significance	***	***	***	***
Post-thawing incubation time (hours):				
0	29.33[a]±0.54	37.78[a]±0.37	25.94[c]±0.78	36.83±0.66
1	23.72[b]±0.54	33.56[b]±0.37	31.89[b]±0.78	34.56±0.66
2	13.22[c]±0.54	30.11[c]±0.37	33.94[a]±0.78	35.94±0.66
Significance	***	***	***	NS

[a,b,c] Values with different superscripts in the same column significantly differ (P< 0.05).
[***] P< 0.001, [**] P< 0.01, [*] P< 0.05 and NS= not significant.

The improvement in cryosurvival spermatozoa values when using moderate warming procedure (50ºC/12s), could be attributed to a reduced recrystallization and cell damage. In pointed out that**,** Amann and Pickett (1987) reported that rapid thaw rate prevents recrystallization and its attendant damages. Also, results in Table (1) revealed that the slower and higher rates of thawing significantly (P<0.001) increased the percentages of completely detached acrosomes (39.56 and 36.44%, respectively) compared to the moderate thawing rate (31.33%).

Decreasing values of cryosurvival spermatozoa with 37ºC/15s (Table 1) may be due to a slow thaw rate allows slower movement of water across membranes, and the metabolism of these sperm cells can be reestablished slowly, whereas a fast thaw rate may require cells to deplete nutrients rapidly in reestablishing metabolic functions and also may lead to frictional damage to the cell membranes because of rapid movement of water across the membranes to re-equilibrate the ion balance. Additionally, a slow thaw rate which allows time for small ice crystals, formed intracellularly during freezing, to recrystallize forming larger crystals that damage the cell structurally, leading to cell death (Mazur, 1984).

Decreasing cryosurvival spermatozoa percentages with increasing thawing temperature to 70ºC/10s (Table 1), may be due to rapid warming can induce osmotic

stress on the spermatozoa because of the abrupt melting of the extra cellular solution, which can cause unbalanced rates of water influx and cryoprotectant egress, and can lead to swelling and lysis of cells (Mazur, 1984). In addition, Holt (2000) reported that increases in post-thawing temperature cause physical disruption of the plasma membrane due to subjecting it to structural rearrangements involving lipids and proteins. Moreover, Cabrita *et al*. (2001) concluded that high thaw temperatures can lead to denaturation of sperm enzymes in the peripheral area of the straws before the inside was thawed, as well as non-homogeneity in the thawing process and reduced viability. Additionally, an increase in the thawing temperature reduced recrystallization of intracellular ice, a process that can lead to the formation of larger and more stable ice crystals that could damage mitochondria (Fiser and Fairfull, 1990).

2. Effect of rabbit breeds on post-thawing sperm quality:

The percentage of post-thawing motility and acrosome status (intact, modified and completely detached) of spermatozoa were significantly (P<0.001) affected by rabbit breeds (Table 1). Irrespective to the thawing rates, semen collected from New Zealand White (NZW) rabbits yielded the higher sperm cryosurvival and intact acrosome compared with those received from Baladi Black (BB) rabbits. This finding is in accordance with Meshreky and Abbas (2001) who found that NZW had the best semen quality compared to Baladi Black rabbit breeds. The difference between breeds may be related to genetic factors. Similarly trends were obtained by Moce *et al.* (2003) observed significant differences between frozen-thawing spermatozoa of three rabbit lines in fertility rate (62 and 68% versus 45% for lines A, V and R, P<0.01). Nakagata *et al.* (1995) also found spermatozoa of Chinese mouse strain has a lower tolerance to cryopreservation than the Czechoslovakian mouse strain. In addition, Thurston *et al.* (2002) found that the variability of sperm of pigs from different individuals to survive freeze-thawing have indicated that there could be a genetic predisposition for freezability**.**

3. Effect of post-thawing incubation time on sperm quality:

Regardless of the warming procedures used and rabbit breeds, percentages of motility and intact acrosome were decreased (P<0.001) with the advancement of post-thawing incubation time at 37°C for up to 2 hours (Table 1). Similar observation was also reported by Abd El-Salaam (2002) and Meshreky *et al*. (2007). This phenomenon may be attributed to the decrease in the content of adenosine triphosphate, which could inactivated spermatozoa were apparently in capable of resynthesizing. This was accompanied by a precipitous fall in the rate of fructolysis (Salamon, 1970). However, Daader and Zeidan (2008) found that frozen-thaw semen incubated at 37°C showed a significant increase in post-thaw motility after one hour incubation and then a decrease after two hours at a significant level.

4. Effect of interactions on post-thawing sperm quality:

Post-thaw sperm motility, intact and modified acrosome percentages were significantly (P<0.05) affected by the interaction between warming procedures used and post-thaw incubation times, (Table 2). Frozen-semen thaw with moderate warming procedure had higher post-thaw sperm motility and intact acrosome percentages immediately after thawing (36.0 and 44.5%), followed by the same warming procedure after one hour incubation (29.17 and 38.83%), then rapid thaw

rate immediately after thawing (28.5 and 36.83%). The lowest values were recorded with slow warming procedure after 2 hr of incubation (9.17 and 24.33%). Whereas, Ana *et al*. (2003) showed that no significant differences in the seminal parameters evaluated for the different thawing procedures used, except after 2 h of incubation at 42ºC, where significant differences were found.

Table 2. Post-thawing cryosurvival rabbit spermatozoa percentages as affected by interactions among warming procedures, post-thawing incubation time and rabbit breed

Interactions	Post-thawing motility (%)	Acrosome status (%)		
		Intact	Modified	Completely detached
*Thawing procedures * incubation time:*				
37ºC/15s * 0 hr	23.50[bc]±1.9	32.00[cde]±1.7	26.50[bc]±2.3	41.50±3.2
1 hr	19.33[cd]±1.9	28.67[ef]±1.7	33.17[ab]±2.3	38.17±3.2
2 hr	9.17[f]±1.9	24.33[f]±1.7	36.67[a]±2.3	39.00±3.2
50ºC/12s * 0 hr	36.00[a]±1.9	44.50[a]±1.7	28.00[bc]±2.3	29.17±3.2
1 hr	29.17[b]±1.9	38.83[b]±1.7	29.83[abc]±2.3	31.33±3.2
2 hr	17.17[de]±1.9	35.00[bcd]±1.7	31.50[ab]±2.3	33.50±3.2
70ºC/10s * 0 hr	28.50[b]±1.9	36.83[bc]±1.7	23.33[c]±2.3	39.83±3.2
1 hr	22.66[cd]±1.9	33.17[cde]±1.7	32.67[ab]±2.3	34.17±3.2
2 hr	13.33[ef]±1.9	31.00[de]±1.7	33.67[ab]±2.3	35.33±3.2
Significance	*	*	*	NS
*Thawing procedures * rabbit breeds:*				
37ºC/15s * NZW	17.56[c]±2.5	28.22[c]±1.3	37.56[a]±1.7	34.22[b]±1.1
BB	17.11[c]±2.5	28.44[c]±1.3	26.67[c]±1.7	44.89[a]±1.1
50 ºC/12s * NZW	31.44[a]±2.5	43.22[a]±1.3	31.00[bc]±1.7	25.78[c]±1.1
BB	23.44[bc]±2.5	35.67[b]±1.3	28.55[c]±1.7	36.89[b]±1.1
70 ºC/10s * NZW	26.33[ab]±2.5	38.56[b]±1.3	33.78[ab]±1.7	27.67[c]±1.1
BB	16.67[c]±2.5	28.78[c]±1.3	26.00[c]±1.7	45.22[a]±1.1
Significance	***	***	**	**
*Rabbit breeds * post-thawing incubation time:*				
NZW * 0 hr	32.78[a]±1.8	41.56[a]±1.9	27.56[bc]±1.3	30.89±1.7
1 hr	27.67[ab]±1.8	36.33[ab]±1.9	36.22[a]±1.3	27.44±1.7
2 hr	14.89[cd]±1.8	32.11[bc]±1.9	38.56[a]±1.3	29.33±1.7
BB * 0 hr	25.89[b]±1.8	34.00[b]±1.9	24.33[c]±1.3	42.78±1.7
1 hr	19.78[c]±1.8	30.78[bc]±1.9	27.56[bc]±1.3	41.67±1.7
2 hr	11.56[d]±1.8	28.11[c]±1.9	29.33[b]±1.3	42.56±1.7
Significance	*	**	*	NS

[a,b,c] Values with different superscripts in the same column in each interaction differ significantly (P< 0.05).
[***] P< 0.0001, [**] P< 0.001, [*] P< 0.05 and NS= not significant.

Post-thaw sperm survival and acrosome status percentages were affected (P<0.001 or 0.01) by interaction between different thawing procedures and rabbit breeds (Tables 2). The highest percentage of post-thaw sperm motility and intact acrosome percentage (31.44 and 43.22%) were obtained with semen of NZW rabbits using moderate thawing procedure, followed by 26.33 and 38.56% with the same rabbit breed but with thaw rate at 70ºC/10s, then 23.44 and 35.67% with BB rabbits thaw with moderate warming procedure. Similarly, Moce *et al.* (2003) found significant differences between frozen-thawing spermatozoa of three rabbit lines (A, V and R) and thawing rates (50 or 70 ºC for 10-12 second), the best results for all lines were observed when sperm thawed at 50ºC (85, 84 *vs*. 50%). Decreased or increased thawing rate increased (P<0.01) completely detached acrosome percentages

from 36.89 to 44.89 and 45.22%, respectively with moderate thawing rate in BB rabbits, whereas from 25.78 to 34.22 and 27.67% in NZW rabbits (Tables 2).
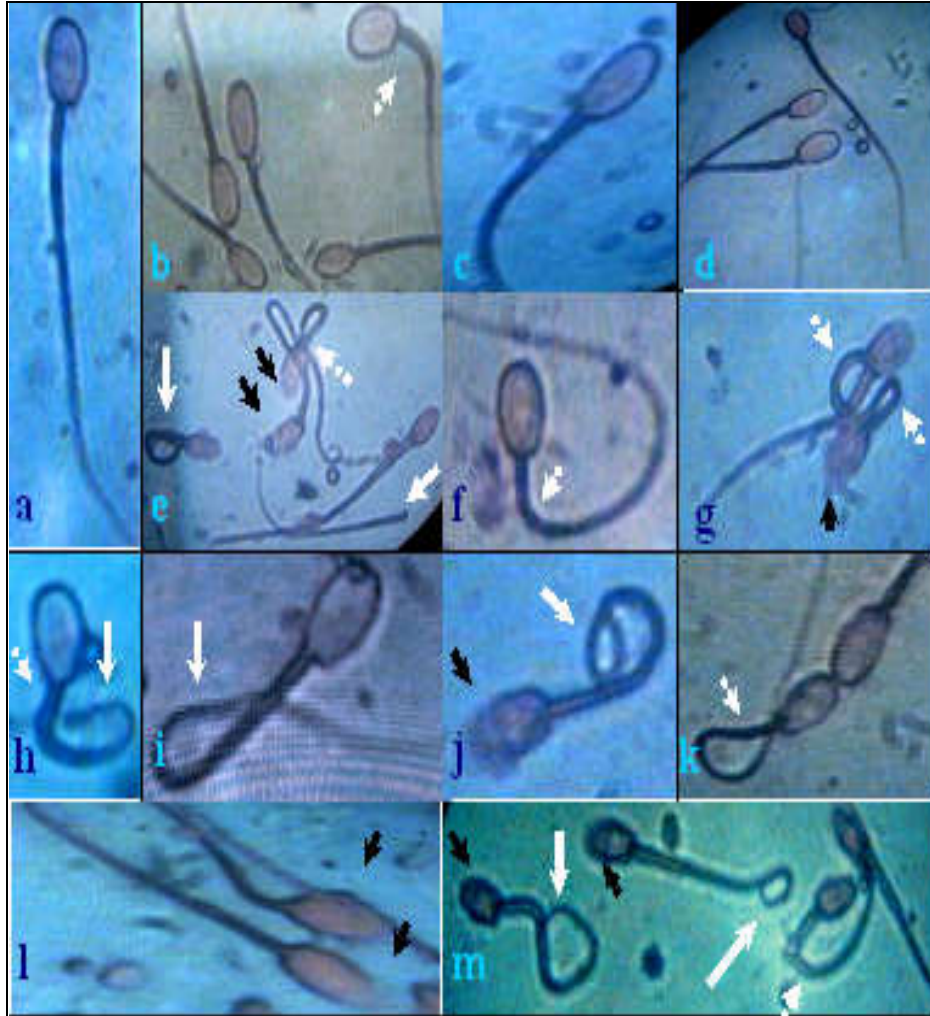
Post-thaw sperm motility, intact and modified acrosome percentages were affected (P<0.01 or 0.05) by interaction between rabbit breeds and post-thaw incubation time (Tables 2). Thawing spermatozoa of NZW or BB rabbits and incubated for 2 h at 37°C, reduced sperm motility (longevity) and intact acrosome percentages, and increased modified acrosome. Semen collected from BB rabbit bucks ranked the second in cryosurvival of spermatozoa after NZW rabbit and gave its lowest values after thawing. Generally, the freeze-thaw process damages the anterior plasma membrane of sperm, which is the normal site of calcium exchange that alters ionic balance and increases intracellular calcium (Bedford *et al.*, 1999). Therefore, the freezing and thawing process can stimulate both physiologic and nonphysiologic capacitation and acrosome reactions (Bradford and Buhr, 2002).

Interaction among different thawing procedures, post-thawing incubation time and rabbit breeds insignificantly affected any of the tested parameters.
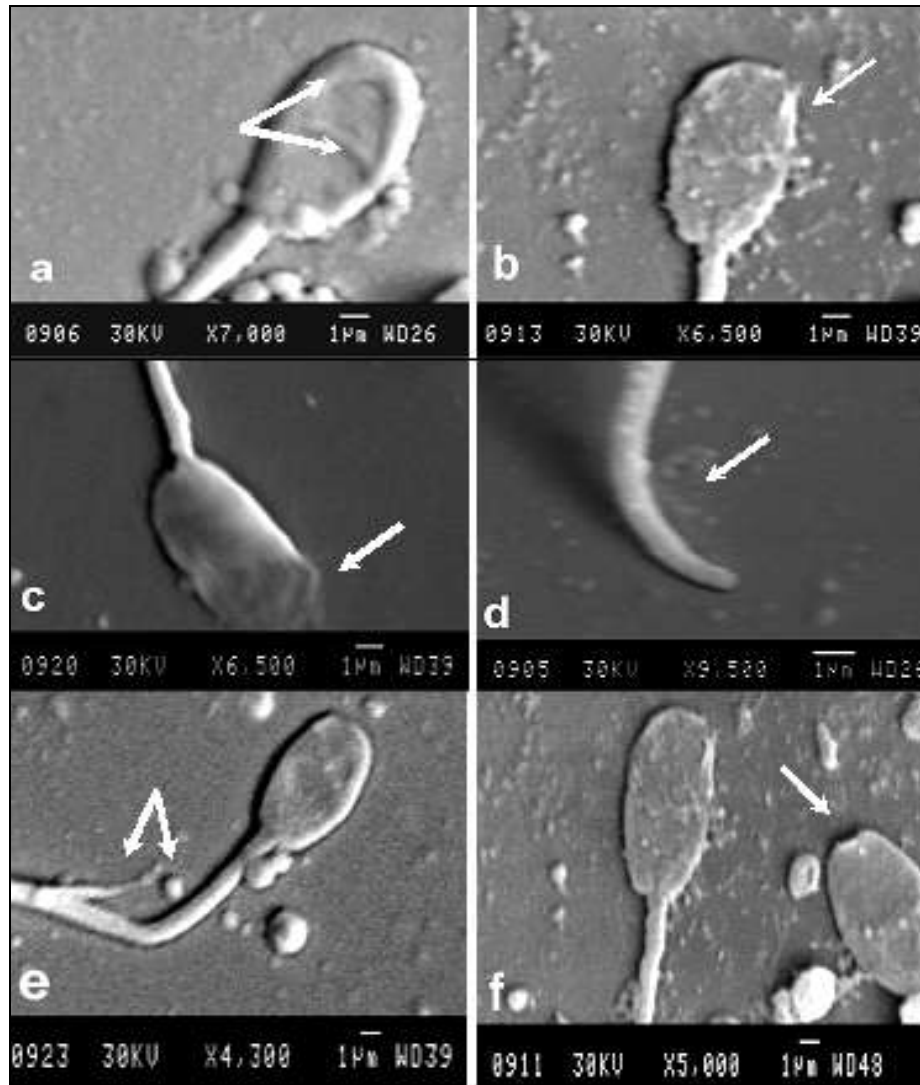
Structures of frozen-thawed spermatozoa

The frozen-thawed spermatozoa of rabbit were analyzed for morphological changes under scanning electron microscope (S.E.M.) and showed either normal spermatozoa without any surface damage (intact acrosome) or ultra structure damage as ruptures of the plasma membrane in the acrosomal region during the cryopreservation stage, large areas of rough or disrupted acrosomal surface (the acrosome covered more than 50% of the head length. Results of S.E.M. confirmed that the semen cryopreservation induced a decrease of intact acrosomes, in addition, increased structure changes in bent midpiece and thickening of the midpiece, coiled tail, and detached tail (Plate 1 & 2). These observations are accordance with those obtained by Liu *et al.* (2004) who found damage in acrosome and sperm tail after freez-drying process in rabbits. In addition, this study documented the dimensions of rabbit sperm. The total length of rabbit spermatozoa was about 35–45 μm with an ovoid head of about 7-10 μm in length and about 5 μm in diameter.

Frozen-thawed semen with moderate warming procedure of NZW rabbits without incubation time showed the lowest damage in sperm acrosome and tail, followed by those thawing in the same warming procedure with the same rabbit breed after one hours of incubation time, then semen of the same rabbit breed with rapid warming procedure (70ºC/10s). After 2 hrs post-thawing incubation, semen of BB rabbits thaw with slow or rapid warming procedures showed the highest percentage of abnormal sperm structure of acrosome and the connection between the sperm head and tail became so fragile that pipetting could easily cause head-tail separation (Plate 1 & 2). These observation are agree with Mazur (1984) who found that mechanical damage or alteration of membrane molecules impedes proper sperm function, and both types of damage are affected by the rates of cooling, freezing, and warming. In the same relation, Polgar *et al.* (2004) observed that the freezing-thawing process increased the number of dead sperm cells from 18% to 46%, at the same time, a significant (P<0.001) damage of acrosomal and tail membrane integrity. Nur *et al.* (2003) also found that thawing temperatures higher than 35ºC improved post-thaw acrosomal integrity or sperm morphology.

Plate 1. Phase-contrast of frozen-thawed rabbit spermatozoa: (a) normal viable sperm with intact acrosome. (b, c and d) viable sperm with gauzy and intact acrosome while non-viable sperm tended to be reddish. (e, g, j and l) the membrane damage in the acrosome. (b, f, g, I and k) bent midpiece. (e, h, J, i and m) coiled tail. (e) detached tail. Black arrows indicate acrosome modified or completely detached and white arrows indicates abnormality in tail sperm, white arrow head indicates abnormality in neck and midpiece regions

Plate 2. Scanning electron photograph of ultrastructure of frozen-thawed rabbit spermatozoa: (a) The sperm head shape is normal ovoid with intact acrosome. (b) Sperm with modified acrosome (head shows partly acrosomal damage). (c) Sperm with completely damaged acrosome (ruptured membrane). (d) Intact and bent tail (e) Sperm with coiled and ruptured tail. (f) Detached head (head-tail separation)

CONCLUSION

It can be concluded that moderate warming procedure (50ºC/12s) provided significantly more cryosurvival spermatozoa compared with the other thawing procedures immediately after thawing, especially in semen of the NZW rabbit bucks.

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تجميد السائل المنوى للارانب: تأثير معدل الإسالة ، سلالة الارانب و فترة التحضين بعد الأسالة

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أجرى هذا العمل لدراسة تأثير ثلاثة من طرق الإسالة (٣٧ °م/١٥ ث أو ٥٠ °م/١٢ ث أو ٧٠ °م/١٠ ث) و فترة التحضين بعد الأسالة (من صفر الى ٢ ساعة) على حيوية (الحركة) الحيوانات المنوية المجمدة و حالة الأكروسوم (كامل، محور، منفصل تماما) معمليا للأرانب النيوزيلاندى الابيض و البلدى الاسود. تم جمع السائل المنوى من الذكور الناضجة (ستة ذكور من كل سلالة) ثلاث مرات أسبوعيا (قذفتين من كل ذكر فى كل مرة). أستخدم فقط السائل المنوى الذى يحتوى على أكثر من ٧٠% حيوية، تم تجميعه و تجميده فى النيتروجين السائل.

تأثرت النسبة المئوية للحركة و الأكروسوم الكامل للحيوانات المنوية للارانب بعد الأسالة معنويا (على مستوى ٠.٠٠٠١ أو ٠.٠٠١ أو ٠.٠٠٥%) بطرق الإسالة المختلفة المستخدمة و سلالة الارانب و فترة التحضين بعد الأسالة و التداخل بينهم. لوحظ تحسن في قيم الحركة و الأكروسوم الكامل بعد الإسالة عند استخدام معدل الإسالة المتوسطة (٥٠ °م/١٢ ث) و كان ٢٧.٤٤ و ٣٩.٤٤% مقارنة بمعدل الإسالة السريع (٧٠ °م/١٠ ث) و كان ٢١.٥٠ و ٣٣.٦٧% و معدل الإسالة البطيء (٣٧ °م/١٥ ث) و كان ١٧.٣٣ و ٢٨.٣٣% على التوالى. سجلت أعلى نسبة مئوية من الحركة و الأكروسوم الكامل بعد الإسالة (٣٦.٠٠ و ٤٤.٥٠% على التوالى) عند أستخدام طريقة الإسالة المتوسطة مباشرة بعد الاسالة، يلية ٢٩.١٧ و ٣٨.٨٣% على التوالى مع نفس معدل الاسالة بعد ساعة واحدة تحضين، ثم ٢٨.٥٠ و ٣٦.٨٣% على التوالى مع معدل اسالة عند ٧٠ °م/١٠ ث بدون مدة تحضين. لوحظت أعلى نسبة تركيبات شاذة للحيوانات المنوية المجمدة بعد الأسالة فى الارانب البلدى الاسود عند أستخدام معدلات الاسالة البطيئة أو السريعة مقارنة بالارانب النيوزيلاندى الابيض. يمكن أستنتاج أن معدل الإسالة المتوسط (٥٠ °م/١٢ ث) قد أعطى أفضل نسبة حيوية مقارنة بمعدلات الاسالة الاخرى و خصوصاً مع السائل المنوى لذكورالأرانب النيوزيلاندي الأبيض.