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EFFECT OF USING TWO METHODS OF STAINING ON SPERM MORPHOLOGY CHARACTERS OF BULL AND BUCK SPERM AFTER FREEZING.

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ABSTRACT

Two different methods of staining (Harris hematoxylin&Modified Bryan`s sperm stain) were used to explore sperm morphology changes after freezing for both bull and buck sperm. Morphology sperm characters were concerned with state of the following sperm organelles; head, med piece, acrosome, tail and cytoplasmic droplet. Observations of abnormal sperm morphology were higher and differed significantly for rabbit sperm than bull sperm after freezing. In addition both bull, buck sperm morphology differed significantly before and after freezing. It could be concluded that, rabbit sperm is more sensitive for freezing process than bull sperm and it is still needed for more appropriate freezing media to avoid freezing injuries at cellular and sub cellular levels.

Key words : Rabbit – Buck – Bull - Sperm – Staining – Acrosome – Frozen semen

INTRODUCTION

Freezing process can affect sperm morphology in different ways. Hyperosmotic media is the feature of frozen semen by the solute concentration which results as pure water is withdrawn from suspension media both inside and outside the cells (Salisbury, et al., 1978). Ice crystals formation both inside or outside sperm cells can alter and destruct sperm constitution. Rabbit sperm cell is quit different from bull sperm in morphology and its resistance to freezing techniques (Courtens and Theau-Clement, 1996; Weitze and Petzoldt, 1992). Bull sperm could be considered to be ideal cell for freezing than sperm from a great number of other domestic animals (Vengust *et al.,* 1981). Still far rabbit sperm do not persist and outrun both physical and chemical changes during freezing process. This is very noticeable from low rates of fertility using rabbit frozen semen (Awad *et al.,* 2000). The objective of this study is to point out and determine physical changes in bull and rabbit sperm cells after and before freezing using two different methods of staining.

MATERIALS AND METHODS

Freezing procedure:

Semen of three Holstein-Friesian bulls and 3 White New Zealand bucks was collected and pooled separately for subsequent deep-freezing. Freezing media for bull semen based on the following ingredients according to Steinbach and Foote (1967). tris hydroxymethylaminomethane 3.028 gram, glucose 1.25 g, citric acid 1.7 g, Egg yolk 20 ml, glycerol 8ml, 30,000 IU procaiene penicillin-G, 10,000 IU sodium penicillin-G and 50,000 micrograms

streptomycin based (as sulphate). Whereas extender for freezing buck semen was prepared as the following according to Kishk (2001). Tris 3.03 g, Egg yoik 20 ml, glycerol 3ml, citric acid 1.675 g, D-glucose 1.25 g and the aforementioned levels of antibiotics. Extenders for freezing both bull and buck semen were divided into two portions. Portion A was free of glycerol and portion B contained double percentage of glycerol for each extender. Portion B was added after reaching extended semen 5 °C in refrigerator and left for 2 hours after packing in plastic straws (0.5 ml) for glycerolation period. Liquid Nitrogen (LN) at - 196 °C was used for semen freezing in two steps criteria. First step was carried out at LN vapor in a foam container using a metal rack which kept 5 cm over surface of LN for 5 minutes. After then all the straws were plunged in LN to reach – 196 °C. After one week of storage at –196 °C, a straw of each species of semen was thawed at 37 °C for 1 minute for subsequent sperm staining.

Semen Smear staining:

Two different stains were used in this experiment (Harris hematoxylin & Modified Bryan's sperm stain). First stain is Harris hematoxylin stain. The stain was prepared as the following order: I- dissolve 2 gram hematoxylin stain (crystals) in 1 liter distilled water and heat slowly up to 90 °C until complete dissolving. The solution color will be dark red. II- Add 1 gram citric acid to change pH to acidic condition. III- Add 160 gram ammonium sulphate and 6 gram mercuric oxide and heat once again slowly until complete dissolving at 90 °C. The stain color will be dark yellowish. Immediately plunge the stain container in a cold- water bath and filter after the solution become cold. Store in dark- brown bottles at room temperature and let the stain for 2 days. Dilute the required amount with an equal amount of distilled water and filter again. Staining procedure was carried out in 3 successive steps. 1- Fixation: sperm smear was fixed in ethyl alcohol 95% for 5 minutes. After that, let the slides for drying at room temperature for 1 hour. 2- Staining: fixed smear was plunged in staining jar for 5 minutes only. 3- Rinsing: stained smear was rinsed in distilled water for 2 minutes. After that, keep the slides at room temperature for drying for next microscopic examination. Whereas Brayan's modified stain was prepared as the following: 1500 ml acetic acid 1%, eosin yellow 0.5 gram, fast green 0.5 gram and naphthol yellow S 0.5 gram, mix the previous ingredients and store in stoppered bottle and filter the stain before use.

Sperm examination:

Morphology of stained sperm was described for normal and abnormal (malformation) sperm cells. Each sperm was subjected to examination of head region, acrosome membranes, med piece part, tail piece and cytoplasmic droplet. Percentages of forementioned characters were measured basing on an account of 300 sperm / semen smear slide. For each kind of semen smear before and after freezing of bull and buck semen, some microscopic photos were taken using Zeise computerized microscope with connected camera to obtain a magnification 1000 X with oil lens.

Statistical analysis:

Percentages of examined criteria were analyzed to test significant differences using t-tests for paired samples of SPSS Program. T-tests were carried out to test significant differences of sperm morphology between frozen bull semen and unfrozen bull semen and the same for buck sperm morphology.

RESULTS AND DISCUSSION

Sperm head morphology

Data which are presented in Tables 1& 2 showed the significant differences (P < 0.01) between unfrozen and frozen semen as regards sperm morphology of rabbit sperm but there were no significant differences in respect to bull semen. The morphology of sperm head region did not differ significantly between frozen bull sperm and frozen buck sperm. Where the percentage of intact head was 82 and 75% for frozen bull and buck sperm respectively. The percentage of intact head was 97 and 98% for unfrozen bull and buck sperm, respectively and sperm morphology differed significantly (P < 0.01) between two species. The importance of head region for evaluation sperm morphology after freezing lies in its content of nucleus (genetic material) and acrosome apparatus which enables sperm to achieve following fertilization process (Yanagimachi, 1988). It is noticeable that bull sperm is more resistant to deep freezing technique (Vengust *et al.,* 1981). The sperm resistance to freezing process can be observed from the percentages of broken head for bull and buck sperm as shown in Table 1. ure 6 A&B can also prove to what extent the buck sperm may loss its motility apparatus through freezing process. Whereas acrosome region of bull sperm was more sensitive to freezing where percentages of lost acrosome were 12 and 2% for bull and buck sperm, respectively as shown in Table 1& ures 1, 2& 5.

Swallowing acrosome percentage was higher for bull sperm (Table 1) than for buck sperm especially after freezing while it was not observed for unfrozen semen for both kinds of two species. The major significance of acrosome region can be attributed to its enzyme content which pave the entrance for sperm to across egg investments and to achieve fertilization process (Meizel, 1978). The opposite situation was recorded as for swallowing acrosome for unfrozen semen as shown in Table 1. Swallowing acrosome is a needed step to reach acrosome reaction or acrosome loss. This phenomena in turn is needed prior to fertilization occurrence. Whereas swallowing head is another phenomena in which it explains the existence of a degree of hypotonic media around the sperm cell. Such these conditions, which may face sperm cell especially during thawing process was reported by (Courtens and Theau-Clement, 1996). Elongated head or tapered head can denote that the sperm cell may encounter a degree of hypertonic solution effects, which lead to sperm dehydration. Sperm dehydration can produce some changes at the sperm head level especially sperm cell membrane and acrosome membranes (Curry, 2000). Ice crystals formation during freezing process can deform and alter sperm cell membranes. Some of these injuries are the broken plasma membranes, defective acrosomes and acrosome

misshaping as shown in ures 1,3,7& 8. Such high concentrations modify both the conformations of proteins and the relations between nucleoproteins and DNA (Courtens *et al.,* 1989). A situation which does no favour fertility performances in the rabbit (Courtens *et al.,* 1989; 1994).

Table 1: Effect of freezing and thawing procedure on sperm morphology at head and acrosome level of bull and buck sperm.

Head (h.) Morphology	Frozen Sperm (%) Bull ^{A**}	Frozen Sperm (%) Buck ^{A**}	Unfrozen Sperm (%) Bull ^{A**}	Unfrozen Sperm (%) Buck ^{B**}
Intact h.	82	75	97	98
Broken h.	4	20	0	2
Deformed h.	4	0	0	0
Tapered h.	3	2	2	0
Swallowing h.	4	2	1	0
Circle h.	1	0	0	0
Small h.	2	1	0	0
Acrosome (A.) Morhpology	**Frozen Sperm (%)** Bull ^{A**}	Buck ^{A**}	**Unfrozen Sperm (%)** Bull ^{A**}	Buck ^{B**}
Intact A.	75	98	96	98
Lost A.	12	2	4	2
Sallowing A.	13	0	0	0

** Different letters show highly significant differences ($P < 0.01$) using t-test for paired samples.

Morphology of med piece, tail piece and cytoplasmic droplet

As respect to med piece l evel, the obtained results s howed a high degree of med piece destruction as for buck sperm after freezing (Table 2). This deformation of med piece can be attributed mainly to lack of sperm protection of rabbit preservation media and not to the congenital sperm malformation as shown in Table 2. In which the addition of glycerol in one step seems also deleterious at least in some animals (Courtens and Theau-Clement, 1996), where degenerated med piece reached 32% for rabbit frozen sperm and only 1% for frozen bull sperm. The micro folded plasma membranes might result first from a hypoosmotic shock in spermatozoa which, being already dehydrated, are surrounded by a second diluent with a lower molarity. After penetration of glycerol in sperm cells, a new osmotic state is obtained, and the extended membranes tend to return to their original position, close to the underlying structures (Courtens and Theau-Clement, 1996).

Membranes of sperm cell at the level of tail piece are more resistible to freezing process than that at the head level (Courtens and Theau-Clement, 1996). It was recognized that the percentage of broken tail in this experiment for frozen buck sperm reached 32% (Table 2). Tail significance of tail piece is enabling sperm cell to exhibit movement ability. While frozen bull sperm was noticed to contain 14% bent tail as shown in Table 2& 4. Both bent and broken tail sperm can not reach fertilization site. In addition sperm ripining as

indicated from removal of cytoplasmic droplet was differed significantly between unfrozen bull sperm and unfrozen rabbit sperm. Unfrozen Buck sperm contained 19% sperm with cytoplasmic droplet while it was only 8% for unfrozen bull sperm as shown in Table 2. The same situation was almost recognized as for frozen sperm in which buck sperm contained the double percentage of sperm which contained cytoplasmic droplet (20%) than that of frozen bull sperm (10%) as shown in Table 2.

Table 2: Effect of freezing and thawing procedure on sperm morphology at med piece, tail pieace and cytoplasmic droplet level of bull and buck sperm.

Med piece (MP.) Morphology	Frozen Sperm (%)		Unfrozen Sperm (%)	
	Bull ^{A**}	Buck ^{A**}	Bull ^{A**}	Buck ^{B**}
Intact MP.	99	68	99	98
Degenerated MP.	1	32	1	2
Tail piece (TP.) Morphology	**Frozen Sperm (%)**		**Unfrozen Sperm (%)**	
	Bull ^{A**}	**Buck ^{A**}**	**Bull ^{A**}**	**Buck ^{B**}**
Intact TP.	76	65	92	90
Bent TP.	14	3	4	2
Short TP.	7	0	1	0
Broken TP.	1	32	3	8
Degenerated TP.	2	0	0	0
Cytoplasmic Droplet(CD.) Existance	**Frozen Sperm (%)**		**Unfrozen Sperm (%)**	
	Bull ^{A**}	**Buck ^{A**}**	**Bull^{A**}**	**Buck ^{B**}**
Free of CD.	92	81	90	80
Contained CD.	8	19	10	20

** Different letters show highly significant differences (P $P < 0.01$) using t-test for paired samples.

Staining appearance

Both staining methods (Harris hematoxylin&Modified Bryan's sperm stain) could result clear and apparent sperm staining with distinct background. Where sperm head had darkly stained to a great extent. While sperm staining was the opposite situation as regards acrosome structure. Acrosome membranes should be visible as a lighter region at the apical zone of the sperm head. Outside the acrosome membranes, it is very obvious to denote sperm cell membrane with a colorless appearance. It envelops sperm cell from all directions and it is thick specially around the normal sperm head as shown in ures 1, 2and 4.

It could be concluded that, both freezing and thawing techniques adversily affect sperm morphology specially rabbit sperm more than bull sperm. Biological freezing media for rabbit sperm cryopresevation still need more modification to prevent both hypo and hyperosmotic conditions which encounter sperm cell during both freezing and thawing procedure.

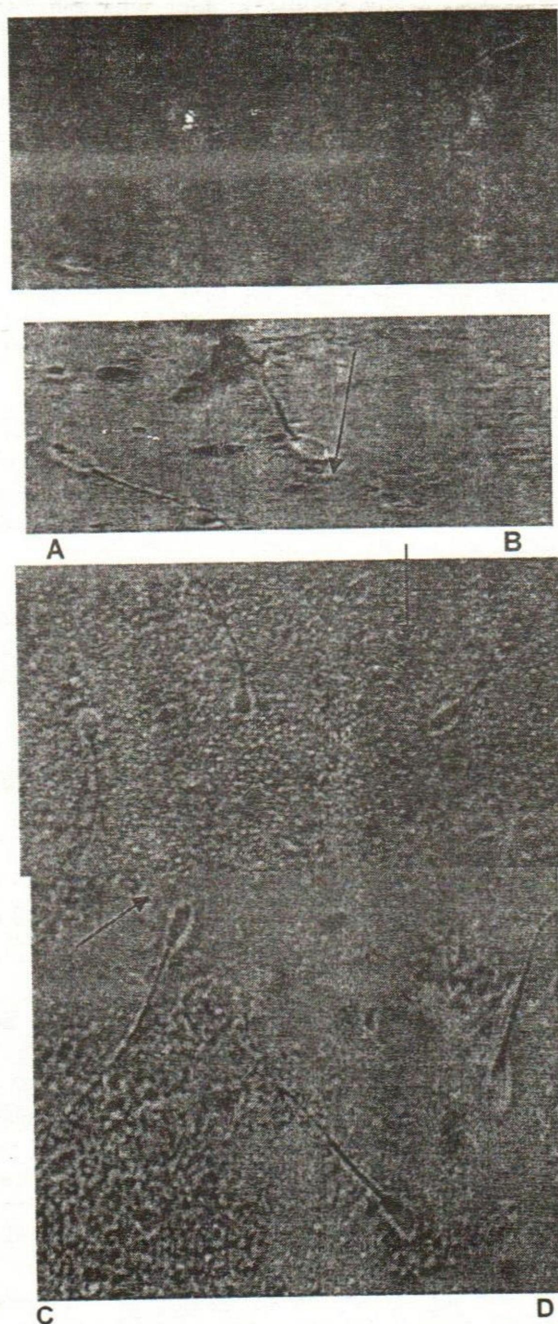
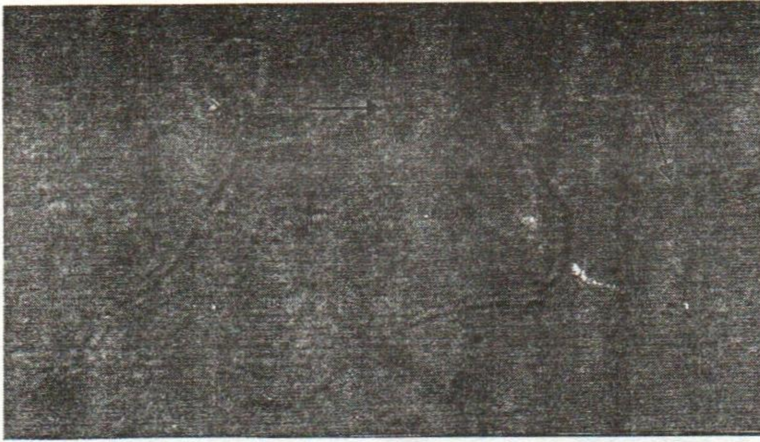
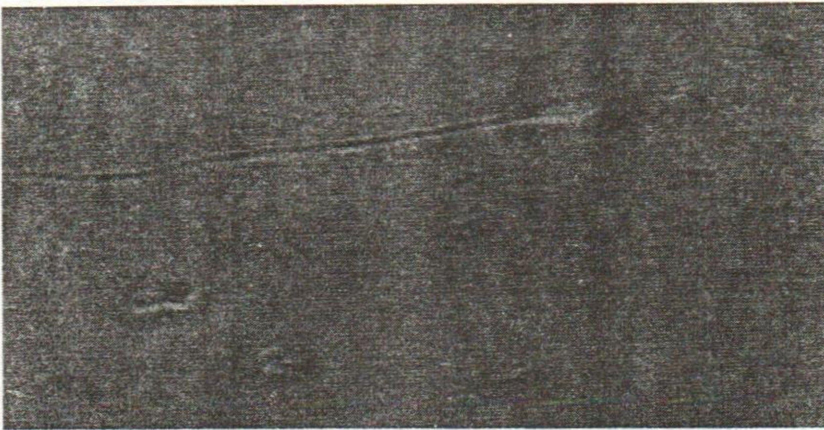


Fig.1A, B, C& D. Showing reacted (arrow) and non-reacted acrosome of frozen bull sperm using modified Harris stain (1000 x magnification by Zeise phase contrast microscope).





A B

Fig. 2 A& B. Represents different head sizes of frozen bull sperm (arrow is showing tapered or elongated head) according to freezing process using modified Harris stain (1000 x magnification by Zeise Phase contrast microscope). And changes in head region showing equatorial segments (arrow).

A B

Fig. 3 A & B . S howing s wallowing h ead o f f rozen bull sperm process using modified Harris stain (1000 x magnification by Zeise Phase contrast microscope).

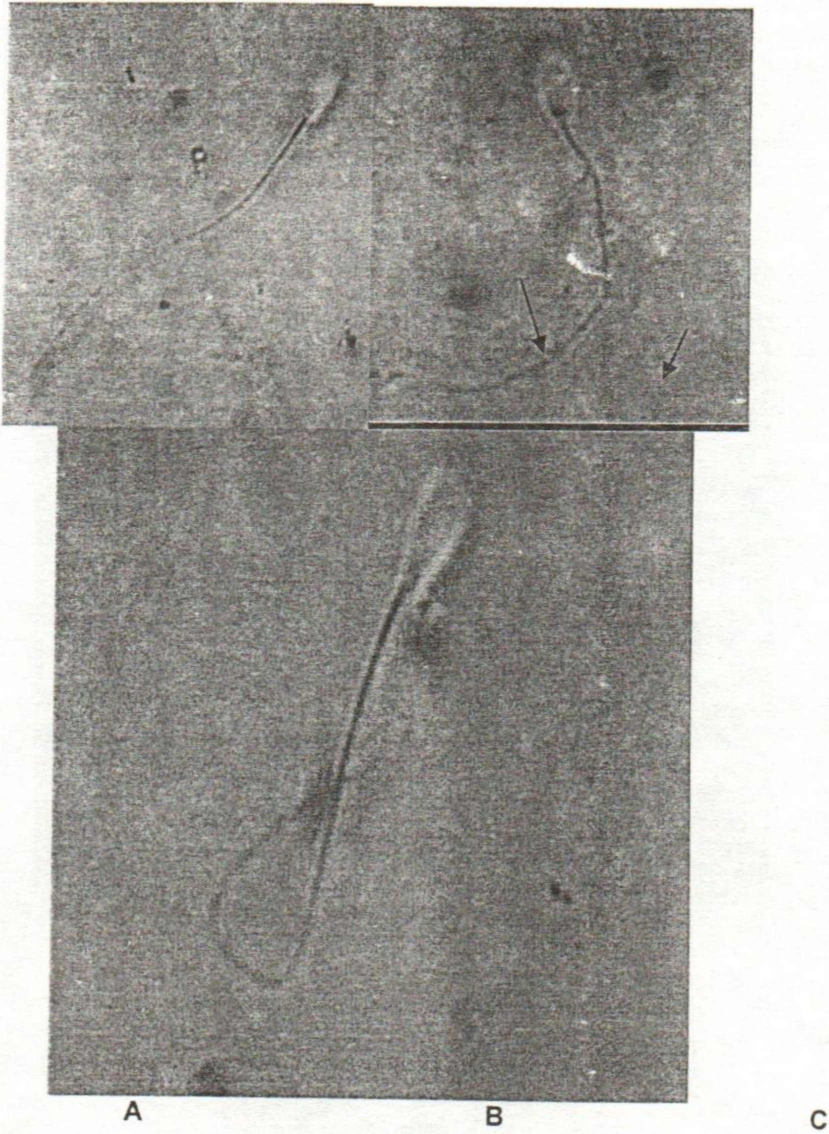


Fig. 4 A, B& C. Represent normal bull sperm (A), broken tail sperm at arrow (B) and bent tail sperm (C) after freezing using modified Harris stain (1000 x magnification by Zeise Phase contrast microscope).

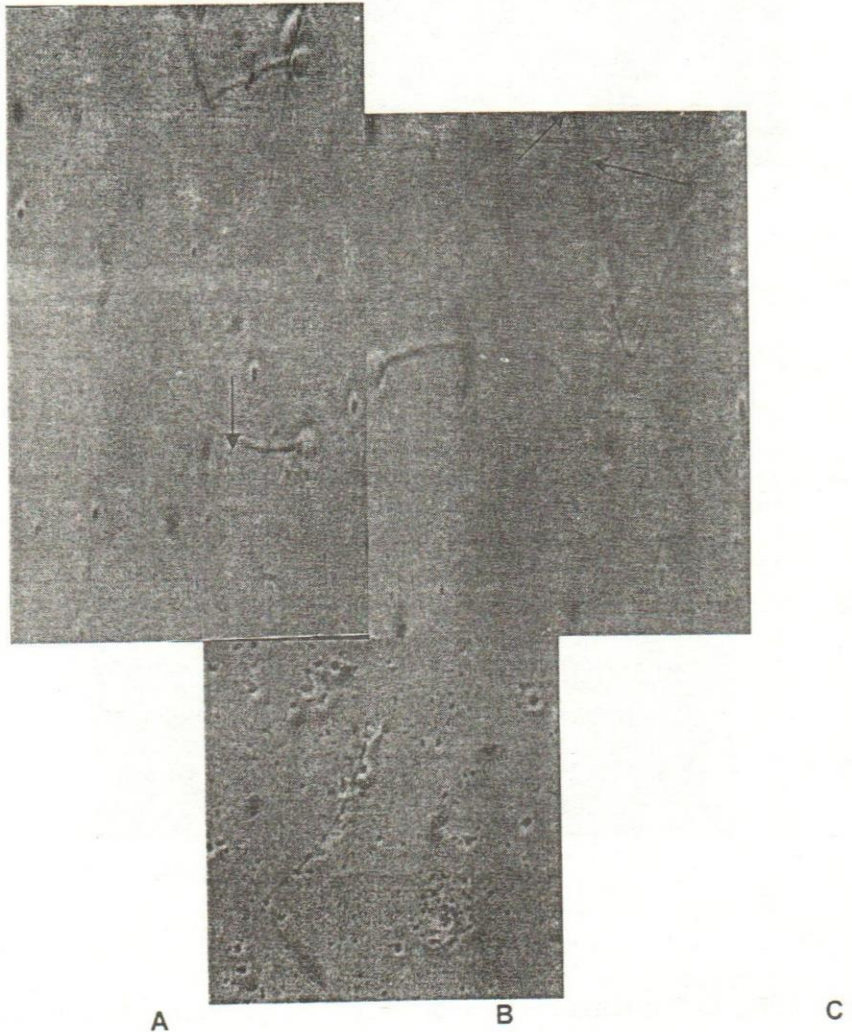
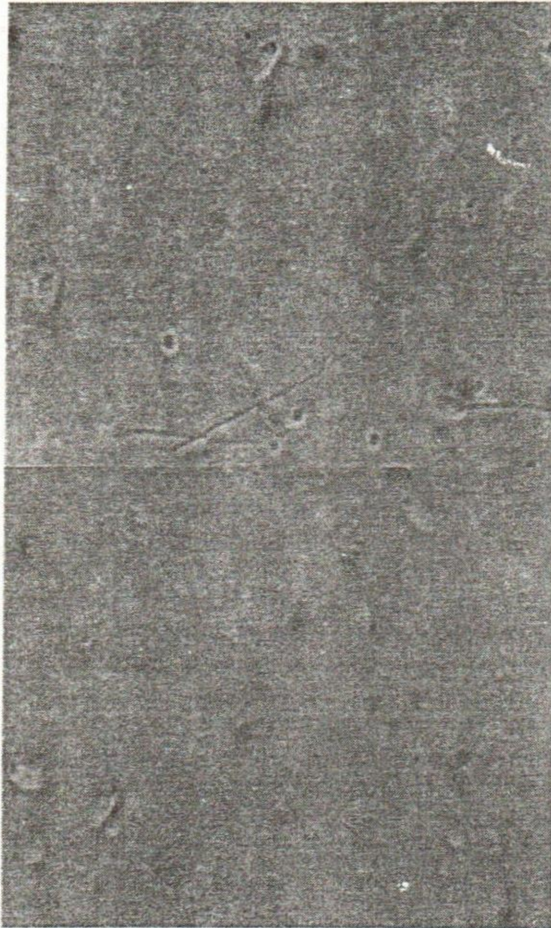
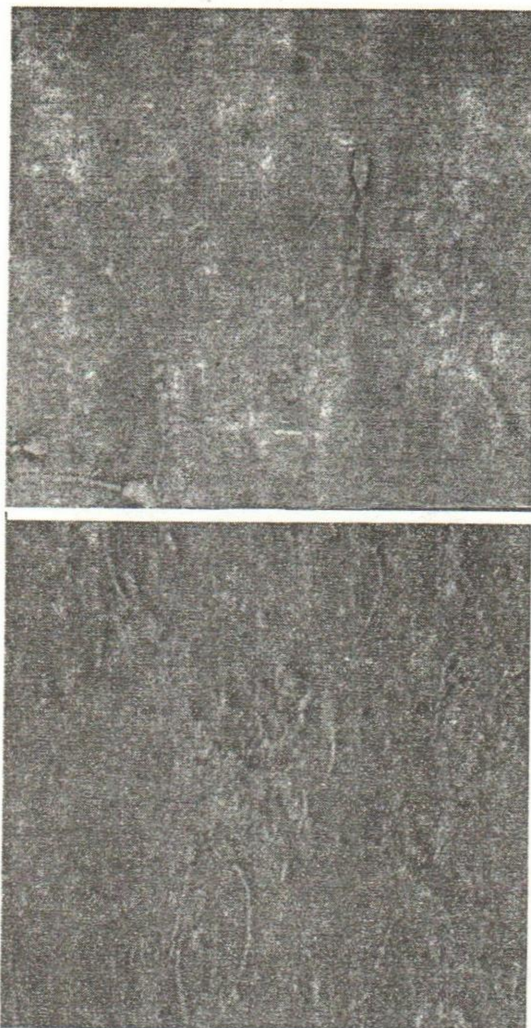


Fig. 5. A, B & C. Showing reacted (arrow) and non-reacted acrosome of frozen rabbit sperm using modified Harris stain (1000 x magnification by Zeise phase contrast microscope).



A B

Fig. 6 A& B. Represents major effects of freezing process on rabbit sperm where there is a lot of broken heads and tails rabbit s perm a s shown i n A& B u sing m odified Harris stain (1000 x magnification by Zeise phase contrast microscope).



A B

Fig. 7 A& B: Different head sperm sizes for frozen rabbit sperm A and raw rabbit sperm B. Notice dimension of head sperm region of frozen rabbit sperm is less than head sperm of raw rabbit sperm. These smears were stained by modified Harris stain A and by Modified Bryan`s sperm stain B (1000 x magnification by Zeise phase contrast microscope).

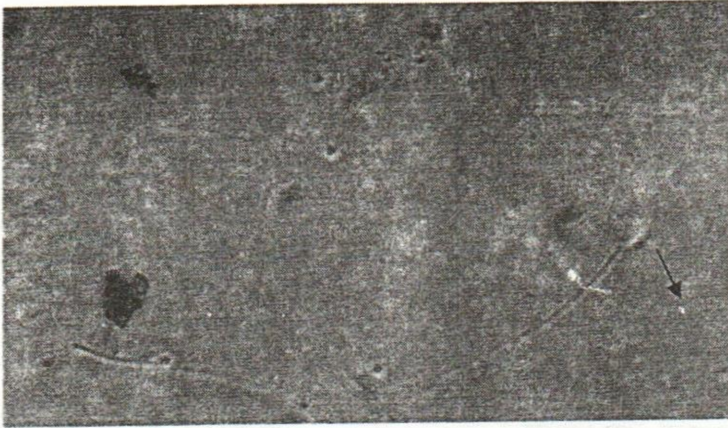


Fig. 8: Shows small rabbit sperm head dimension of frozen rabbit sperm (arrow) compared with rabbit raw sperm or frozen rabbit sperm as shown in 7 using modified Harris stain (1000 x magnification by Zeise phase contrast microscope).

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تأثير استخدام نوعين من الصبغات على الشكل المورفولوجى للحيوان المنوى بعد التجميد فى كل من ذكور الماشية والأرانب

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تم استخدام نوعين مختلفين من الصبغات وهم صبغة Harris hematoxylinوصبغة Modified Bryan`s . وذلك لدراسة التغيرات فى الشكل المورفولوجى بعد عملية التجميد للسائل المنوى لذكور الأبقار والأرانب كل على حدة. والصفات المورفلوجية التى تمت دراستها للحيوان المنوى هى : منطقة رأس الحيوان المنوى ، المنطقة الوسطى ، منطقة الأكروسوم ، منطقة الذيل ، والنقطة السيتوبلازمية. والنتائج المتحصل عليها أوضحت أن نسبة الصفات المورفلوجية الغير طبيعية قد زادت بدرجة معنوية وذلك بالنسبة للسائل المنوى الخاص بالأرانب عنة بالنسبة لذكر الماشية وذلك بعد التجميد. بالإضافة إلى ذلك فأن كل من الحيوانات المنوية لكل من الأرانب والماشية اختلفت فى صفاتها المورفلوجية قبل التجميد عنة بعد عملية التجميد بدرجة معنوية. ويمكن القول بأن الحيوان المنوى لذكور الأرانب أكثر حساسية لعملية التجميد مقارنة بذكور الماشية مما يتطلب معه المزيد من الدراسات فى مجال بيئات تجميد الحيوانات المنوية (المخففات) فى الأرانب وذلك لتجنب الأضرار التى تحدث نتيجة لعملية التجميد على المستوى الخلوى والتحت خلوى (العضيات).