

FUNCTIONAL CHARACTERISTICS AND CHROMATIN DAMAGE OF BULL SPERMATOZOA AS AFFECTED BY DIFFERENT FREEZING STEPS

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ABSTRACT

The present study aimed to define functional changes as sperm motility, dead spermatozoa and sperm abnormalities and chromatin damage of sperm by toluidine blue stain, acridine orange stain and flowcytometry during different stages of freezing semen. Semen ejaculates from five Friesian bulls were pooled, diluted in tris-citric acid extender, cooled to 4°C over 2 hours, equilibrated at 4°C for 4 hours and frozen immediately into a liquid nitrogen (-196°C). Samples were evaluated just after dilution (at 37°C), at 4 hours as equilibration period, and after thawing at 37°C for 30s in water bath. The obtained results showed that, the percentages of sperm motility were significantly ($P<0.05$) decreased, while the percentages of dead spermatozoa and sperm abnormalities revealed significantly ($P<0.05$) increased with the different stages of raw semen, diluted semen, cooled semen, equilibrated semen and frozen-thawed semen. In addition, the percentage of chromatin damage was significantly ($P<0.05$) higher during thawing after freezing using toluidine blue, acridine orange and flowcytometry techniques as compared to other steps of freezing bull semen. In conclusion, the present study indicated marked difference in impairment of semen quality and chromatin damage during different steps of Friesian bull frozen spermatozoa, in particular, from equilibration to freezing/thawing.

Keywords: Freezing, Bull semen, Chromatin, Toluidine blue, Acridine orange, Flowcytometry

INTRODUCTION

Semen freezing is a well-established procedure used in the human and veterinary assisted reproduction technology applications. Over the last 50 years ago, it was used for genetic improvement of beef and dairy cattle. It is also used to control venereal diseases and facilitate management of bovine herd fertility. In human, it is usually associated with male fertility preservation such as those associated with malignancy (Nangia *et al.*, 2013).

Spermatozoa are characterized by plasma membrane fluidity and low water content which makes it more resistant to damage compared to other cell types. Freezing have been shown to induce deleterious changes of spermatozoa structure and function (Watson, 2000). During this process, extensive chemical and physical damage occur to the spermatozoa. This

involves thermal stress by the change in temperature during cooling, freezing and thawing, as well as, osmotic stress caused by the addition of molar concentrations of cryoprotective agents and crystallization, that result in protein denaturation, shrinkage and irreversible membrane collapse (Watson, 2000). Therefore, phospholipids and cryoprotective agents and certain dilution, equilibration and cooling procedures are required to avoid cold shock and reduce crystallization to minimize sperm damage.

Conventional sperm evaluation parameters using in artificial insemination centers for raw semen evaluation of sires and post-thawing assessment of frozen semen are usually limited to assessment of post-thawing motility and morphology. Evaluation of sperm motility either microscopic examination or by sperm analyzers have been used as the main parameter to determine sperm quality and predict fertility in humans and animals (Sanchez-Partida *et al.*, 1999). In bovine no correlation was found between post-thaw motility and fertility defined as non-return rate at 56 days post-insemination (Soderquist *et al.*, 1991). Therefore, better evaluation of different freezing protocols and their improvement require employment of more efficient tests.

Freezing semen procedure can also cause DNA damage and thus impaired sperm function. Various studies have evaluated the impact of freezing on chromatin structure in human, with contradictory results (Paoli *et al.*, 2014). Sperm DNA damage has been recognized as an important indicator of sperm quality, and has a great clinical significance in the assessment of sperm selection in human (Novotny *et al.*, 2013). Sperm DNA damage was significantly higher with morphological abnormalities and was related to the cryo-storage technique and to the duration of storage of human semen (Fortunato *et al.*, 2013). In buffaloes (Mahmoud *et al.*, 2015) concluded that, DNA damage evaluation can provide reassurance about genomic normalcy and guide the development of improved methods of selecting spermatozoa with intact DNA to be used in artificial insemination.

Assessment of sperm DNA damage is widely determined using Sperm Chromatin Structure Assay (SCSA), which is based on the acridine orange (AO) which is usually assessed by flowcytometry. It is generally accepted that when DNA fragmentation exceeds a threshold of 30%, chances for successful pregnancy are very low (Evenson *et al.*, 1999 and Bungum *et al.*, 2004). Alternatively, Toluidine Blue (TB) test has been shown to be correlated to SCSA results (Erenpreiss *et al.*, 2004).

There are several potential damaging effects of freezing that cannot be necessarily detected by routine assessment of post-thawing motility. In bovine, the effect of freezing semen processes on sperm chromatin integrity has not been extensively examined although sperm chromatin structure assay, was suggested to be a valuable tool of prognostic relation to sire fertility after AI. Fertility parameters (non-return to estrous rates, and calving rates) were positively correlated with the percentage of morphologically normal spermatozoa and negatively correlated with the percentage of DNA fragmentation (Nagy *et al.*, 2013). Therefore, the present study aimed to evaluate the damaging effects induced at each processing step of freezing on

post-thawing of Friesian bull quality semen. Assessment of chromatin damage in spermatozoa was also investigated.

MATERIALS AND METHODS

The experimental work was carried out at Animal Production Department, Faculty of Agriculture, Mansoura University, in cooperation with Animal production Research Institute, Agricultural Research Center, Egypt, during the period from January till May, 2015.

Semen collection:

Semen ejaculates were collected from five healthy, fertile Friesian bulls, 4-6 years old and 400-500 kg live body weight raised under similar environmental conditions of feeding and managements at the International livestock Management Training Center, Sakha, Kafrelsheikh Governorate, Egypt. Semen was collected twice a week for 20 weeks. Semen was collected using an artificial vagina (Neustadt/Aisch, Müller, Nürnberg, Germany) between 8 and 9 a.m. pre-warmed to 42°C. The percentage of progressive motility of spermatozoa was determined for each ejaculate using a phase contrast microscope at x 200 magnification. Only ejaculates with $\geq 70\%$ mass motility were used for freezing process.

Freezing process:

Immediately after ejaculate collection, semen was evaluated, diluted at 37°C with Tris-yolk fructose (TYF) extender (containing 30.28 mg/ml tris amino methane, 16.75 mg/ml citric acid anhydrous, 12.5 mg/ml fructose, 7% (v/v) glycerol, 20% (v/v) egg yolk, 5 IU/ml penicillin and 5 µg/ml streptomycin). The extension rate was 1 semen: 20 extender. The diluted semen was kept at 4°C for 4 h as an equilibration period, then automatically filled in 0.25 ml French straws (IVM technologies, L' Aigle, France). Thereafter, straws were exposed to nitrogen vapors at level of 4 cm distance above liquid nitrogen for 10 min, then frozen in liquid nitrogen at -196°C according to the method described by Salisbury *et al.* (1978). Frozen-thawed semen samples were evaluated immediately after dilution at 37°C, cooled at 4°C for equilibration (4 hours), and thawed at 37°C for 30 seconds in water bath.

Semen evaluation:

Semen was evaluated for the percentages of progressive motility, dead and abnormality of spermatozoa in raw, diluted, cooled, equilibrated and post-thawed semen.

Sperm progressive motility (%):

It was estimated by adding one drop of the diluted fresh semen with physiological saline (0.9 % sodium citrate) on the dry, clean and pre-warmed (37 °C) glass slides. The drop of the extended semen was covered by a warmed cover slip and immediately examined using light power examination (x 400). Percentage of sperm progressive motility was calculated according to Salisbury *et al.* (1978).

Dead spermatozoa (%):

It was determined using eosin-nigrosin staining procedure of Hackett and Macpherson (1965) and then examined on microscope at (x 400).

Sperm abnormalities (%):

The morphological abnormalities of spermatozoa were determined in the same smears prepared for live/dead ratio under oil immersion using 100 x objective of a light microscope.

Chromatin damage:

Semen was evaluated for chromatin damage in diluted, cooled, equilibrated and post-thawed semen.

Toluidine blue staining:

Toluidine blue staining was performed as previously described by Erenpreiss *et al.* (2004). Smears obtained during different steps of freezing semen were fixed in ethanol-acetic acid (3:1, v/v) for 1 min and 70% ethanol for 3 min. Smears were hydrolyzed for 20 min in 4 mM Chloridric acid, rinsed in distilled water and air-dried. One drop of 0.025% toluidine blue in McIlvaine buffer (sodium citrate-phosphate, pH 4) was placed over each smear and then cover slipped. Smears were evaluated with magnification power (x 1000). The percentage of chromatin damage was estimated by evaluating 300 sperm cells in each smear. Spermatozoa stained as green to light blue were considered to have normal chromatin, while those stained dark blue to violet were considered to have damaged chromatin.

Acridine orange (AO) fluorescence staining:

AS reported by Tejada *et al.* (1984), air-dried smears were fixed overnight in methanol-glacial acetic acid (3:1) at room temperature (RT). The slides were removed from the fixative and allowed to dry for a few minutes before staining with AO (0.19 mg/ml, pH 2.5) for 5 minutes at RT. Staining solution was prepared daily from a stock solution consisting of 0.1 mg/ml AO (Sigma) and stored in the dark at 4°C. To prepare the staining solution, 10 ml of the stock solution was added to 40 mL of 0.1 M citric acid and 2.5 ml of 0.3 M Na₂HPO₄·7H₂O. All solutions were maintained at RT. After staining, the slides were gently rinsed in distilled water, mounted by (DABCO) and sealed under a cover slip with nail polish. Sperm cell heads with good DNA integrity had green fluorescence, and those with damaged DNA were stained orange-red. Samples were scored within 1 hour after staining.

Sperm Chromatin Structure Assay (SCSA):

SCSA was applied following the procedure as the method described by Tsarev *et al.* (2009). Briefly, 200 µl of diluted semen from each sample at each time point/step was diluted to a concentration of 1 x 10⁶ sperm/ml with a buffer composed of 0.01 M Tris-HCl, 0.15 M NaCl and 1 mM EDTA (pH 7.4) and treated with an acid detergent solution (pH 1.2) containing 0.1% Triton X-100, 0.15 mol/l NaCl and 0.08 N HCl for 30s. Spermatozoa were then stained with 6 µg/ml purified AO in a phosphate-citrate buffer (pH 6.0). Cells were analyzed using a flowcytometer (Becton Dickinson, Sunny vale, CA, USA), equipped with an air-cooled argon ion laser. A total of 10000 events were accumulated for each measurement at a flow rate of 200–300 cells/s. Acridine orange (AO) that is intercalated in double-stranded DNA emits green fluorescence, whereas AO associated with single-stranded DNA emits red

fluorescence. The extent of DNA denaturation was expressed in terms of DNA fractionation index (DFI), which is the ratio of red to total (red plus green) fluorescence intensity.

Statistical analysis

Data were statically analysis by one way (ANOVA), using General Liner Model (GLM) procedures of SAS (2004) and Duncan's New Multiple Range Test (Duncan, 1955) was used to test the significant differences among means .Percentage values were transformed to arc-sin values before the statistical analysis.

RESULTS

Semen quality (functional characteristics):

Results presented in Table (1) revealed significant (P<0.05) reduction in progressive motility by advancing semen processing from collection (raw semen) up to freezing (thawed semen). However, both dead and abnormal sperm percentages showed significantly (P<0.05) an opposite trend of change. It is of interest to note that the most deleterious effects on sperm characteristics studied was observed during freezing (differences between equilibrated and post-thwing semen).

Table 1. Changes in motility, livability and abnormality of spermatozoa during freezing process of Friesian bull semen.

Semen processing	Progressive motility (%)	Dead sperm (%)	Abnormal sperm (%)
Raw semen (37°C)	77.0±1.7 ^a	23.0±0.7 ^d	19.0±0.8 ^d
Diluted semen (37°C)	71.0±0.8 ^b	26.0±0.4 ^d	20.0±1.2 ^{cd}
Cooled semen (4°C for 2 h)	67.0±1.1 ^c	30.0±1.6 ^c	22.0±1.0 ^c
Equilibrated semen (4°C, 4 h)	63.0±1.7 ^d	35.0±1.3 ^b	24.0±0.5 ^b
Frozen-thawed semen	50.8±2.7 ^e	45.0±2.2 ^a	29.0±1.5 ^a

a-e Values with different superscripts within the same column are significantly different (P<0.05)

Chromatin damage:

Data of determining chromatin damage using techniques of toluidine blue (TB), acridine orange (AO) and flowcytometry presented in Table (2) showed that freezing steps had significant effect (P<0.05) on the percentage of chromatin damage in spermatozoa.

Values of TB (Fig. 1), AO and flowcytometry significantly (P<0.05) increased by advancing step of semen freezing from dilution up to freezing/thawing processes. The highest (P<0.05) rate of chromatin damage was observed between equilibrated and freezing semen (Table 2).

Table 2. Effect of different processing steps of freezing semen on sperm chromatin integrity using Toluidine blue staining (TB), Acridine orange (AO) and Flowcytometry.

Item	TB (%)	AO (%)	Flowcytometry (%)
Diluted semen	1.70±0.4 ^a	1.80±0.3 ^a	2.79
Cooled semen	2.20±0.3 ^{ab}	2.00±0.4 ^a	3.75
Equilibrated semen	2.80±0.4 ^b	2.80±0.3 ^b	4.88
Frozen-thawed semen	4.50±0.3 ^c	5.30±0.6 ^c	8.42

a-c Values with different superscripts within the same column are significantly different ($P<0.05$)

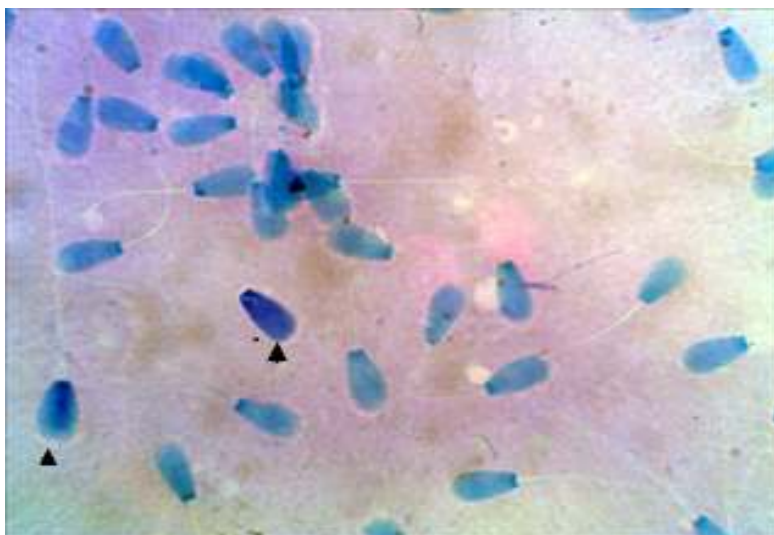


Fig. (1): Bull spermatozoa showing chromatin status by Toluidine Blue staining sperm heads with intact chromatin were light blue and those of fragmented chromatin and abnormal compaction were deep violet (black arrow)

DISCUSSION

The current study aimed to investigate DNA damage (chromatin damage) using Toluidine Blue stain (TB), Acridine orange (AO) and Flowcytometry, in association with each step of freezing bull semen process in relation to changes in percentages of motility, dead and abnormality of spermatozoa in bull semen. Tris-based extenders and standard cryopreservation procedures were conducted in this study.

Based on the obtained results, there was a step-wise reduction in sperm motility, associated with an increase in dead and abnormal spermatozoa mainly in the form of bent and coiled tail, detached heads and / or destroyed heads in post-thawed semen. These morphological

abnormalities have been previously linked to cold shock and were observed when cold shock was induced in bull semen by cooling and storing in the absence of egg yolk, which was associated with reduced fertilization rate *in vitro* (Ducha *et al.*, 2012).

Functionally, although motility and viability were reduced, tris-egg-yolk-glycerol used in this study protected semen preserve viable spermsatozoa available after thawing which are normally capable of fertilization. It is worthy noting that the reduction in motility was in relation with increasing abnormality and decreasing viability of spermatozoa. Also, these changes was in parallel with increasing chromatin damage by advancing freezing steps, in particular, from equilibration to thawing.

Mitochondrial function and sufficient ATP production has been correlated to sperm motility and hyperactivity (Ho *et al.*, 2002) and is also linked to higher fertilization potential *in vitro* (Kasai *et al.*, 2002). A gradual decrease in the metabolic activity of spermatozoa was showed during storage at cold shock temperature could limit the production of detrimental by-products, which might reduce sperm function but metabolic activity influence essential sperm functions such as motility. Sperm motility was significantly higher in spermatozoa with higher inner mitochondrial membrane potential and flowcytometry in human. This was also linked to higher fertilization potential *in vitro* (Kasai *et al.*, 2002).

The mechanism of the generation of DNA fragmentation and chromatin damage after freezing is has not been clearly elucidated, but mainly attributed to oxidative stress and activation of caspases (Novotny *et al.*, 2013 and Paoli *et al.*, 2014). This is particularly important, since this DNA damage have been shown to affect genes that are crucial for fertilization and early embryo development (Valcarce *et al.*, 2013) as induced DNA damage in murine spermatozoa have been shown to persist after intracellular sperm injection (ICSI) fertilization and no repair could be observed with DNA synthesis in the zygote (Yamauchi *et al.*, 2012). This may explain the relation between sperm DNA damage and unexplained recurrent spontaneous abortion (Zhang *et al.*, 2012).

Percentage of spermatozoa with chromatin defects positive to chromomycin A3 indicating protamine deficiency showed significant negative correlation with fertilization rate following ICSI (Iranpour, 2014). In addition, chromatin assessment with Diff-Quik staining kit has been shown to indicate embryo quality and pregnancy outcomes following *in vitro* fertilization (Tavares *et al.*, 2013). However, this correlation was not observed when aniline blue and acridine orange staining were used (Iranpour, 2014). In ovine, it has been shown that deterioration in chromatin stability may vary according with different semen extenders and that a pronounced decline in pregnancy rate (PR) was observed when percentages of decondensed and destabilized chromatin have reached thresholds of 10.5-30 % and 4-9%, respectively (Khalifa and Lymberopoulos, 2013).

Reducing sperm DNA damage and protecting mitochondrial functions should be assessed and targeted when improving freezing bull semen protocols used for AI. Generally, aging (Rybar *et al.*, 2011), inter individual

variation (Oleszczuk *et al.*, 2011), or pollution (Calogero *et al.*, 2011) have been shown to impact chromatin integrity in human, which is a factor that can substantially affect fertility in men, rather than by basic sperm parameters.

In conclusion, the present study indicated marked difference in impairment of semen quality and chromatin damage during different steps of Friesian bull frozen spermatozoa, in particular, from equilibration to freezing/thawing.

REFERENCES

- Bartak, V., and Raboch, J. (1966). The motility of spermatozoa and male fertility. *Cesk Gynekol*, 31(8): 572-576.
- Bungum, M.; Humaidan, P.; Spano, M.; Jepson, K.; Bungum, L. and Giwercman, A. (2004). The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. *Hum. Reprod.*, 19 (6): 1401-1408
- Calogero, A.E.; La Vignera, S.; Condorelli, R.A.; Perdichizzi, A.; Valenti, D.; Asero, P.; Carbone, U.; Boggia, B.; De Rosa, N.; Lombardi, G.; D'Agata, R.; Vicari, L.O.; Vicari, E. and De Rosa, M. (2011). Environmental car exhaust pollution damages human sperm chromatin and DNA. *J. Endocrinol Invest* 34 (6): 139-143.
- Ducha, N.; Susilawati, T.; Aulanni'am, Wahyuningsih, S. and Pangestu, M. (2012) Ultrastructure and fertilizing ability of Limousin bull sperm after storage in CEP-2 extender with and without egg yolk. *Pakistan J. Biol. Sci.*, 15(20), 979-985.
- Duncan, D.B. (1955). Multiple range and multiple F test. *Biometrics*, 11:1-420.
- Mahmoud, K.Gh.M.; El-sokary, A.A.E.; Abdel-Ghaffar, A.E.; Abou El-Roos, M.E.A. and Ahmed, Y.F. (2015). Analysis of chromatin integrity and DNA damage in cryopreserved buffalo semen. *Iranian Journal of Veterinary Research*, 16: 161-166.
- Erenpreiss, J.; Jepson, K.; Giwercman, A.; Tsarev, I.; Erenpreisa, J. and Spano, M. (2004). Toluidine blue cytometry test for sperm DNA conformation: comparison with the flow cytometric sperm chromatin structure and TUNEL assays. *Hum. Reprod.*, 19 (10): 2277-2282.
- Evenson, D.P.; Jost, L.K.; Marshall, D.; Zinaman, M.J.; Clegg, E.; Purvis, K.; de Angelis, P. and Claussen, O. P. (1999). Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Hum. Reprod.*, 14 (4): 1039-49
- Fortunato, A.; Leo, R. and Liguori, F. (2013). Effects of cryostorage on human sperm chromatin integrity. *Zygote*, 21(4): 330-6
- Hackett, A.J. and Macpherson, J.W. (1965). Some staining procedures for spermatozoa. *The Canadian. Veterinary Journal*, 6 (3): 55.
- Ho, H.C.; Granish, K.A. and Suarez, S.S. (2002). Hyperactivated motility of bull sperm is triggered at the axoneme by Ca²⁺ and not cAMP. *Dev. Biol.*, 250 (1): 208-217.

- Iranpour, F.G. (2014). Impact of sperm chromatin evaluation on fertilization rate in intracytoplasmic sperm injection. *Adv. Biomed. Res.*, 3: 229.
- Kalthur, G.; Raj, S.; Thiyagarajan, A.; Kumar, S.; Kumar, P. and Adiga, S. K. (2011). Vitamin E supplementation in semen-freezing medium improves the motility and protects sperm from freeze-thaw-induced DNA damage. *Fertil. Steril.*, 95 (3): 1149-1151.
- Kasai, T.; Ogawa, K.; Mizuno, K.; Nagai, S.; Uchida, Y.; Ohta, S.; Fujie, M.; Suzuki, K.; Hirata, S. and Hoshi, K. (2002) Relationship between sperm mitochondrial membrane potential, sperm motility, and fertility potential. *Asian. J. Androl.*, 4(2): 97-103.
- Khalifa, T. and Lymberopoulos, A. (2013). Changeability of sperm chromatin structure during liquid storage of ovine semen in milk-egg yolk- and soybean lecithin-based extenders and their relationships to field-fertility. *Cell Tissue Bank*, 14 (4): 687-698.
- Kotdawala, A.P.; Kumar, S.; Salian, S.R.; Thankachan, P.; Govindraj, K.; Kumar, P.; Kalthur, G. and Adiga, S.K. (2012). Addition of zinc to human ejaculate prior to cryopreservation prevents freeze-thaw-induced DNA damage and preserves sperm function. *J. Assist. Reprod. Genet.*, 29(12): 1447-1453.
- Menon, A.G.; Thundathil, J.C.; Wilde, R.; Kastelic, J.P. and Barkema, H.W. (2011). Validating the assessment of bull sperm morphology by veterinary practitioners. *Canadian Vet. J.* 52 (4): 407-408.
- Moskovtsev, S.I. and Librach, C.L. (2013). Methods of sperm vitality assessment. *Methods Mol. Biol.*, 927: 13-19
- Nagy, S.; Johannisson, A.; Wahlsten, T.; Ijas, R.; Andersson, M. and Rodriguez-Martinez, H. (2013). Sperm chromatin structure and sperm morphology: their association with fertility in AI-dairy Ayrshire sires. *Theriogenology*, 79(8): 1153-1161.
- Nangia, A.K.; Krieg, S.A. and Kim, S.S. (2013). Clinical guidelines for sperm cryopreservation in cancer patients. *Fertil. Steril* 100(5): 1203-1209.
- Novotny, J.; Aziz, N.; Rybar, R.; Brezinova, J.; Kopecka, V.; Filipcikova, R.; Reruchova, M. and Oborna, I. (2013). Relationship between reactive oxygen species production in human semen and sperm DNA damage assessed by Sperm Chromatin Structure Assay. *Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech Repub.*, 157(4): 383-386
- Oleszczuk, K.; Giwercman, A. and Bungum, M. (2011). Intra-individual variation of the sperm chromatin structure assay DNA fragmentation index in men from infertile couples. *Hum. Reprod.*, 26(12): 3244-3248.
- Paoli, D.; Lombardo, F.; Lenzi, A. and Gandini, L. (2014). Sperm cryopreservation: effects on chromatin structure. *Adv. Exp. Med. Biol.*, 791: 137-150
- Rybar, R.; Kopecka, V.; Prinosilova, P.; Markova, P. and Rubes, J. (2011) Male obesity and age in relationship to semen parameters and sperm chromatin integrity. *Andrologia*, 43(4):286-291
- Salisbury, G.W.; Van Demark, N.L. and Lodge, J.R. (1978) 'Physiology of reproduction and artificial insemination of cattle (W.H. Freeman and Co., San Francisco, USA.

- Sanchez-Partida, L.G.; Windsor, D.P.; Eppleston, J.; Setchell, B.P. and Maxwell, W.M. (1999). Fertility and its relationship to motility characteristics of spermatozoa in ewes after cervical, transcervical, and intrauterine insemination with frozen-thawed ram semen. *J. Androl.*, 20(2): 280-288.
- SAS (2004). SAS/Stat. Users Guide Statics, Ver., 6.06 4th Ed. SAS Institute Inc. Cary. NC.
- Soderquist, L.; Rodriguez-Martinez, H. and Janson, L. (1991). Post-thaw motility, ATP content and cytochrome C oxidase activity of A.I. bull spermatozoa in relation to fertility. *Zentralbl Veterinarmed*, 38(3): 165-174.
- Sousa, A.P.; Amaral, A.; Baptista, M.; Tavares, R.; Caballero Campo, P.; Caballero Peregrin, P.; Freitas, A.; Paiva, A.; Almeida-Santos, T.; and Ramalho-Santos, J. (2011). Not all sperm are equal: functional mitochondria characterize a subpopulation of human sperm with better fertilization potential. *PLoS One*, 6(3):
- Tavares, R.S.; Silva, A.F.; Lourenco, B.; Almeida-Santos, T.; Sousa, A.P. and Ramalho-Santos, J. (2013). Evaluation of human sperm chromatin status after selection using a modified Diff-Quik stain indicates embryo quality and pregnancy outcomes following in vitro fertilization. *Andrology*, 1(6): 830-837.
- Tejada, R.I.; Mitchell, J.C.; Norman, A.; Marik, J.J. and Friedman, S. (1984) A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. *Fertil. Steril*, 42(1): 87-91.
- Tsarev, I.; Bungum, M.; Giwercman, A.; Erenpreisa, J.; Ebessen, T.; Ernst, E. and Erenpreiss, J. (2009). Evaluation of male fertility potential by Toluidine Blue test for sperm chromatin structure assessment. *Hum. Reprod.*, 24(7): 1569-1574.
- Valcarce, D.G.; Carton-Garcia, F.; Riesco, M.F.; Herraiez, M.P. and Robles, V. (2013). Analysis of DNA damage after human sperm cryopreservation in genes crucial for fertilization and early embryo development. *Andrology*, 1(5):723-730.
- Watson, P.F. (2000). The causes of reduced fertility with cryopreserved semen. *Anim Reprod. Sci.*, 60-61: 481-492
- Yamauchi, Y.; Riel, J.M. and Ward, M.A. (2012). Paternal DNA damage resulting from various sperm treatments persists after fertilization and is similar before and after DNA replication. *J. Androl.*, 33(2): 229-238.
- Zhang, L.; Wang, L.; Zhang, X.; Xu, G.; Zhang, W.; Wang, K.; Wang, Q.; Qiu, Y.; Li, J. and Gai, L. (2012). Sperm chromatin integrity may predict future fertility for unexplained recurrent spontaneous abortion patients. *Int. J. Androl.*, 35(5): 752-757.

تأثير مراحل التجميد المختلفه علي الخصائص الوظيفيه وضرر الكروماتين فى الحيوانات المنويه للطلائق
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٤ - قسم التوليد والتناسل والتلقيح الإصطناعي، كلية الطب البيطري، جامعة القاهرة، الجيزة ١٢٢١١، مصر.

أجريت هذه الدراسة في معمل الفسيولوجي والتكنولوجيا الحيوية بقسم إنتاج الحيوان بكلية الزراعة جامعه المنصوره بالتعاون مع معمل السائل المنوي بالمركز الدولي للتدريب علي رعايه الحيوان بسخا- معهد بحوث الانتاج الحيواني. وكان الهدف من البحث هو دراسه تأثير الحفظ بالتجميد علي الخصائص الوظيفية للسائل المنوي (النسبه المؤيه للحركه التقدمية - الميت -الشواذ) وكذلك سلامه الكروماتين وذلك خلال المراحل المختلفه لتجميد السائل المنوى (بعد الجمع طازجا - بعد التخفيف على درجة حراره الغرفه - بعد التبريد لمده ساعتين - بعد فتره الموازنه لمده ٤ ساعات علي درجة حراره ٤م - بعد التجميد والإساله). وكانت أهم النتائج المتحصل عليها هي:
١- إنخفضت النسبه المنويه لحركة الحيوانات المنويه معنوياً وارتفعت نسبة الحيوانات المنوية الميتة والشاذه بتقدم مرحلة التجميد من بعد الجمع مباشرة وحتى الإساله. وكانت أعلى التغيرات خلال عملية التجميد.
٢- ارتفعت النسبه المؤيه لتكسر الكروماتين بتقدم مراحل التجميد من بعد الجمع مباشرة وحتى الإساله. وكانت أعلى التغيرات خلال عملية التجميد.
نستخلص من هذه النتائج أن هناك إختلافات ملحوظه فى جودة السائل المنوى والضرر الحادث فى الكروماتين خلال مراحل التجميد المختلفه للسائل المنوى فى طلائق الأبقار.