Plant Archives Volume 20 No. 2, 2020 pp. 6545-6550 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

COMPARISON OF DNA BY ACRIDINE ORANGE ASSAY IN DIFFERENT STAGES OF CRYOPRESERVATION OF EPIDIDYMAL SPERM OF BUFFALO BULL

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Abstract

The sperm cells extracted from the testes are of good quality and movement. Pentoxyfylline (PTX) is added to maintain sperm cells for a period in the different stage of cryopreservation, after which the safety of the DNA is examined. The aim of this research was to evaluate the DNA of the sperm in the testicle by adding PTX, and to compare it with fresh sample, equilibrated sample (5°C) and Frozen thawed Sample, which is used to enhance sperm motility in IVF. Epididymal sperm samples taken from the massacre of 33 bull buffaloes were divided into 4 parts; T control had only Tris-citric egg yolk-glycerol, T1 had PTX at a dose of 1.0 m mol/ml, T2 had PTX at a dose of 0.5 m mol/ml and T3 had PTX at a dose of 10.0mmol/ml. All samples were kept cooled at 4°C to equilibrate. After equilibration straws were placed over (2-3 cm) liquid nitrogen vapors for 5-10 minutes and then plunged in liquid nitrogen and stored at -196. At each stage, sperm chromatin quality was evaluated. Chromatin quality was assessed by Orange acridine. Statistical analysis was performed using unidirectional variance analysis (ANOVA). A value of P less than 0.05 was accepted as a statistically significant difference. Showed significantly improved sperm chromatin quality when adding PTX in the control group and lowering the chromatin quality in a frozen sample compared to the control phase and equilibration sample. Adding PTX to Epididymal sperm samples can improve sperm chromatin quality and maintain sperm during the freezing period in concentration 10µl sperm it may be because it can mimic the sperm condition in the body during late spermatogenesis. The consumption of PTX is significantly increase the maturity and percentage of sperms with double-stranded and healthy DNA integrity was ultimately improves.

Key Words: Epididymal Sperm, Buffalo, Bull, pentoxyfylline, DNA integrity

Introduction

Pentoxifylline (PTX) functions as an inhibitor of methylxanthine phosphor-diesterase. It decreases anions of superoxide and inhibits factor-alpha (TNF-alpha) tumor necrosis responsible for DNA fragmentation and apoptosis or programmed cell death (Van Furth *et al.*, 1997; Peeker *et al.*, 1997). It also increases intracellular cAMP (Yovich, 1993), stimulates motility of sperm and improves fertilization (Numabe *et al.*, 2001; Henkel and Schill, 2003). In addition, Zhang *et al.* (2005) showed that clinical implementation of PTX leads to decreased lipid peroxidation associated with sperm membrane damage and DNA apoptosis and toxic reactive oxygen species scavenging. Many ingredients were used in laboratory and national species to study sperm hyperactivation using PTX as variables promoting sperm motility (Numabe *et al.*, 2001).

Water buffaloes are abundant in southern Iraq and reproductive biology limits complete buffalo production, as fertility in this population is significantly lower than in animals (Drost, 2007). The integrity of sperm DNA is critical to the development of viable embryos after the fertilization process has been completed (Andrabi, 2008). Higher sperm DNA damage has been noted to reduce the growth of the embryo (Benchaib *et al.*, 2003 and Yildiz *et al.*, 2007). Severe harm to the integrity of the spermatozoa DNA decreased its capacity for fertilization. Interestingly, sperm with seemingly ordinary motility, movement features, intact plasma membrane and organelles could effectively fertilize the oocytes and produce embryos. The freezing and thawing of spermatozoa lead to significant changes in sperm chromatin, including increases in unstable DNA (Evenson *et al.*, 2002). Responsible for these damages which include: chemicals and toxins; also impaired the sperm DNA is then marked with AO (acridine orange) metachromatic DNA stain. When intercalated into

ordinary sperm chromatin, AO fluoresces green with double-stranded DNA, while AO fluoresces red with single-stranded DNA.

Materials and Methods

Experimental Samples

Samples were collected in the current study from the Basrah slaughterhouse from December 2018 until the end of August 2019, with an average of 3-4 visits per week for male adult buffalo.

Collect of sperm from the tail of Epididymis

Thirty-three (33) samples testis of adult male buffalo from a slaughterhouse in Basrah after slaughtering the animal. The testis with attached epididymis were transferred in a cool box at 4°C - 6°C (Lone *et al.,* 2011), to Laboratory at the Research Unit Center of college of Veterinary Medicine/ Basrah University.

Collection of Epididymal fluid

Once of collected samples are reached to the laboratory, the organ washed with distilled water, then with normal saline containing antibiotic after that removing of tunica vaginitis using sterile scissor, then theepididymis were separated from the testicle, the tail of epididymis was removed from the entire epididymis and placed in petri dish ,then injected with 3 ml Tyrode Albumin Lactate Pyruvate (TALP) which prepared using sterile needle gauge (no. 23), connected to a 5 ml syringe, then sterileblade use for sliced of tail of epididymisfor small pieces according to (Lone *et al.,* 2011). Then the tissue was removed and the remaining medium was centrifugedat 7000 rpm for 2 minute to create a sperm pellet (Barati *et al.,* 2009). Supernatant was removed from sperm.

Epididymal sperm processing

Tris-citric egg yolk-glycerol (TCEG) extender was added the concentration or density was further adjusted to 451-813 x 10^6 sperm/ml. The plastic tube containing the epididymal sperm, the diluted Epididymal sperm will divide into 4 parts1. T control contains only (Tris-citric egg yolk-glycerol (TCEG). 2. T1 add pentoxyfylline with (1.0) mmol/ml. 3. T2 add pentoxyfylline with (0.5) mmol/ml. 4.T3 add pentoxyfylline with (10.0mmol/ml).was placed in a glass beaker containing water and the beaker was placed in a refrigerator for Cooling 5°C The all diluted Epididymal sperm containing different concentration of antioxidant were transferred into the cold beaker, allow to reach the stabilize degree 5°C in about 1-1.5 hour to control the time of cooling diluted Epididymal sperm in cold beaker, ice cubes were added to the beaker when temperature of the water in the beaker reached 20°C, so should be bellow 5°C in a controlled manner, this can be done by the aid of sensitive thermometer to determine the degree of the temperature). Equilibration: Diluted Epididymal sperm containing different concentration of antioxidant at a temperature of 5°C was performed for 4 hours at the same temperature (5 C), diluted Epididymal sperm was filled in French medium straw (0.5 ml) and stopper was applied using a heat press and preservation at 5°C till equilibration time, and evaluated in the same measured way as after diluted Epididymal sperm. Freezing -196 c of straws, Straws were kept horizontally, then placed in a container containing liquid nitrogen to be exposed to liquid nitrogen vapor (2-3 cm) in the form for 5-10 minutes (Yu *et al.,* 2002). Then collect the straw on each shelf and quickly dip it into special cups containing liquid nitrogen and these cups were transferred to a liquid nitrogen container. After 48 hours of storage, thawing is carried out by placing the straw into a water bath at (massage straw by hand) 37°C for 30 second then straws were cut to removes the straw vacuum first drop and put the second one on the slide to start the diluted Epididymal sperm was evaluated in the same measured way as after diluted Epididymal sperm and after cooling sample.

Assessment of Sperm DNA Integrity

Preparation of acridine orange fixative (Carnoy's solution); Carnoy's fixative provides a better predictive value for DNA damage to semen using acridine orange (AO.) stain, Carnoy's solution consists of (3) parts methanol/(1) part glacial acetic acid (Martins *et al.,* 2007).

Preparation of acridine orange (AO) stain; Acridine orange was used to prepare staining solution from a stock solution consisting of one g m acridine orange (AO.) in (100ml) of distilled water and stored in the dark at 4°C. Then (1 ml) of stock solution was added to (40 mL) of 0.1M Citric acid and (2.5. mL) of 0.3M Na2Hpo4.7H2O and the pH was adjusted to (2.5) before staining. All solutions were maintained at room temperature (Varghese *et al.,* 2011).

Sperm Preparation and AO Staining

a. A thin smear of diluted Epididymal sperm (10µl straw) was prepared.

b. Smear was fixed in Carnoy s solution overnight.

c. Slides were then washed in air and put in tampon solution (80 mM of citric acid and 15 mM of sodium acid) PH 2.5 at 75 C for 5 minutes.

d. The slide was Stained with Acridine orange AO for 5 minutes as Fig. 1.

e. The slide was rinsed in water and covered with cover slip.

f. The slide was read on a fluorescence microscope using 490 nm excitation filter and 530 nm barrier filter.

g. A total of 100 sperms were evaluated in each prepared slide ,Sperm cell heads with usual DNA Integrity (double-stranded) had green fluorescence indicated sperms with intact (non- damaged) DNA, whereas those with single-stranded DNA had orange, yellow, or red fluorescence indicated sperm with damaged DNA.

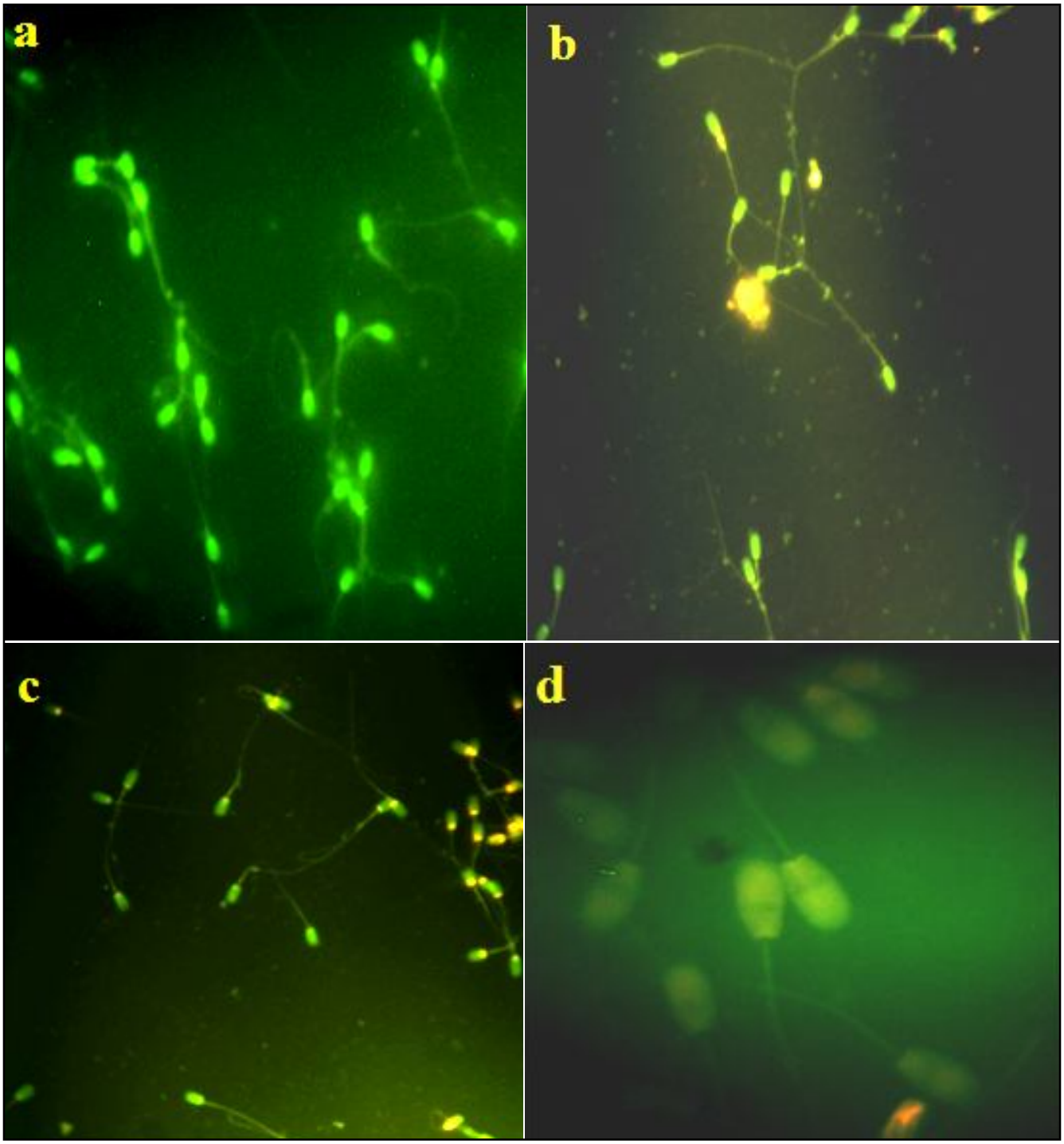

Fig. 1: Fluorescence microscopy displaying buffalo spermatozoa treatment with Pentoxifylline of acridine orange stain.
a. After dilution spermatozoa treatment with Pentoxifylline intact normal DNA with green colour(10X). **b.** After cooling Spermatozoontreatment with Pentoxifyllinedamaged DNA of dead sperm as shown by yellow or red colours (10×). **c.** Post thawing of Spermatozoon treatment with Pentoxifylline damaged DNA of dead sperm as shown by yellow or red colors (10×). **d.** Post thawing of Spermatozoon treatment with Pentoxifylline intact normal DNA with green color (40×).

Statistical analysis

All data analysis has performed using unidirectional variance analysis (ANOVA). A value of P less than 0.05 was accepted as a statistically significant difference.

Results

The result of DNA integrity Percentage of Epididymal sperm treated with different concentrations of pentoxifylline table 1, after dilution were in T1 (90.44±0.66%), T2 (92.11±0.54%), T3 (93.79±0.64%), and T4 (96.36±0.40%) respectively. The result indicate there is significant difference in T4 in comparison with others with highest significant (p> 0.05) value was in T4 (%), T3 (93.79±0.64%) and T4 (96.36±0.40%) respectively, but there is non-significant difference between T1 (90.44±0.66) and T2 (92.11±0.54) as Fig. 1.a.

Values of DNA integrity Percentage with different concentrations of pentoxifylline after cooling were in T1 (86.42±0.56%), T2 (88.43±0.49%), T3 (90.27±0.39%) and T4 (92.33±0.49%) respectively table 1. There is highest significant (p> 0.05) value in DNA integrity Percentage in T4 (%), T2 (88.43±0.49 %) and lowest significant (p> 0.05) was in T1 (86.42±0.56%) in comparison with othersT2 and T3as Fig. 1.b.

The result DNA integrity Percentage in post thawing table 1 were in T1 (37.27±0.96%), T2 (63.57±0.55%), T3(71.51±0.61%) and T4 (77.94±0.60%) respectively. There is significant difference between in all treatment with highest significant (p> 0.05) value in T4 (77.94±0.60%) and lowest significant (p> 0.05) was in T1 (37.27±0.96%) as Fig. 1.c and d.

Regarding the effect of different steps of Epididymal sperm processing (after dilution, after cooling and post thawing) within each treatment, there is gradually significant (p> 0.05) decreased in DNA integrity Percentage in all treatment, with highest significant (p> 0.05) value in after dilution and lowest significant (p> 0.05) value in post thawing.

Table 1: Effect of different concentrations of pentoxifylline on DNA integrity percentage during different steps of processing (after dilution, after cooling and post thawing) (Mean± SE).

Concentration of pentoxifylline	Step of freezing		
	after dilution	After cooling	Post thawing
Control -Tris T1	90.44±0.66Ca	86.42±0.56Cb	37.27±0.96Dc
T2- 1mM/ml	92.11±0.54 BCa	88.43±0.49Bb	63.57±0.55Cc
T3 -5mM/ml	93.79±0.64Ba	90.27±0.39Bb	71.51±0.61Bc
T4-10mM/ml	96.36±0.40Aa	92.33±0.49Ab	77.94±0.60Ac

Different capital letters Means significant (p<0.05) different within column

Different small letters Means significant (p<0.05) different between column

Discussion

The result of DNA integrity Percentage of Epididymal sperm treated with different concentrations of pentoxifylline, there is gradually significant (p> 0.05) decreased in DNA integrity Percentage in all treatment, with highest significant (p> 0.05) value in after dilution and lowest significant (p> 0.05) value in post thawing. This result in agreement with Evrim *et al.,* (2015) who reported that increase DNA damage in treated with pentoxifylline. Vernocchi, *et al.,* (2014) mentioned that sperm containing fragmented DNA may be important parameter of semen quality. Numabe *et al.,* (2001) and Zang *et al.,* (2005) were showed that pentoxifylline leads to reduced lipid peroxidation associated sperm membrane damage and DNA apoptosis and scavenges the toxic reactive oxygen species (ROS). Mukhopadhyay *et al.,* (2011) reported that significant differences in the percentage of nuclear sperm DNA in the fresh and frozen thaw semen samples. This result study, Koonjaenak *et al.,* (2007) reported that previously frozen buffalo there the sperm DNA integrity has been severely damaged by cryopreservation. While, Kumar, *et al.,* (2011) recorded that cryopreservation reduce the DNA integrity of buffalo spermatozoa by reducing the antioxidant potential of semen. Said, (2003) and Sakkas *et al.,* (2002) found that temperature variation during (cooling ,freezing and thawing) may cause adverse changes in sperm functions and structure (nucleus and membrane) that may affect fertility; while disagreed with Duru *et al.,* (2001), Anzar *et al.,* (2002), and Hallap *et al.,* (2005) who found that an unchanged DNA integrity rate during freezing and thawing process in swamp buffalo bull, human and stallion. Said *et al.,* (2010) reported that DNA integrity is a concern during cell freezing because cryopreservation easily changes mitochondrial membrane properties and increase the production of ROS, which may subsequently result in the oxidation of DNA, producing high frequencies of single and double strand DNA breaks. This suggested the critical role of antioxidant in reducing DNA intact during cryopreservation of semen (Bucak *et al.,* 2010). While, Baumber *et al.,* (2003) reported that ROS molecules were mainly responsible for causing sperm DNA damage. However, the potential role of antioxidants in preventing DNA damage induced by ROS during cryopreservation Branco *et al.,* (2010) ROS released during cooling induce oxidative DNA damage in bovine spermatozoa reported by Sakkas

et al., (2002). Also, in post thaw buffalo epididymal fluid treatment with pentoxifylline samples was observed 78.93% DNA integrity ,this is lower than to the finding reported in stallion recorded by Kasimanickam *et al.,* (2007) and in boar (Frajer and Strzezk, 2007). On the other hand, previous observation process in buffalo (koonjaenak *et al.,* 2007; Kadirvel *et al.,* 2012) and in cattle (Anzar *et al.,* 2002). However, sperm DNA is to be altered or damaged during cryopreservation in human (Donnelly *et al.,* 2001) and in ram (Peris *et al.,* 2004). Thus, buffalo sperm DNA seems to be compact nature of the sperm nucleus and DNA during cryopreservation. Sperm DNA integrity is important for the success of natural or assisted fertilization including normal development of the embryo. Lopes, *et al.,* (1998) and Jian-hong *et al.,* (2008) suggested that evaluation of sperm DNA integrity may aid in determining the quality of frozen buffalo bull semen. Abd El- fatah *et al.,* (2008) and Mahmoud *et al.,* (2015) found that significant increase in DNA damage in buffalo frozen semen than fresh. Sakkas *et al.,* (2002) showed that the ROS released during cooling induce oxidative DNA damage in bovine spermatozoa.

Conclusion

(1) Adding of PTX to Epididymal sperm samples can improve sperm chromatin quality and maintain sperm during the freezing period in concentration 10µ sperm it may be because it can mimic the sperm condition in the body during late spermatogenesis. (2) PTX consumption significantly increases the sperm maturity and percentage of sperms with double-stranded and healthy DNA integrity which ultimately improved.

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