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Short Communication

Optimization of Acridine Orange Staining for Buffalo Sperm, Cryopreserved in Egg Yolk based Extender to Detect DNA Fragmentation

Mushtaq Ahmad[1,*], Mehboob Ahmed[1,2] and Nasim Ahmad[1]

[1]*Department of Theriogenology, Faculty of Veterinary Science, University of Veterinary and Animal Sciences, Outfall Road, Lahore 54000, Pakistan*
[2]*Livestock and Dairy Development Department, Punjab, Pakistan*

ABSTRACT

The integral genome of sperm is crucial for successful fertilization and embryonic development. Different techniques have been used to determine the integrity of DNA in which acridine orange (AO) assay is easy and less costly. Though used in many species but the variability exists in technique. Therefore, the present study was aimed to optimize the steps of AO staining protocol for buffalo sperm which has not been explored yet. Semen was collected from two mature buffalo bulls, diluted in tris citrate egg yolk based extender and cryopreserved using standard protocol. The semen straws were thawed at 37°C and stained using different protocols as followed: Protocol 1 (washing effect); washing either with normal saline (NS) or phosphate buffered saline (PBS), Protocol II (heating effect during incubation in tampon solution); smears were immersed in tampon either at room temperature or at 60°C, Protocol III (concentration of dye in staining solution); slides were stained with 1, 5, 200, 500, or 1000µg/mL solution. The slides were observed, intensity of staining and debris presence in samples. The debris presence was reduced ($P<0.05$) due to washing of semen sample. Intensity of staining increased ($P<0.05$) due to washing and heating during incubation in tampon solution. However, single and double washing and type of buffer did not affect the staining intensity. Higher concentration (1000µg/mL) stained the fragmented DNA better than lower ($P<0.05$) concentrations. However, double stranded DNA was stained with lower concentration of dye in staining solution. In conclusion, the buffalo sperm should be stained with higher concentration of AO stain in washed sample either with NS or PBS and heated incubation in tampon solution for better assessment of fragmented DNA.

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Mushtaq A designed the study and wrote the manuscript. Mehboob A executed the experiments and NA supervised the study.

Key words
Acridine orange, DNA fragmentation, Buffalo, Sperm.

Successful fertilization of mammalian gametes depends upon complete union of their intact and functional genome. Nuclear matrix of sperm containing condensed and compact DNA (O'brien and Zini, 2005) has been reported as basic component to form normal and viable embryos (Khalifa *et al*., 2008) and subsequent their development (Schulte *et al*., 2010; Zhang *et al*., 2008). Integrity of genome has been related as determinant of male fertility in various species (Fernandez-Santos *et al*., 2009; Minervini *et al*., 2013; Zini and Libman, 2006). DNA fragmentation may result from defective spermatogenesis, abnormal genome packaging, exposure to high ratio of reactive oxygen species (ROS) in ejaculate, toxins and processing of sperm cell (Chohan *et al*., 2006). Cryopreservation of sperm cell also leads to DNA fragmentation due to increased oxidative stress, augmenting the apoptosis and necrotic pathways of sperm death and physical and biochemical changes (Ahmad *et al*., 2015; Anzar *et al*., 2002).

The development of strategies is necessary to determine the DNA fragmentation in order to enhance reproductive outcomes of artificial reproductive technologies and determination of fertility potential of males by obtaining sperm either from testis or cryopreserved storage (Evenson and Wixon, 2006). For this purpose, TdT-mediated-dUTP nick end labeling (TUNEL) assay, single cell gel electrophoresis (COMET) assay and sperm chromatin dispersion test (SCD) are being used as advanced techniques (Chohan *et al*., 2006). However, acridine orange (AO) assay is simple cytochemical method which can be used as clinically significant microscopic procedure to determine DNA fragmentation (Eggert-Kruse *et al*., 1996). Acridine orange possesses fluorescent metachromatic property, shows green fluorescence when bound as monomer to double stranded DNA and fluoresces yellow to red when bound to

* Corresponding author: dr.mushtaq85@yahoo.com; mushtaq@uvas.edu.pk
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single stranded DNA (fragmented DNA) depending upon intensity of fragmentation (Virant-Klun *et al*., 2002). As AO assay produces variable results and interpretations due to differences in procedure involved, species effect and intensity of DNA fragmentation, so it needs further standardization of this technique.

Therefore, the objective of the present study was to standardize the steps of acridine orange assay to detect DNA fragmentation of buffalo (*Bubalis bubalis*) sperm, effectively.

Material and methods

A total of fourteen ejaculates were collected from two mature buffalo (*Bubalis bubalis*) bulls (age 4-5 years) using artificial vagina (temperature 42°C, IMV France). The semen samples which met the minimum standards (motility >60% and concentration >350×10^6 sperm/mL) were pooled, diluted with Tris-based extender containing Tris (24.2g/L; MP Biomedicals LLC, Illkirch, France), citric acid monohydrate (14g/L; Merck, Darmstadt, Germany), egg yolk (200mL/L), fructose (10g/L; Sigma-Aldrich Chemicals, St. Louis, MO, USA), glycerol (70 mL/L; BDH Laboratory Supplies, Poole, England), and antibiotics (benzyl penicillin 100000 i.u./L and streptomycin sulphate 10mg/L, Sigma) at 37ºC to final concentration of 50×10^6/mL. It was filled into 0.5mL French semen straws, cooled to +4°C over 90 min, equilibrated for 2 h, frozen in nitrogen vapour for 7min and plunged into liquid nitrogen until analyzed. For analysis, two straws per replicate were thawed at 37°C, pooled and processed for acridine orange staining.

The serial steps of staining procedure included 1) smear formation of semen, 2) fixation of smear in carnoy's solution (methanol: glacial acetic acid in 3:1), 3) incubation in tampon solution (0.1M Citric acid and 0.3M Na_2HPO_4 in 16:1 ratio; pH= 2.5) for 7 min and 4) staining with working solution of acridine orange and later visualization under fluorescent microscope with suitable filter. In this study, three steps of staining protocol were considered as limiting factors which needs to be optimized for better staining of fragmented DNA *i.e.* sperm preparation before smear formation (I), temperature of incubation in tampon solution (II) and concentration of working solution of stain (III) for intact and fragmented DNA of buffalo sperm.

In the protocol I of sperm preparation, the semen sample was divided into three aliquots. First and second aliquot were washed either with phosphate buffered saline (PBS) or normal saline (NS). It was carried out either in single or double for each washing solution to compare the effect of frequency of washing on sperm preparation. Third aliquot was considered as control in which washing was not performed. The washing was applied by centrifuging the mixture of 100 µL semen and 900 µL of washing solution (PBS or NS) at 1000 rpm for 5 min. Pellet was suspended in 100 µL of same buffer for further processing. For protocol II, the semen sample was divided into two aliquots. The first aliquot (control) was incubated at room temperature while second was incubated at 60°C in tampon solution. In protocol III, the processed samples were stained with different concentrations of staining solutions of AO *i.e.* 1, 5, 200, 500 and 1000 µg/mL at separate slides in dark for 5 min. The stained slides were evaluated immediately under fluorescent microscope (400X; CX 41, Olympus, Japan) with an excitation filter at 460-570 nm and an emission filter at 460-610 nm. Presence of debris and intensity of staining of each pattern (green, yellow-orange and red) was scored as 1-5.

The data for presence of debris particles and intensity of sperm staining with intact, partially and completely fragmented DNA were presented as mean ± SEM. Each parameter was assessed through score of 1-5. All the statistical analyses were performed using SAS Enterprise Guide (Version 4.2; SAS Inst. Inc., Cary, NC, USA). The effect of washing, frequency of washing, heating of tampon solution and concentration of acridine orange was compared using non parametric Mann-Whitney test. A probability level of <0.05 was considered as significant.

Results and discussion

To the best of our knowledge, this is first report on standardization of AO assay for buffalo sperm. Although, this assay is being used commonly in various species based upon protocol as described by Tejada *et al*. (1984), yet different scientists modified it for different species and microscopic or flow cytometric method of detection (Fernandez-Santos *et al*., 2009; Virant-Klun *et al*., 2002). For buffalo sperm, it was crucial to standardize this assay to enhance the validity and potential outcome of this assay. We anticipated that washing of semen sample, heating of smear and concentration of dye would affect the staining efficiency for buffalo sperm.

The results for comparison of washing with PBS, NS and without washing on presence of stained debris and intensity of staining of sperm have been presented in Supplementary Table S1. Debris particles were reduced (P=0.0002) due to washing with both solutions of PBS and NS. However, they were equally reduced by PBS and NS (1.77±0.22 and 1.66±0.16, respectively). Intensity of sperm staining was not affected (P=0.369) due to washing with PBS and NS. The results for effect of frequency of washing of semen sample before semen preparation on score of presence of stained debris particles in the slide and intensity of sperm staining have been presented in Supplementary Table S2.

Table I.- Effect of concentration of acridine orange in working solution on staining score (1-5) of intact (green), partially fragmented (yellow-orange) and completely fragmented (red) DNA of cryopreserved buffalo sperm.

DNA staining	Concentration of Acridine orange (µg/mL)					P-value
	1	2	200	500	1000	
Green	2.17±0.31[a]	3.83±0.31[b]	4.17±0.17[b]	4.33±0.21[b]	4.33±0.21[b]	0.001
Yellow-orange	0.00±0.00[a]	0.50±0.22[a]	2.00±0.37[b]	3.67±0.21[c]	4.33±0.21[c]	0.002
Red	0.00±0.00[a]	0.17±0.17[a]	0.33±0.33[a]	1.67±0.76[a]	4.17±0.17[b]	0.001

Different small letters ([a-c]) show significant difference between concentrations in a row of each parameter.

The present study depicted better staining due to washing of semen sample and removal of egg yolk based extender. Washing has been used by researchers before preparation of smear as in human (Chohan *et al*., 2006; Eggert-Kruse *et al*., 1996), bull (Carreira *et al*., 2012) and buffalo (Kadirvel *et al*., 2009). AO can lodge into various substances bacteria, algae, leukocytes and debris present in egg yolk. Washing helps to remove these debris molecules; thus differentiated sperm head with other confusing bodies in the smear. It depends at purity and centrifuged egg yolk based extender. Frequency of washing didn't affect intensity of staining in present study.

The results for effect of concentration of stain (µg/mL) in working solution on staining score (1-5) of intact (green), partially fragmented (yellow-orange) and completely fragmented (red) DNA of sperm, are presented in Table I. The score of staining of intact DNA was observed very less (P=0.001) when stained with 1µg/mL working solution. However, it was found equal with higher concentrations (5, 200, 500 and 1000 µg/mL) of working solution. Partial and complete fragmented DNA was not stained with 1µg/mL concentration. The score of yellow to orange fluorescence was observed higher (P=0.002) when stained with 500 and 1000 µg/mL working solution (3.67±0.21 and 4.33±0.21, respectively) than 5 and 200µg/mL solutions (0.50±0.22 and 2.00±0.37, respectively). It was found similar in 500 and 1000 µg/mL staining solution. The score for red fluorescence (complete fragmentation of DNA) was observed highest (4.17±0.17; P=0.001) in 1000 µg/mL than all other concentrations of working solutions. Intensity of sperm staining was increased (P=0.017) when tampon solution was heated on slide to 60°C for 5-7 min (3.20±0.20) than sample without heating (2.44±0.17) during staining procedures.

The concentration of dye is crucial to stain fragmented DNA. The present study evidenced that higher concentration is required to stain fragmented DNA (single stranded DNA). It has been reported that intact DNA required less stain/base pair ratio to intercalate and manifest green fluorescence while higher stain/base pair ratio (about 100µM) for start of appearance of red fluorescence after stacking of AO to phosphates of ssDNA (fragmented DNA). In current study, fragmented DNA started to appear better under fluorescence microscope when stain was used at 200 µg/mL concentration in staining solution. As the fragmentation increased, the higher concentration of stain proved better results in terms of staining intensity which is necessary to visualize accurately in microscopy.

In conclusion, the buffalo sperm can be stained with higher concentration of AO stain (1000µg/mL) to detect DNA fragmentation effectively; after washing of egg yolk based extender either with normal or phosphate buffered saline and using heat during the incubation in tampon solution.

Supplementary material

There is supplementary material associated with this article. Access the material online at: http://dx.doi.org/10.17582/journal.pjz/2017.49.5.sc6

Statement of conflict of interest

Authors have declared no conflict of interest.

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