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Effectiveness of Giemsa and Modified Trypan Blue-Giemsa Staining for the Assessment of Acrosome Integrity in Bull and Buck semen

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ABSTRACT

The estimation of acrosomal integrity is crucial for predicting the fertility of spermatozoa. The semen quality analysis demands assessment techniques to be reliable, quick and easy to perform for routine application in semen station. A study was conducted to assess the acrosomal integrity of Kangayam cattle and Salem Black goat using Giemsa stain and modified Trypan Blue-Giemsa stain. For this procedure, 70 numbers of frozen semen straws of Kangayam bull and Salem Black bucks were stained. The staining quality and acrosomal integrity were assessed at the interval of 3, 5 and 24 hours interval up to 5 days, and evaluated as acrosomal intact and non-intact spermatozoa. In Kangayam bull semen, the quality of the staining was good and better in trypan blue – Giemsa stain than Giemsa alone but for Salem Black buck semen Giemsa showed better staining quality than trypan blue – Giemsa combination. However, the working solution of Giemsa once prepared can be used for 4 days but the trypan blue-Giemsa stain can be used for only 24 hours once prepared. Hence, stain is required to be prepared on daily basis for routine semen evaluation in semen stations. As a conclusion trypan blue – Giemsa staining method is recommended for better staining of acrosome in bull semen and Giemsa alone in Buck semen.
Key words: Acrosomal integrity, Buck, Bull, Giemsa, Trypan blue.
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INTRODUCTION

Only few single sperm viability parameters show a significant correlation with fertility of semen samples. Therefore, there is need to explore functional *in vitro* fertility tests having capability to disclose the ability of spermatozoa to undergo complicated processes such as capacitation, binding with zona pellucida, acrosome reaction, fertil-

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ization of oocyte and induction of embryo development. Several assays have been developed to estimate semen quality, often expensive and time-consuming, and many studies have correlated *in vitro* assays with fertility in bulls (Ostermeier *et al.,* 2001; Kastelic and Thundathil, 2008). A sperm cell must possess many attributes to fertilize an oocyte (Graham and Moce, 2005), as intact and functional plasma and acrosomal membranes. Various assays are used for evaluating different sperm parameters such as concentration, motility, membrane integrity or viability and morphology. No single assay reliably predicts fertility of the spermatozoa and female effect on sperm cells (Aitken, 2006).

Spermatozoa have three types of membranes which are plasma membrane, mitochondrial membrane and acrosomal membrane. These membranes contain certain polyunsaturated fatty acids (PUFA) and hence they are very susceptible to oxidative stress, especially during freezing process (Chelucci *et al.,* 2015). Oxidative stress is one of the factors resulting in cell damage due to Reactive Oxygen Species (ROS) (Rath *et al.,* 2009). Lipid peroxidation of PUFA present in membrane resulting in formation of ROS that causes changes in membrane function by altering the morphology and decreasing the metabolism, motility and fertility of sperm (Insani *et al.,* 2014). There are some *in vitro* fertility tests for estimation of membrane integrity and functional integrity such as hypo-osmotic swelling test and acrosomal integrity which can be used for evaluation of semen. In all mammals, the capacitation and the subsequent acrosome reaction of spermatozoa represent essential steps for successful fertilization and formation of a zygote. The determination of the ability of spermatozoa to activate the acrosome reaction is supposed to be a useful parameter in evaluating infertility. One of the key processes in mammalian fertilization is the acrosome reaction (AR) usually triggered in spermatozoa upon their biding to the zona pellucida of the egg. The AR involves fusion between the plasma membrane and the underlying outer acrosomal membrane, which results in the release of the acrosomal contents. Acrosomes play a crucial role in the process of fertilization. Spermatozoa binding to the zona pellucida will stimulate acrosome reaction and cause the release and the activation of acrosome enzymes, allowing spermatozoa to penetrate the zona pellucida. The acrosomal integrity of a spermatozoa can be assessed by light microscope using various staining methods such as Giemsa staining technique and triple staining technique (trypan blue, Bismark brown and rose bengal) (Talbot and Chacon, 1981).

The aim of this study to assess the acrosomal integrity of Kangayam bull and Salem Black buck semen using Giemsa staining and modified Trypan blue-Giemsa staining and to assess the quality of stains.

MATERIALS AND METHODS

The semen straws were prepared from the Kangayam bull and Salem Black buck which were maintained at Frozen Semen Bank, Department of Veterinary Gynaecology and Obstetrics, Veterinary College and Research Institute, Namakkal. Seventy samples of frozen semen straws were used for each staining procedure. The frozen semen straws were removed from liquid nitrogen containers and thawed using a water bath at the temperature of 37ºC for 30 seconds. Thirty-five straws were utilized for Giemsa staining technique. The staining procedure was carried out by a making a neat smear on a clean glass slide from a drop of thawed semen. Then the slide is warmed using a warming plate at the temperature of 37ºC for 10 minutes. The preparation was fixed using methanol for 10 minutes and then rinsed in running tap water. Then the slide was stained in a Giemsa working solution (3 ml absolute Giemsa, 2 ml Phosphate Buffered Saline (PBS) and 35 ml of distilled water) by immersing it in a staining jar. The slide was rinsed in running tap water and dried again and examined under the microscope (Prihantoko *et al.,* 2020). The acrosomal integrity was assessed at an interval of 3, 5 and 24 hours. The working solution was reused for up to 5 days to assess the keeping quality and quality of staining of the working solution.

Another 35 straws were utilized for modified Trypan blue-Giemsa staining technique. The staining procedure was carried out by placing a drop of thawed semen and was mixed with a drop of Trypan blue (0.4 %) on a clean glass slide. A neat smear was made on a clean glass slide. Then the smear was dried under air and fixed using ethanol for 10 minutes. Then the slide was air dried and immersed in a staining jar containing Giemsa working solution composed of 3 ml Giemsa stock solution and 27 ml distilled water. The slide was rinsed in running tap water and dried again and examined under the microscope. The acrosomal integrity was assessed at an interval of 3, 5 and 24 hours. Then the working solution was reused to assess the acrosomal integrity for up to 5 days to assess the keeping quality and quality of staining of the working solution.

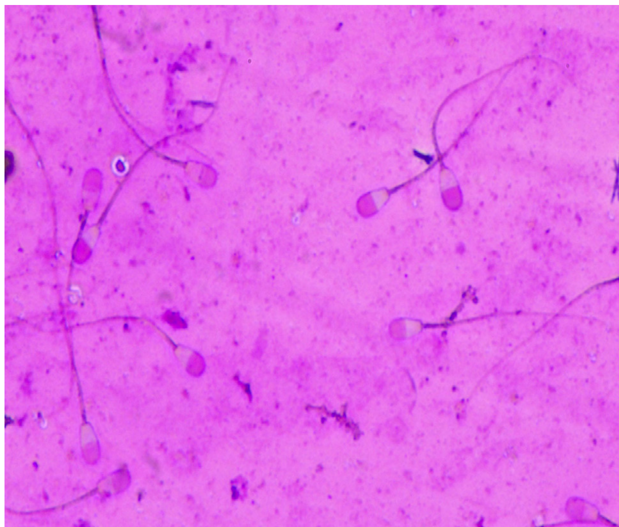
RESULTS AND DISCUSSION

Acrosomal integrity of spermatozoa of Kangayam bull and Salem Black buck was determined by light microscopy

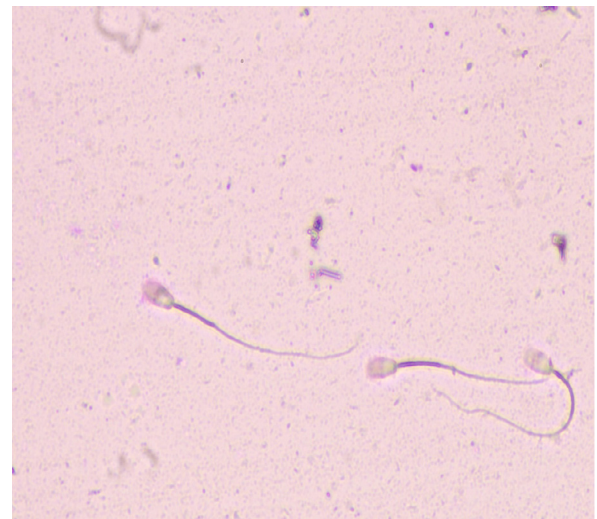
using Giemsa stain and Modified Trypan blue-Giemsa stain. A distinct apical ridge was present in intact spermatozoa. The spermatozoa with intact acrosome of both species were stained purple whereas spermatozoa lacking an acrosome were pale purple or lavender colour. The mean acrosomal integrity (%) of Kangayam bull semen and Salem Black buck semen at an interval of 3, 5 and 24 hours were depicted in the Table 1.

Table 1: The mean acrosomal integrity (%) of Kangayam bull and Salem Black buck semen

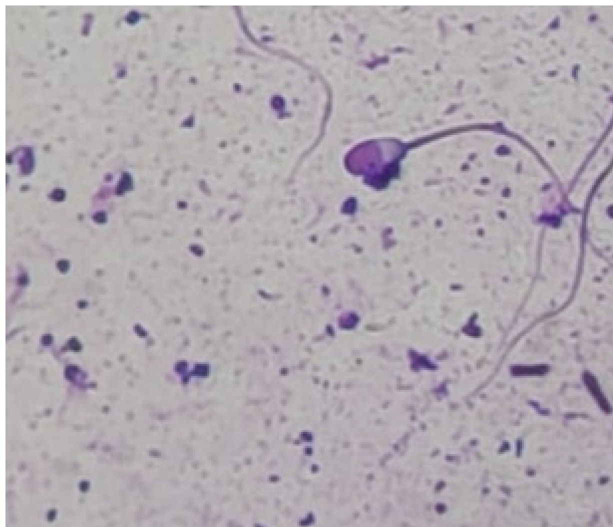
Species	Mean acrosomal integrity (%) at an interval of 3 hours	Mean acrosomal integrity (%) at an interval of 5 hours	Mean acrosomal integrity (%) at an interval of 24 hours
Kangayam bull semen	94.12±1.43	93.44±2.02	93.09±1.96
Salem Black buck semen	91.89±1.84	90.02±1.23	89.65±2.79



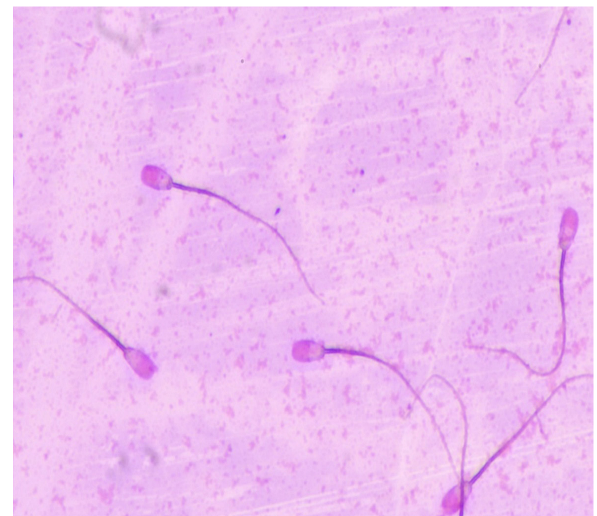
1.A- Acrosome intact - TBG stain at 3 hours



1.B- Acrosome intact - TBG on subsequent usage



1.C- Acrosome intact - Giemsa stain at 3 hours



1.D- Acrosome intact - Giemsa stain on subsequent usage

Fig. 1: Acrosome integrity of a Kangayam bull semen

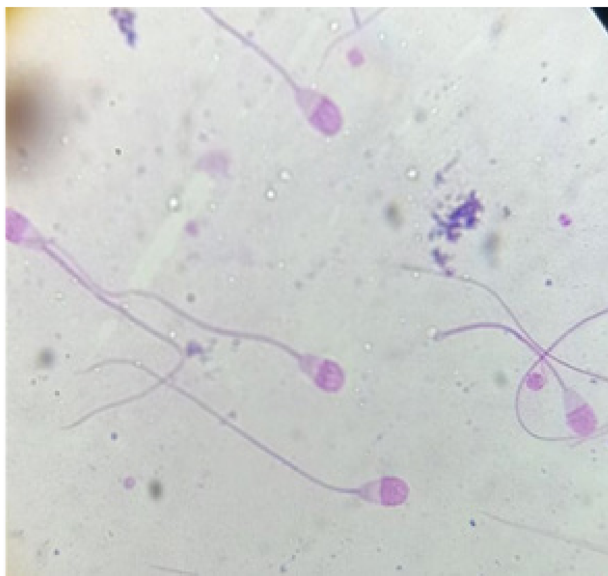


Fig. 2: A- Acrosome intact - Giemsa stain at 3 hours

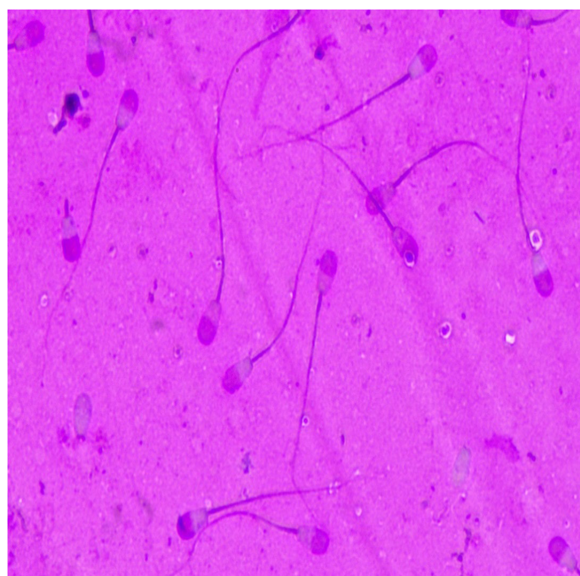


Fig 2: B- Acrosome intact - TBG stain at 3 hours

Fig. 2: Acrosome integrity of a Salem Black buck semen

The microscopic examination of semen smears using modified trypan blue giemsa stain and giemsa stain revealed intact and non intact acrosome of both the species. However, the modified trypan blue giemsa stain offers better staining quality and intensity of staining acrosome of Kangeyam bull was more when compared to the Giemsa stain but for staining of buck sperm Trypan blue Giemsa gave better results than Giemsa. Regarding the reusable properties of stains, modified TBJ lacks the affinity to stain the acrosome after 24 hours of preparation, however Giemsa can be reused for staining upto 2-3 days without affecting the results. Therefore, for better staining, Trypan blue-Giemsa should be freshly prepared.

Acrosome is a cap like structure on the head of the spermatozoa. It covers approximately 60-70 per cent of bovine sperm head (Patrat *et al.,* 2000). Acrosome contains enzymes which are instrumental in penetration of the ovum. Therefore, acrosome integrity needs to be maintained to achieve normal fertility. Significant changes in the plasma membrane and outer acrosomal membrane are observed during capacitation (Jankovičová *et al.,* 2008). During the process of capacitation, the seminal proteins are removed from the spermatozoa in the female genital tract which is necessary for the acrosomal reaction to happen. Acrosomal reaction involves the fusion of outer acrosomal membrane with the sperm plasma membrane. This fusion results in the formation of vesicles called as "Vesiculation". It results in release of acrosomal enzymes like hyaluronidase, etc which are important for sperm penetration. Neild *et al.,* (2005) suggested that acrosome reaction is a process of releasing a penetrating enzyme that allows spermatozoa to penetrate zona pellucida and to fertilize oocytes. However, if the acrosome reaction takes place before spermatozoa reach fertilization site, the spermatozoa will lose their ability to fertilize oocytes. Spermatozoa must be acrosome-intact to have the ability to fertilize oocytes. The sperm must be able to undergo these changes in the female reproductive tract to attain optimum fertilization potential, which requires the acrosome to be structurally intact and biochemically functional. Various physical and chemical factors influence the acrosome integrity (Chelucci *et al.,* 2015). Acrosome can also be damaged during the process of freezing and thawing. Hence, evaluation of acrosomal integrity should be integral part of semen quality assessment which can be used for detecting acrosomal abnormalities, evaluating the effect of cryopreservation and evaluation of acrosomal status during *in vitro* capacitation (Huo *et al.,* 2002).

Acrosome intactness can be tested using Giemsa and modified TBG staining illustrated in Figure 1 and Figure 2. The results of spermatozoa TBG staining showed that Giemsa staining has the ability to bind to membrane proteins. So, it can be assumed that the Giemsa can stain the membrane proteins. The damaged acrosome can be observed on the basis of the results of spermatozoa staining, whether the acrosome absorb color or not. Prihantoko *et al.,* 2020 reported that the TBG staining was not quite effective in assessing frozen semen because the extender components were also stained, which caused the difficulty in analyzing the results of the staining. Additionally, the analysis of the TBG staining results was more time consuming that that of simple Giemsa stain.

CONCLUSION

Modified Trypan blue – Giemsa staining method is recommended for better staining of acrosome in bull semen and Giemsa alone in Buck semen.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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