DIGITAL ANALYSIS OF TESTICULAR ULTRASOUND IMAGE CAN CLASSIFY BUFFALO BULLS WITH HIGH SPERM PRODUCTION CAPACITY

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ABSTRACT

The objective of the present study was to measure pixel intensity mean (Echogenicity) and pixel intensity standard deviation (Heterogeneity) of testicular parenchyma in constantly low or high sperm concentration producing bulls. The average sperm concentration/ml in ejaculate over thirteen month's period (≥100 ejaculates) was recorded. On the basis of sperm concentration, bulls were grouped into; Low sperm concentration (Group A, n=6: age 4.5 to 6 years) and High sperm concentration (Group $B_{1,}$ n=6: age 4.5 to 6 years and $B_{2,}$ n=3: age 7 to 7.5 years) for this experiment. Digital image analysis of ultrasound scan images was done to measure echogenicity and heterogeneity by using ImageJ software. There was no significant difference in echogenicity values between groups, whereas image heterogeneity values of Group A showed statistically significant ($P<0.05$) lower in comparison with B_1 and $B_{2.}$ However, between Group B_1 and B_2 there was no difference. No correlation was observed between the echogenicity and heterogeneity values in any of the groups. Heterogeneity of echo structure may indicate the seminiferous tubule diameter, sertoli cell population and fluid density within tubules. In conclusion, lower sperm output was observed in testes that were less heterogenic at the tissue level. The heterogeneity values in bulls for rejection at the time of BSE (Breeding soundness evaluation) should be studied in more detail to have more insight and to incorporate in BSE of Murrah bull.

Keywords: *Bubalus bubalis*, buffaloes, digital image, pixel intensity, testes, ultrasound

INTRODUCTION

Dairy industry extensively depends on the artificial insemination, leading to the selection of potential fertile bulls with optimal sperm production. Assessment of reproductive function in the bull is performed by conducting a BSE (Breeding soundness examination). Additional to routine examination, use of ultrasonography can add to accuracy. The visual assessment of testicular ultrasonogram is valuable only if testicular pathology exists (Barth *et al*., 2008). The

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minor changes in testicular parenchyma density cannot be visualized by the human eye. However, digital image analysis allows the measurement of changes at tissue level which is not visualized in the organ. The measurement of echogenicity in terms of pixel intensity is possible by using digital image analysis (Ivancic and Mai, 2008). This allows understanding the characteristics of tissue (Cardilli *et al*., 2010). In previous studies the ultrasonographic heterogeneity had also been assessed by calculation of standard deviation of pixel intensity (Hershkovitz *et al*., 2010). The testes ultrasound pixel heterogeneity has been directly correlated with tissue biochemical composition (Omer *et al*., 2012). There are only a few studies demonstrating the relationship between quantitative measurement of testis echogenicity and semen quality (Artega *et al*., 2005). Various other studies have reported on visual ultrasonographic image analysis in correlation with semen quality and mild testicular degeneration, but did not get significant results (Eilts *et al*., 1988; Sidibe *et al*., 1992). In one study, it is reported that pixel intensity was a good indicator of pubertal and mature stages as there will be certain changes in seminiferous tubules under the hormonal influence (Brito *et al*., 2012). The objective of the present study was to check that, there would be a difference in ultrasonographic pixel intensity mean (Echogenicity) or pixel intensity standard deviation (Heterogeneity) in constantly low or high sperm concentration producing bulls.

MATERIALS AND METHODS

Bull selection

For the study, the average sperm concentration/ml in ejaculate over thirteen month's period (≥100 ejaculates) was recorded and fifteen Murrah buffalo bulls aged between 4.5 to 7.5 years with body weight 653.46±24.16 kg (Mean±SE) and scrotal circumference of 30.33±0.96 cm (Mean±SE) kept under uniform housing, feeding and management were selected. Based on sperm concentration animals were grouped (n=6) into low, (n=6) high sperm concentration producing bulls of 4.5 to 6 years age and additionally extra bulls selected (n=3, with duplicate images) of age 7 to 7.5 years whose sperm concentration in ejaculate was also high. Hence, the three groups were, low concentration producing bulls (A), high concentration producing bulls (B_1) and high concentration producing bulls with greater age (B_2) (Figure 1.). All the bulls were selected based on following criteria; (1) clinical healthy, (2) over 4.5 years of age, (3) reproductively active with regular semen collection, (4) no history of previous scrotal or testicular injury.

Testicular ultrasonography and digital image analysis for pixel intensity

B-mode ultrasound scanner with a 6.5 MHz linear transducer (KX2600, Kaixin veterinary ultrasound, Xuzhou Kaixin Electronic Instrument Co. Ltd. China) was used to image testis of each bull. The same ultrasound was used for each examination and all settings such as gain, TGC (Time gain compensation), depth and contrast were kept constant. Testes were prepared as per standard procedure and all the examination was performed by the same examiner. The transducer was held both longitudinal and transverse on testis; the image was frozen and stored when mediastinum was clear. Therefore, each bull will have four ultrasound images.Digital image analysis for pixel intensity was performed using image analysis software (ImageJ, National Institutes of Health

(NIH), MD, USA). The testicular pixel intensity (PI) mean and PI standard deviation of images in longitudinal view were analyzed by drawing six rectangles in the parenchyma, three above and three below the mediastinum where parenchyma appeared homogenous. The same procedure was repeated on the images of the transverse view by drawing four rectangles (Figure 2). The macro initially established, remained the same for all the images. Pixel intensity (PI) (according to shade on a 1 to 255 grey scale) was measured as mean (Echogenicity) and standard deviation (Heterogeneity) within the selected areas. Each testicle had ten areas of measurement, mean of the ten echogenicity values and heterogeneity values indicate the overall echogenicity and heterogeneity of that testis respectively.

Statistical analysis

Data were analyzed using the SPSS 22 software package by one-way analysis of variance (ANOVA) and Tukey's Studentized Range (HSD) test. Relationship between the PI mean and PI standard deviation was investigated using Pearson's correlation tests. The results were reported as the mean values for each set of data ± SE and considered significant at $P<0.05$.

RESULTS AND DISCUSSION

There were no differences between pixel intensity mean and PI standard deviation of left and right testis. The comparison of PI mean (Echogenicity) between the groups showed no statistically significant difference as determined by one way ANOVA (Table 1). Whereas, a comparison of PI standard deviation (Heterogeneity) of Group A with B_1 and B_2 showed statistically significant lower value. However, between Group B_1 and B_2 there was no difference (Table 1). No correlation was observed between the echogenicity and heterogeneity in any of the group.

This study considered bulls, only after calculation of average concentration (Low or high) of ≥100 ejaculates over thirteen months period. This eliminates the influence of other factors in the variation of sperm concentration in ejaculate. Bulls of Group A producing 776.64±27.54 (Mean ± SE) millions/ml sperms were considered low concentration, as average concentration reported is 998.91±10.90 (Mean±SE) millions/ml in Murrah buffalo bulls (Bhakat *et al*., 2011). This inturn affects the profitability of semen station as there will be a decrease in a certain number of doses in comparison with >1000 millions/ml. All the bulls considered had normal testes and ultrasonographic homogenous parenchyma with no fibrotic lesions.

We assessed the testicular parenchymal echogenicity by measurement of pixel intensity mean in various regions of interest, similar to other workers (Tomlinson *et al*., 2017). Also measured heterogeneity by measuring the standard deviation of the pixel intensity of the region of interest as reported in the dog (Moxon *et al*., 2015). It is appropriate to consider the many regions of interest and taking a range of values for pixel intensity rather than to directly obtain a single mean value. Brito *et al*. (2012) reported that testicular echogenicity was associated with sperm production, seminiferous tubule, epithelial area, and sperm morphology, but these associations were not constant. In our study, comparison of the echogenicity between the groups did not show any significant difference. Ahmadi *et al.* (2012) also reported in their study that there was no significant relationship between testicular echogenicity and semen quality in fertile males. No studies reported the relationship

between testicular echogenicity and sperm output. However, our study demonstrated that in Group A, PI standard deviation (heterogeneity) was significantly lower ($P<0.05$) from Group B_1 and B_2 i.e. less heterogenous testes (Group A) = less sperm concentration in ejaculate. Heterogeneity is measured as the variation in pixel intensity within small sampling windows and it may be due to the changes in the density of fluid containing seminiferous tubules (Moxon *et al*., 2015). Thus testes with less heterogeneity may have lower fluid density and reduced sperm output. Moxon *et al.* (2015) defined those testes with anechoic parenchyma with prominent echogenic stipplings were recorded as high heterogeneity and reported that mean testicular heterogeneity was positively correlated with total spermatozoa output in dogs.

The higher heterogeneity also may be due to parenchyma had greater seminiferous tubule diameter and more sertoli cells. Hence, the management of early calf hood nutrition appears to be important, as the growth of seminiferous tubule and sertoli cells in pre-pubertal life is important for spermatogenesis. A positive effect of the plane of nutrition during calf hood on early puberty onset was reported by Byrne *et al.* (2018). The intent of balanced nutrition is not only to achieve early puberty and also to produce optimum volume of semen with higher concentration post-puberty. Restricted diet significantly decreases the number of spermatogenic cell series at all stage and sertoli cells (Davies *et al*., 1957). Calves on a high plane of nutrition had larger testis, greater seminiferous tubule diameter, more mature spermatogenic cells and more sertoli cells (English *et al*., 2018). The sertoli cells are important for the nourishment of germ cells for production of spermatozoa. Testis of larger diameter with adequate number of sertoli cells and larger seminiferous tubule produce high sperm concentration. This indicates bulls with lower heterogeneity in this study may have a lower population of sertoli cells and lesser seminiferous tubule diameter due to suboptimal nutrition during pre-puberty. The appearance of no heterogeneity difference between Group B_1 and B_2 indicates fixation of sertoli cells number at puberty and does not change as age advances.

In conclusion, testicular echogenicity and heterogeneity can be measured by digital analysis of B-mode scan images. This study found significantly lower PI standard deviation (Heterogeneity) value in Group A with B_1 and B_2. Such that lower sperm output was observed in testes that were less heterogenic at the tissue level. Heterogeneity may indicate the seminiferous tubule diameter, sertoli cell population and fluid density within tubules which has to be cross-validated with histology/FNAC. The bull calf and pre-puberty period management with optimal nutrition are important for the development of testicular parenchyma at puberty and greater sperm output. The heterogeneity values of testes in bulls at the time of BSE (Breeding soundness evaluation) with low sperm output should be further studied in depth for its significance and for incorporation in BSE of Murrah bull at an early age.

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Table 1. Data represent mean (±SE) pixel intensity mean (echogenicity) and PI standard deviation (heterogeneity) between the groups.

Experimental group	Mean (±SE) Pixel intensity (PI) mean (Echogenicity)	Mean (±SE) PI standard deviation (Heterogeneity)
Low concentration (A: n=6)	52.117±1.82	9.039±0.23[a]
High concentration (B_1: n=6)	49.177±1.65	9.905±0.16[b]
High concentration (B_2: n=3)	47.787±2.12	9.802±0.17[b]

PI is found to be significant at P>0.05

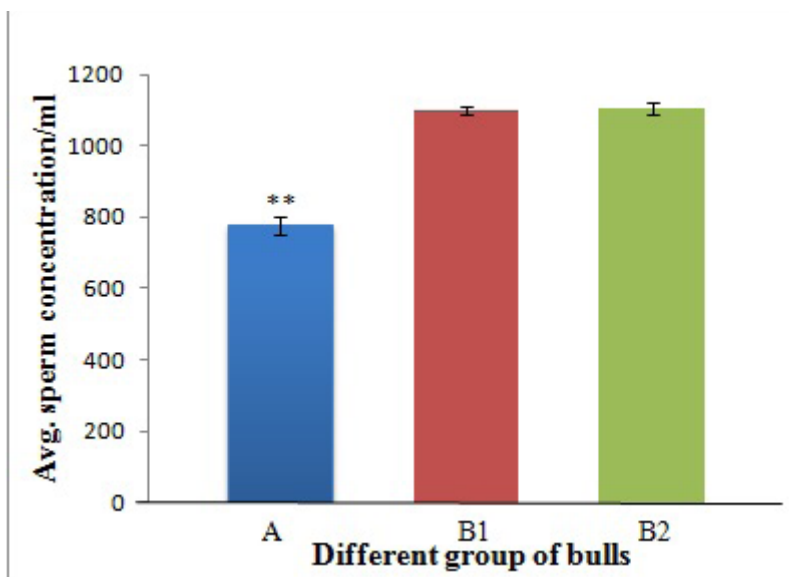

Figure 1. Different groups of bulls selected (A and B_1: low and high sperm concentration respectively of 4.5 to 6 years age), (B_2: high sperm concentration of 7 to 7.5 years age) by considering Avg. sperm concentration/ml for ≥100 ejaculates over period of thirteen months. Error bars represent standard error of mean. Two asterisks mean a significant interaction (**P<0.01).

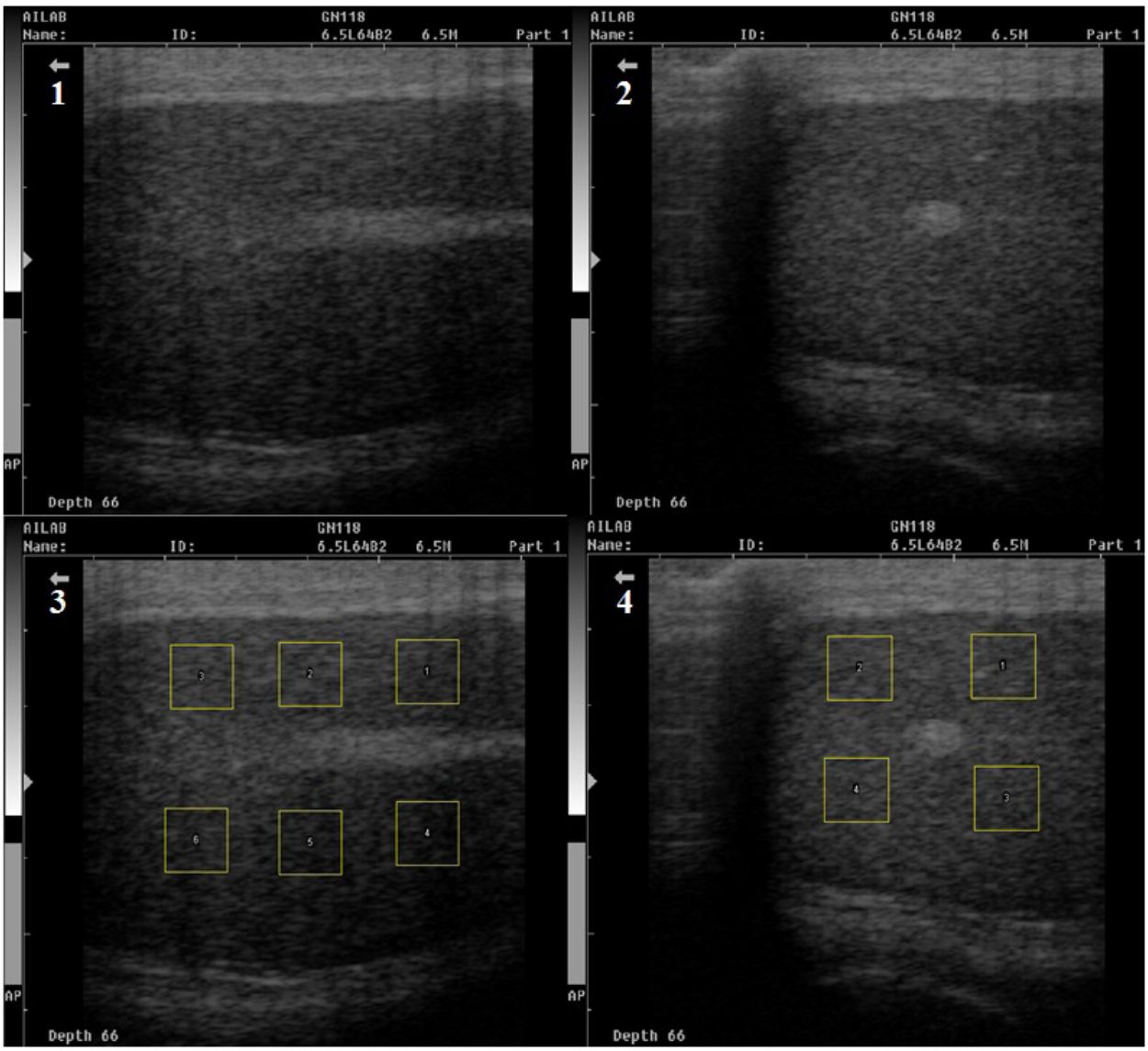

Figure 2. Ultrasonographic appearance of testicular images with mediastinum in longitudinal (1 and 3) and transverse plane (2 and 4). The macro selected for pixel intensity analysis in image J software corresponding to images (1 and 2) can be seen in (3 and 4) indicated by rectangle.

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