IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS)
e-ISSN: 2319-2380, p-ISSN: 2319-2372. Volume 14, Issue 4 Ser. I (April 2021), PP 55-61
www.iosrjournals.org

Association of fertility and *in-vitro* analysis of sperm structural and functional parameters, reactive oxygen species, oesteopontin and ubiquitin of crossbred bulls in India

S. Rangasamy[1*], S. Balasubramanian[2], K. Kulasekar[3], G. Dhinakar Raj G[4], A. Palanisammi[5], D. Reena[6], D. Gopikrishnan[7]and K. Chitra[8]

[1]*Department of Veterinary Gynaecology and Obstetrics, Faculty of Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai-07, Tamilnadu, India*
[2]*Directorate of Clinics, Director of Clinics, Tamil Nadu Veterinary and Animal Sciences University, Chennai-07, Tamilnadu, India*
[3]*Department of Veterinary Gynaecology and Obstetrics, Retired faculty of Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai-07, Tamilnadu, India*
[4]*Director of Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Chennai–51, Tamilnadu, India*
[5]*Dean of Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Tirunelveli - 627 358, Tamilnadu, India*
[6]*Department of Clinics, Faculty of Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai-07, Tamilnadu, India*
[7]*Department of Veterinary Gynaecology and Obstetrics, Faculty of Veterinary College and Research Institute, Tamil Nadu Veterinary and Animal Sciences University, Namakkal-637002, Tamilnadu, India*
[8]*Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Chennai–51, Tamilnadu, India*
***Corresponding Author**: S. Rangasamy, Department of Veterinary Gynaecology and Obstetrics, Faculty of Madras Veterinary College,Tamil Nadu Veterinary and Animal Sciences University, Chennai-600 007, Tamilnadu, India*

Abstract:
***Background**: In cattle, male infertility or subfertility is mainly contributed by the molecular and cellular defects of the spermatozoa affecting the fertilizing ability of the bull's ejaculate on the whole. Hence précised screening of ejaculates for elimination of subfertile or infertile bulls are warranted for a successful progeny testing program, breeding program or semen preservation. The objective of this study was to elucidate bull to bull variation in sperm structural and functional parameters, functional protein expression and associated fertility of crossbred bulls in Tamil Nadu, India.*
***Materials and Methods**: Forty ejaculates from six healthy Jersey crossbred bulls were collected using artificial vagina (AV). After initial screening for motility and morphology ejaculates were ejaculates were divided into two aliquots and one aliquot was frozen using egg-yolk extender and stored at -196 °C. Fresh semen samples initially and post-thaw frozen samples at a later time were evaluated. Sperm viability, acrosome integrity, sperm DNA fragmentation index (%DFI) were evaluated using flow cytometry. In addition, hypo-osmotic swelling, oxidative stress and expression of osteopontin (OPN) and ubiquitin in sperm were determined.*
***Results**: The results showed that viability, acrosome integrity, %DFI, hypo-osmotic swelling, oxidative stress and expression of osteopontin (OPN) and ubiquitin (UBI) were different among bulls (P<0.01) for fresh and frozen semen. The fresh semen had greater viability, acrosome integrity, HOST, oxidative stress and osteopontin; and lesser %DFI and ubiquitin compared with frozen semen (P<0.05). Bulls with greater viability, acrosome integrity, HOST, oxidative stress and OPN and lesser %DFI and UBI in their frozen semen sired more calves.*
***Conclusion:** Bulls with less fractionated sperm structural and functional defects, ROS and UBI, and more OPN and less UBI protein expression in their frozen sperm had greater fertility. These variations in sperm parameters contributed to observed differences in the fertility.*

***Keywords:** Cross-bred bull; Sperm; DNA; Membrane; Protein; Fertility*

Date of Submission: 14-04-2021 Date of Acceptance: 28-04-2021

I. Introduction

In cattle, extensive fertility data and progeny records have revealed the inability of a subset of high-merit bulls to produce successful full-term pregnancies, despite the acceptable sperm motility and morphology levels of their spermatozoa. This observation underlines the molecular and cellular defects affecting the fertilizing ability of spermatozoa and contribute to subfertility or infertility (Amann and DeJarnette, 2012; Amann *et al*., 2018). Accurate screening of the ejaculates will assist elimination of bulls with a low fertility prior to their entrance to progeny testing program, breeding program or preservation of semen, thus cost effective for bull enterprises.

Sperm abnormalities were classified based on the location of defects (head, tail, mid piece) or site of origin (primary: testis; secondary: epididymis; tertiary: accessory glands / post ejaculation). The significance of specific sperm abnormalities were better understood from the results of mating trials, analysis of non-return rates to artificial insemination (Phillips *et al*., 2004) and in-vitro fertilization (Gadea, 2005; Kipper *et al*., 2017) with semen containing high percentages of sperm with individual classes of abnormalities. There was undoubtedly a correlation between sperm functional and structural parameters, and fertility. Since, determination of sperm morphology by light microscope differed in result from person to person on the same sample, computer aided sperm analysis (Hirai *et al*., 2001) and flow cytometry based evaluation methods are applied in laboratories. Similar approaches could be applied to the selection of bulls.

Functional and structural parameters and functional sperm proteins are correlated to field fertility (Kasimanickam *et al*., 2006; Kasimanickam *et al*., 2012). Functional sperm parameters are reportedly affected by reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and/or hydroxyl radical (•OH). The lipid peroxidation process caused by ROS such as H_2O_2 is the limiting factor of the lifetime of mammalian spermatozoa. The H_2O_2 diffuses through sperm membranes and reacts with transition metals to form a highly reactive hydroxyl radical that damages the sperm structural and functional parameters. Hence the present study was carried out to elucidate the bull to bull variation in sperm structural and functional parameters, functional protein expression and associated fertility of crossbred bulls in Tamil Nadu, India.

II. Material And Methods

The protocol was approved by the Institutional Animal Care and Use Committee at Tamil Nadu Veterinary and Animal Sciences University,

Animals and semen handling

Six crossbred bulls from TANUVAS licensee Unit, Chennai, India (aged 2 to 8 yrs; weight approximately 350 kg) were utilized for the study. All the bulls were apparently healthy with normal reproductive status and maintained under optimum feeding and management conditions. Forty ejaculates from each bulls were collected using artificial vagina (AV). After initial screening for motility and morphology ejaculates were transported to Centralized Embryo Biotechnology Unit and processed within 1 h of collection. Only ejaculates with a minimum of 60% initial motility and 80% morphology were used. The ejaculates were divided into two aliquots and one aliquot was frozen using egg-yolk extender and stored at -196 °C. Randomly selected fresh semen samples initially and corresponding frozen samples at a later time were evaluated.

Sperm viability assay

Sperm viability was determined using propidium Iodide (PI) method as outlined by Graham et al. (1990). Briefly, 10 µl volume of PI was added to 400 µl semen sample suspension (2×10^6/mL) and incubated for 5 min. One mL of sheath fluid was added and filtered through a 40 µm pore size cell strainer to remove any large debris. Flow cytometry analysis was performed using Beckman Coulter (MoFloTMXDP) fitted with a beveled tip. The PI was excited at 488 nm by an argon laser at 100 mW of power. Fluorescence emission was measured with a 515 nm long-pass filter and with a 610 nm long-pass filter for PI detection and the results were analyzed using density plots.

Acrosome integrity assay

Acrosome integrity of the bull sperm was evaluated by previously outlined method by Graham et al. (1990) using fluorescein isothiocynate (FITC) labelled peanut agglutinin (PNA). Briefly, 10 µl of PI and 20 µl FITC labelled PNA were added to the semen samples to give a ratio of 0.5 µg lectin per million cells. One mL of sheath fluid was added and filtered through a 40 µm pore size cell strainer to remove any large debris. Flow cytometric analysis was performed with 560 nm beam-splitting filter and a 525 nm band-pass filter for FITC-PNA detection. The percentage of the acrosome reacted sperms was analyzed from the density plot obtained.

Sperm chromatin structure assay (SCSA)

The SCSA was evaluated by Acridine orange (AO) using previously described (Kasimanickam *et al*., 2006; Mahfouz *et al*., 2009). Briefly, 100 µL containing 1 million spermatozoa was added to 1mL of ice cold PBS (pH 7.4) and centrifuged at 2000 rpm for 5 min. The pellet was re-suspended in ice-cold TNE (0.01 mol/l Tris-HCl, 0.15 mol/l NaCl and 1 mmol/l ethylene diamine tetra acetic acid; EDTA), pH 7.4 and again centrifuged at 2000 rpm for 5 min. The pellet was then re-suspended in 200 µL of ice cold TNE with 10% glycerol and immediately fixed in 70% ethanol for 30 min. The fixed samples were treated for 30 sec with 400 µL of a solution of 0.1 per cent Triton X-100, 0.15 mol/l NaCl and 0.08 N HCl, pH 1.2. After 30 sec., 1.2 ml of staining buffer (6 µg/ml AO, 37 mmol/l citric acid, 126 mmol/l Na2HPO4, 1 mmol/l disodium EDTA, 0.15 mol/NaCl, pH 6.0) was admixed to the tube and filtered through a 40 µm pore size cell strainer to remove any large debris. Flow cytometric analysis was performed with excitation by a 488 nm wavelength light source, AO bound to double-stranded DNA fluoresced green (515-530 nm) and AO bound to single stranded DNA fluoresced red (630 nm or greater). The results were analyzed and interpreted based on the percentage of DNA fragmentation or DNA fragmentation index (DFI).

Reactive oxygen species (ROS) / oxidative stress (OS) assay

Reactive oxygen species (ROS) / oxidative stress (OS) in sperm was assessed by using CellRox ® Deep Red reagent fluorescent probe (2.5 mM, Life Technologies, New York, USA) as per manufacturer's instructions (KorkMaz *et al*., 2017). Briefly, 2.5 mM CellRox® Deep Red reagent fluorescent probe was diluted in dimethyl sulphoxide for a final concentration of 1 mM (working solution) and stored at -20 °C in dark. During use, this working solution was kept in the dark at 37°C. Two hundred µL of semen samples diluted in Sp-TALP (25×10^6 sperm/mL) were added to 0.5 µL of CellRox® and incubated at 37°C for 30 min. After incubation the solution was centrifuged at 2000 rpm for 5 min, the supernatant was removed, and the pellet re-suspended in one mL of sheath fluid, filtered through a 40 µm pore size cell strainer to remove any large debris and the samples were analyzed by flow cytometry to evaluate the oxidative stress. The samples were excited at 640 nm by an argon laser at 100 mW of power. Fluorescence emission was measured with a 655 nm long-pass filter CellRox® detection. The percentage of ROS / OS was assessed using the density plot.

Expression of osteopontin (OPN)

Sperm OPN expression (Souza *et al*., 2008; Erikson *et al*., 2007)was estimated by flow cytometry analysis. Sp-TALP buffer (100 µL) was added to the 10 µL of sperm (100 X 10^6 /mL) pellet. To that, 6 µL of anti-osteopontin antibody was added and incubated at 4 °C for 1 h. Washed with BD wash buffer (BD Biosciences, India) and centrifuged at 1500 rpm for 5 min at 4 °C. Fluorochrome conjugated secondary antibody (anti-mouse) was added 5 µL for OPN and incubated in 4°C for 1 h. Then, the sperm solution was washed twice with BD sperm wash buffer and centrifuged at 1500 rpm for 5 min at 4°C. To the pellet 1mL of sheath fluid was added and the content was filtered with 40 µm pore size cell strainer and the samples were analyzed by flow cytometry. The samples were excited at 488 nm and fluorescence emission was measured at 561 nm (Capkova *et al*., 2016). The percentage of stained and unstained sperms were analysed from the density plot obtained.

Expression of ubiquitin (UBI)

Ubiquitin (UBI) expression in sperm was estimated using the following protocol (Sutovsky *et al*., 2001). Sp-TALP buffer (100 µL) was added to the 10 µL of semen samples (100 X 10^6 per mL). To that, 6 µL of anti-ubiquitin antibody was added and incubated at 4°C for 1 h. Washed twice with BD wash buffer and centrifuging at 1500 rpm for 5 min at 4 °C. Fluorochrome conjugated secondary antibody (anti-mouse) 5 µl was added for UBI and incubated in 4 °C for 1 h. After 1 h, the sperm solution was washed twice with BD wash buffer and centrifuged at 1500 rpm for 5 min at 4 °C. To the pellet, 1 mL of sheath fluid was added and the content was filtered with 40 µm pore size cell strainer and the samples were analyzed by flow cytometry. The samples were excited at 488 nm and fluorescence emission was measured at 561 nm. The percentages of the stained and unstained sperms were analyzed from the density plot obtained.

Artificial insemination with frozen semen

Jersey crossbred cows (n=60) were administered with TRIU-B, a progesterone impregnated device [Virbac Animal Health, Mumbai, India; each device comprises of 3 medicated rings (green) containing 186 mg of each and an additional ring (pink) containing 400mg progesterone] intravaginally on Day 0 day and 500 µg Cloprostenol (Pragma, 2 mL; Intas Pharmaceuticals, Ahmedabad, India) IM on Day 8. TRIU-B removed on Day 9 and artificially

inseminated (10 cows per bull) at a fixed time (FTAI) on Day 11 at 56 h and again on Day 12 at 72 h from device removal. The cows were examined for pregnancy 45 days after second AI by ultrasonography [Esaote, MyLab™ 30 Vet GOLD Platform, 5 MHz linear transducer, Chennai, India].

Statistical analysis

All sperm assay parameters were evaluated for a normal distribution with a Shapiro-Wilk test and asymmetry and kurtosis using the UNIVARIATE procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC) with the NORMAL option. Logarithmic, log(x+1), or angular, arcsin (x/100) transformations were performed when necessary. Parametric variables of sperm assay parameters subjected to analysis of variance using the GLM procedure of SAS, and means were compared with a Tukey test. Variables, bulls (n=6) and semen type (fresh vs. frozen semen) were included. Correlations between fresh and frozen semen and bull performance score and conception rate were analyzed using Pearson Correlation coefficient. For all analyses, P values ≤ 0.05 were considered significant.

III. Result

The sperm viability (%) assessed by plasma membrane integrity varied among bulls ($P<0.01$; Table 1) and varied between fresh and frozen semen ($P<0.01$; Supplementary table 1). Among bulls, the range of % viability varied from 73.0 to 85.5% for fresh semen and from 52.7 to 65.0% for frozen semen. The % acrosome integrity for viable sperm varied among bulls ($P<0.01$; Supplementary table 2) and varied between fresh and frozen semen ($P<0.01$; Supplementary table 2). The range of % acrosome integrity varied from 62.4 to 81.0 % for fresh semen and from 47.2 to 55.6 % for frozen semen. The % HOST varied among bulls ($P<0.01$; Supplementary table 3) and varied between fresh and frozen semen ($P<0.01$; Supplementary table 3). Among bulls, the range of % HOST varied from 70.4 to 83.2 for fresh semen and from 52.5 to 57.3 for frozen semen.

The % DFI varied among bulls ($P<0.01$; Supplementary table 4) and varied between fresh and frozen semen ($P<0.01$; Supplementary table 4). Among bulls, the range of % DFI varied from 14.0 to 21.6 % for fresh semen and from 17.1 to 23.0 % for frozen semen.

The % of sperm under oxidative stress varied among bulls ($P<0.01$; Supplementary table 5) and varied between fresh and frozen semen ($P<0.01$; Supplementary table 5). Among bulls, the range of % sperm under oxidative stress varied from 10.2 to 14.8 % for fresh semen and from 19.8 to 27.4 % for frozen semen. The % of sperm expressed OPN varied among bulls ($P<0.01$; Supplementary table 6) and varied between fresh and frozen semen ($P<0.01$; Supplementary table 6). Among bulls, the range of % sperm expressed OPN varied from 86.2 to 95.1 for fresh semen and from 88.4 to 93.1 for frozen semen. Similarly, the % of sperm expressed UBI varied among bulls ($P<0.01$; Supplementary table 6) and varied between fresh and frozen semen ($P<0.01$; Table 4). Among bulls, the range of % sperm expressed UBI varied from 3.7 to 13.2 for fresh semen and from 4.8 to 13.2 for frozen semen. There was a negative correlation between OPN and UBI expression ($r= -0.92$; $P<0.01$)

Bull performance score (BPS) and conception rate

The mean value of bull performance score (BPS) for fresh and frozen thawed semen is presented in table 1. The BPS was calculated by summing the proportion of all semen parameters (viability, acrosome integrity, 1 - DFI, 1 - OS, HOST and OPN).

For fresh semen, bull number six had the highest BPS of 5.205 followed by bull number two (5.103), one (4.993), three (4.926), four (4.737) and five (4.555). For frozen-thawed semen, bull number six had the highest BPS of 4.946 followed by bull number two (4.860), one (4.738), three (4.670), four (4.511) and five (4.375). The correlation of BPS for fresh and frozen semen was not significant ($r=0.81$; table 1; $P=0.1$).

The average conception rate for bull 6 is 70% and for 5 is 50%. All other bull had an average of 50% CR. The correlation of BPS and conception rate (number of cows pregnant/total number of cows) was positive ($r=0.85$; Table 1; $P<0.05$).

Table 1: Bull performance scores (BPS), and association between fresh and frozen semen BPS and between frozen semen BPS and conception rate.

Bull	Fresh Semen							Frozen thawed semen							Conception rate
	OPN	Viability	Acrosomal Integrity	DFI	ROS	HOST	BPS	OPN	Viability	Acrosomal integrity	DFI	ROS	HOST	BPS	
6	0.951	0.855	0.810	0.140	0.102	0.832	5.205	0.931	0.650	0.556	0.171	0.198	0.573	4.946	70
2	0.946	0.830	0.783	0.157	0.111	0.813	5.103	0.926	0.626	0.551	0.187	0.250	0.570	4.860	60
1	0.915	0.815	0.753	0.167	0.124	0.801	4.993	0.915	0.620	0.542	0.191	0.254	0.548	4.738	60
3	0.904	0.807	0.728	0.171	0.130	0.787	4.926	0.904	0.573	0.486	0.198	0.260	0.530	4.670	60
4	0.894	0.780	0.653	0.196	0.147	0.754	4.737	0.900	0.533	0.478	0.225	0.273	0.528	4.511	60
5	0.862	0.730	0.624	0.216	0.148	0.704	4.555	0.884	0.527	0.472	0.230	0.274	0.525	4.375	50
Mean value of BPS							4.92 ± 0.098	Mean value of BPS						4.68 ± 0.087	
Fresh Vs Frozen thawed semen r = 0.81, *P* = 0.1012 Not significant								Frozen thawed semen Vs Conception rate Correlation co-efficient: r = 0.845, *P* = 0.034 Significant at 5 % level							

OPN, Osteopondin; DFI, Sperm DNA fragmentation index; ROS, Reactive oxygen species; HOST, Hypoosmatic swelling test

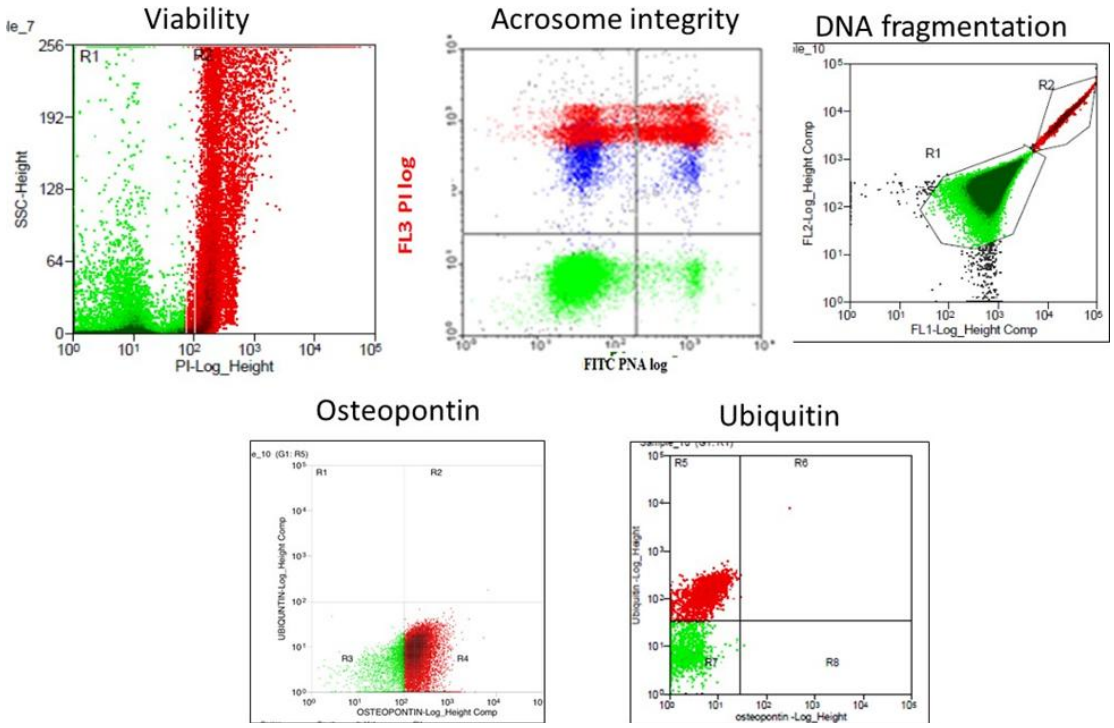

Figure 1. Flow cytometry images of sperm viability, acrosome integrity, %DFI, and expressions of Osteopondin and Ubiquitin.

IV. Discussion

This current study demonstrated huge variation in sperm parameters assessed by flow cytometry and variation in sperm protein expression and fertility. Bulls with less fractionated sperm structural and functional defects and ROS, and more OPN and less UBI protein expression in their sperm had greater fertility.

Garner *et al.* (1986) reported a significant negative correlation with the PI positive sperm and the percentage of motile sperm. They also reported significant negative correlation between PI stained sperm and sperm with intact acrosome. In the present study, flow cytometric analysis revealed viable and acrosomal intact sperms were found to be lower in frozen compared with fresh semen. Nagy *et al*. (2003) reported that

cryopreservation led to acrosomal damage in a sub population of sperm; further those sperms also have disrupted plasma membranes.

Sperm chromatin was a complex of nucleic acids and proteins, histones primarily and condensed during cell division. Any damage to sperm chromatin may impair the capability of the spermatozoa to fertilize, causes impaired embryonic development and decrease IVF or insemination success (Kasimanickam *et al*., 2006; Walters *et al*., 2006). Bochenek et al. (2001) reported 23.8% DNA fragmented spermatozoa in mature bulls in the breeding program. It should be noted that, SCSA using flow cytometry was performed on six crossbred bulls that were already qualified as stud bulls in the present study. Highly significant difference between crossbred bulls in the % DFI was noted.

Membranes of bull spermatozoa are rich in unsaturated fatty acids and therefore very sensitive to oxygen induced damages. Oxidation of the lipids plays a crucial rule in the function of spermatozoa. Mild peroxidation of the membrane lipids is under normal conditions necessary for capacitation of spermatozoa, which is an important prerequisite for the acrosome reaction and successful fertilization (Kodama *et al*., 1996 and Agarwal and Allamanoni, 2004). When there is an imbalance between ROS and anti-oxidants, attack on sperm membranes and DNA by oxidant results in lipid peroxidation, negatively affecting sperm quality consequently affecting fertility (Brouwers and Gadella, 2003; Kasimanickam *et al*., 2007). It has been shown that in bulls with increased ROS and lipid peroxidation in the semen, there is increased incidence of premature sperm membrane destabilization and sperm DNA fragmentation and subsequently suffers with reduced calving rate.

In the present study, the sperm functional parameters and functional proteins studied were greater in fresh semen compared with frozen semen. It should be noted that this fresh semen reflects natural service fertility of bulls, compared to frozen semen reflects the fertility of the semen after processing, dilution, freezing and thawing (Van Doormaal, 1993). In general, spermatozoa that survive handling appear to have similar surface properties to ejaculated spermatozoa. However, handling and cryopreservation may cause changes in sperm surface properties (Leahy and Gadella, 2011). This can include either one or more of the following: phase separation of lipids in the sperm plasma membrane, acrosome loss, lethal ROS production and apoptotic- like changes such as extended phospholipid scrambling and de-polarization of the inner mitochondrial membrane and concomitant lipid peroxidation and DNA damage. Moreover, at the tail, these changes also result in ROS induced tyrosine phosphorylation (which usually activates hyperactivated motility) despite the fact that the spermatozoa are immotile. The end result is plasma membrane damage, the release of protein and vesicles from the sperm membrane and cell deterioration.

In the current study, variation in OPN and UBI were observed. Frozen semen showed less OPN and more UBI. In the current study, bulls with greater fertility had a positive correlation with amount of OPN and negative association with the amount of UBI. More OPN and less UBI had higher fertility success. Moura *et al*. (2006) reported greater OPN in the accessory sex gland fluid of high fertile bulls than in low fertile bulls. Bulls with the high fertility scores had 2.3 times more OPN than bulls with above average fertility and at least four times more than bulls with below average fertility. Further, Cancel *et al*. (1997) reported that based on protein density, OPN was positively correlated (0.48) with fertility.

Ubiquitination was an apoptotic mechanism in which DNA defective sperm were labeled by inserting a small protein (ubiquitin) into the plasma membrane. Kuster *et al*. (2004) found that sperm ubiquitination correlated with primary and total morphological abnormalities in bulls. In addition, several studies (Sutovsky *et al*., 2002; Sutovsky, 2003 and Kuster et al., 2004) reported that semen samples containing large percentages of ubiquitinated sperm were indicative of poor-quality sample and correlated negatively with fertility.

In the current study we associated sperm parameters and fertility with different approach. We calculated bull performance score including all important sperm assay parameters and then correlated bull performance score with conception rate. Sperm possess multicompartment cell with several organelles and each organelle is important for its function towards successful fertilization and early embryonic development. Thus, it is important to include assays determining all organelles' potential when evaluating an ejaculate for its fertility potential. We acknowledge the study's limitation on the sample size for conception rate calculation.

V. Conclusion

This current study demonstrated variation in sperm parameters assessed by flow cytometry and sperm protein expressions in cross-bred bulls. These variations contributed to observed differences in the fertility. Bulls with less fractionated sperm structural and functional defects and ROS, and more OPN and less UBI protein expression in their sperm had greater (linear association) fertility.

Acknowledgements

The authors thank the Dean, Madras Veterinary College and the Project Director, Translation Research in Veterinary Products and Biologicals, Madhavaram Milk Colony, Chennai – 600 051, India, for the facilities extended to carry out this research.

References

[1]. Amann, R.P. and DeJarnette, J.M. 2012. Impact of genomic selection of AI dairy sires on their likely utilization and methods to estimate fertility: a paradigm shift. Theriogenology. 77:795-817.
[2]. Amann, R.P., Saacke, R.G., Barbato, G.F., Waberski, D. 2018. Measuring male-to-male differences in fertility or effects of semen treatments. Annu Rev Anim Biosci. 6:255-286.
[3]. Bochenek, M. and Smorag Z. 2001. Sperm chromatin structure assay of bulls qualified for artificial insemination. Theriogenology. 56:557-567.
[4]. Erikson, D.W., Way, A.L., Chapman, D.A. and Killian G.J. 2007. Detection of osteopontin on Holstein bull spermatozoa, in cauda epididymal fluid and testis homogenates, and its potential role in bovine fertilization. Reproduction. 133:909-917.
[5]. Gadea, J. 2005. Sperm factors related to in vitro and in vivo porcine fertility. Theriogenology. 63:431-44.
[6]. Graham, J.K., Kunze, E. and Hammerstedt, R.H. 1990. Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. Biol Reprod. 43:55-64.
[7]. Hirai, M., Boersma, A., Hoeflich, A., Wolf, E., Foll, J., Aumüller, T.R. and Braun, J. 2001. Objectively measured sperm motility and sperm head morphometry in boars (Sus scrofa): relation to fertility and seminal plasma growth factors. J Androl. 22:104-110.
[8]. Kasimanickam, R., Kasimanickam, V., Thatcher, C.D., Nebel R.L. and Cassell, B.G. 2007. Relationships among lipid peroxidation, glutathione peroxidase, superoxide dismutase, sperm parameters, and competitive index in dairy bulls. Theriogenology. 67:1004-1012.
[9]. Kasimanickam, R., Nebel, R.L., Peeler, I.D., Silvia, W.L., Wolf, K.T., McAllister, A.J and Cassell, B.G. 2006. Breed differences in competitive indices of Holstein and Jersey bulls and their association with sperm DNA fragmentation index and plasma membrane integrity. Theriogenology. 66:1307-1315.
[10]. Kasimanickam, R., Pelzer, K.D., Kasimanickam. V., Swecker, W.S. annd Thatcher, C.D. 2006. Association of classical semen parameters, sperm DNA fragmentation index, lipid peroxidation and antioxidant enzymatic activity of semen in ram-lambs. Theriogenology. 65:1407-21.
[11]. Kasimanickam, V., Kasimanickam, R., Arangasamy, A., Saberivand, A., Stevenson, J.S. and Kastelic, J.P. 2012. Association between mRNA abundance of functional sperm function proteins and fertility of Holstein bulls. Theriogenology. 78:2007-2019.
[12]. Kipper, B.H., Trevizan, J.T., Carreira, J.T., Carvalho, I.R., Mingoti, G.Z., Beletti, M.E., Perri, S.H.V., Franciscato, D.A., Pierucci, J.C. and Koivisto, M.B. 2017. Sperm morphometry and chromatin condensation in Nelore bulls of different ages and their effects on IVF. Theriogenology. 87:154-160.
[13]. Korkmaz, F., Malama, E., Siuda, M., Leiding, C., Bollwein, H. 2017. Effects of sodium pyruvate on viability, synthesis of reactive oxygen species, lipid peroxidation and DNA integrity of cryopreserved bovine sperm. Anim Reprod Sci. 185:18-27.
[14]. Mahfouz, R.Z., Sharma, R.K., Said, T.M., Erenpreiss, J. and Agarwal, A. 2009. Association of sperm apoptosis and DNA ploidy with sperm chromatin quality in human spermatozoa. Fertil Steril. 91:1110-1118.
[15]. Phillips, N.J., Mcgowan, M.R., Johnston, S.D. and Mayer, D.G. 2004. Relationship between thirty post-thaw spermatozoal characteristics and the field fertility of 11 high-use Australian dairy AI sires. Anim Reprod Sci. 81:47-61.
[16]. Souza, C.E., Moura, A.A., Monaco, E. and Killian, G.J. 2008. Binding patterns of bovine seminal plasma proteins A1/A2, 30 kDa and osteopontin on ejaculated sperm before and after incubation with isthmic and ampullary oviductal fluid. Anim Reprod Sci. 105:72-89.
[17]. Sutovsky, P., Terada, Y. and Schatten, G. 2001. Ubiquitin-based sperm assay for the diagnosis of male factor infertility. Hum Reprod. 16:250-258.
[18]. Walters, A.H., Saacke, R.G., Pearson, R.E. and Gwazdauskas, F.C. 2006. Assessment of pronuclear formation following in vitro fertilization with bovine spermatozoa obtained after thermal insulation of the testis. Theriogenology. 65:1016-1028.
[19]. Leahy, T. and Gadella, B.M. 2011. Sperm surface changes and physiological consequences induced by sperm handling and storage. Reproduction. 142:759.
[20]. Van Doormaal B.J. 1993. Linear evaluations of non-return rates for dairy and beef bulls in Canadian AI. Can J Anim Sci. 73:795-804

S. Rangasamy, et. al. "Association of fertility and in-vitro analysis of sperm structural and functional parameters, reactive oxygen species, oesteopontin and ubiquitin of crossbred bulls in India." *IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS)*, 14(4), 2021, pp. 55-61.