Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2020; 8(6): 312-321

Received: 07-09-2020
Accepted: 09-10-2020

Palanisammi A
Dean, Veterinary College and Research Institute, Tirunelveli, Tamil Nadu, India

V Rajaram
Research Student, Veterinary College and Research Institute, Tirunelveli, Tamil Nadu, India

S Rangasamy
Assistant Professor, Department of Veterinary Gynecology and Obstetrics, Madras Veterinary College, Chennai, Tamil Nadu, India

SN Sivaselvam
Dean (Retd.), Madras Veterinary College, Chennai, Tamil Nadu, India

K Kumanan
Professor and Head (Retd.), Bioinformatics and ARIS cell, Madras Veterinary College, Chennai, Tamil Nadu, India

Corresponding Author:
Palanisammi A
Dean, Veterinary College and Research Institute, Tirunelveli, Tamil Nadu, India

Reciprocity of sperm fertility biomarker using flow cytometry and *in vitro* production of bovine embryos: An inquest

Palanisammi A, V Rajaram, S Rangasamy, SN Sivaselvam and K Kumanan

Abstract
The present study involves exploring the utility of osteopontin as a fertility biomarker and also to study its influence on *in vitro* fertilization. In this study, semen from six Jersey crossbred bulls were collected and sperm parameters like motility, viability, abnormality, plasma membrane integrity, acrosomal integrity, sperm chromatin structure assay and oxidative stress were studied by flow cytometry. There was a significant difference ($P<0.01$) between the bulls in expression of osteopontin. These results were seen in *in vitro* fertilization competency determined by the cleavage rate. Pearson coefficient of correlation for the osteopontin expression with the cleavage rate was found to be 0.97 indicating a very high correlation. A bull performance score (BPS) was calculated for the results obtained with the various parameters such as sperm abnormality, viability, HOST, acrosomal integrity, inverse of DFI, inverse of ROS and osteopontin expression. Coefficient of correlation for the BPS with the cleavage rate was high(r=0.969). These results indicated that osteopontin can be used as a fertility biomarker and can also serve as a potential tool for screening the semen quality by flow cytometry before insemination.

Keywords: biomarker, flow cytometry, osteopontin, semen parameters

Introduction
Scientists have strived hard to develop laboratory assays to accurately predict the fertility potential of a particular male (Amann and Hammerstedt, 1993) [5]. The most accurate method for testing the bull fertility is the insemination of many fertile females, which are time consuming, expensive for routine use and only allows a limited number of bulls to be tested at any given time (Barth and Oko, 1989) [8]. Unfortunately, current laboratory assays do not accurately predict the fertility of bulls and are irreplicable from one study to another (Graham *et al.*, 1980). Therefore, the specialized sperm function tests are better predictors of fertilizing potential of bulls than traditional semen parameters which are assessed by standard semen analysis.

Osteopontin (OPN) a 55 kDa protein, shown to be more prevalent in higher-fertility bulls was determined that are secreted by ampulla and seminal vesicle (Cancel *et al*., 1997) [13]. Bulls with the highest fertility scores had 2.3 times more of osteopontin than bulls with above average fertility and at least four times more than bulls with below average (Moura *et al*., 2006) [35]. Osteopontin prevents the attachment of bacteria to the epithelium by competing for the integrin-binding domains that are present on the epithelial cells. In bulls, OPN may protect the epithelial cells from bacterial infections in the accessory sex glands since the most prevalent pathogenic condition of the accessory sex glands is seminal vesiculitis, which is caused by *Actinomyces pyogenes*, *Hemophilus somnus* and *Escherichia coli* that negatively affect the semen quality (Cavalieri and Van Camp, 1997) [15]. In this context, the present study has been taken up to characterize selective fertility biomarker (osteopontin) in bull semen by flow cytometry and to study the influence of the fertility biomarker on *in vitro* embryo production.

Materials and Methods
Collection of semen samples
Jersey crossbred bulls were maintained at uniform managerial conditions in license unit, Madhavaram Milk Colony, Chennai-51. Semen from six Jersey crossbred bulls were collected

by artificial vagina and the collected semen samples were covered with aluminium foil and kept in the beaker containing water at 37 ºC and transported to Centralized Embryo Biotechnology Unit and Translational Research Platform for Veterinary Bilogicals (TANUVAS), Madhavaram Milk Colony for further analysis.

Sperm preparation

The sperm and seminal plasma were separated immediately after collection by diluting ten times with TALP medium (Sigma Aldrich, USA) and washing twice by centrifugation (1200 rpm for 5 min) to collect the sperm pellet. The sperm pellet was resuspended at 100 × 10^6 cells/ml as per protocol mentioned in Graham *et al*. (1990) [22].

Basic semen evaluation

Sperm motility, abnormality and Hypo-osmotic swelling test (HOST) were determined by procedures explained by Bansal and Bilaspuri (2008) [7], Enciso *et al*., (2011) [16] and Zubair *et al*., (2013) [47] respectively.

Evaluation of sperm for viability by flow cytometry

Sperm viability was determined by propidium iodide (PI) (Sigma Aldrich, USA). A 10 µl volume of PI was added to 400 µl sperm suspension and the sample was incubated for five min. one ml of sheath fluid was added and the contents were filtered through a 40 µm pore size cell strainer (Merck, USA) to remove the large debris and the cells were analysed by flow cytometry to evaluate the percentage of the dead cells. Flow cytometer analysis was performed by using Beckman Coulter fitted with a bevelled tip. The PI was excited at 488nm by an argon laser at 100mW of power. Fluorescence emission was measured with a 515 nm long-pass filter and with a 610 long-pass filter for PI detection (Graham *et al*., 1990) [22].

Evaluation of acrosomal integrity by flow cytometry

Acrosomal integrity of the bull sperm was evaluated by fluorescein isothiocynate (FITC) – labelled with peanut agglutinin (PNA) (Sigma Aldrich, USA) and assayed by flow cytometry to assess the percentage of cells without an intact acrosome. A 10 µl of PI and 20 µl FITC labelled with PNA (Sigma Aldrich, USA) were added to the sperm samples in the ratio of 0.5 µglectin per one million cells. One ml of sheath fluid was added and the contents were filtered through a 40 µm pore size cell strainer (Himedia, USA) to remove the large debris and the cells were analysed by flow cytometry to evaluate the acrosomal damage along with the percentage of the dead cells. Flow cytometric analysis was performed with 560nm beam-splitting filter and a 525nm band-pass filter for FITC-PNA (Sigma Aldrich, USA) detection. Within the population, a sub-population of AR cells bound PNA, resulting in a sharp fluorescent emission in forward scatter.

Sperm chromatin structure assay (SCSA) by flow cytometry

Sperm chromatin structure assay was evaluated by acridine orange (AO) (Sigma Aldrich, USA) that has metachromatic properties of intercalates into double-stranded DNA as a monomer and binds to single-stranded DNA as an aggregate. 100 µl containing one million spermatozoa are suspended in one ml of ice cold PBS (Sigma Aldrich, USA) (pH 7.4) and centrifuged at 600 g for five min. The pellet is resuspended in ice-cold TNE (0.01 mol/LTris-HCl (Sigma Aldrich, USA), 0.15 mol/LNaCl (Sigma Aldrich, USA) and one mmol/Lethylenediaminetetra acetic acid [EDTA] (Sigma Aldrich, USA), pH 7.4) and again centrifuged at 600 g for five min. The pellet was then resuspended in 200 µl of ice cold TNE with 10 per cent glycerol and immediately fixed with 70 per cent ethanol for 30 min. The fixed samples were treated with 400 µl of a solution of 0.1 per cent Triton X-100 (Sigma Aldrich, USA), 0.15 mol/l NaCl and 0.08N HCL, pH 1.2 for 30 sec. After 30 sec, 1.2 ml of staining buffer (6µg/ml AO) was mixed in the tube and the contents were filtered through a 40 µm pore size cell strainer (Himedia, USA) to remove any large debris and the cells were analysed by flow cytometry. After excitation by 488 nm wavelength light source, AO bound to double-stranded DNA fluoresces green (515-530 nm) and the AO bound to single stranded DNA fluoresces red (630nm or greater) (Alessandra *et al*., 2010) [3].

Evaluation of oxidative stress by flow cytometry

Two hundred microliters of semen samples diluted in TALP media (Sigma Aldrich, USA) (25 × 106 sperm /ml) were added to 0.5 µl of CELLROX (Thermo Fisher, USA) in one mM in DMSO (Sigma Aldrich, USA) and incubated for 30 min at 37ºC. After incubation, the solution was centrifuged for five min at 2000g and the supernatant was removed. The pellet was resuspended in one ml of sheath fluid and the contents were filtered through a 40 µm pore size cell strainer (Himedia, USA) to remove any large debris and the cells were analysed by flow cytometry to evaluate the oxidative stress (Alves *et al*., 2015) [4].

Oocyte grading and selection

Oocytes derived through ultrasound guided transvaginal follicular aspiration (TVFA) and ovum pick up (OPU) in Jersey crossbred cows (n=24), between three to eight years of age which were maintained at license unit Madhavaram Milk Colony.

Class I (Grade A)	:	More than four layers of compact cumulus cells
Class II (Grade B)	:	Three to four layers of compact cumulus cells
Class III (Grade C)	:	One to two layers of cumulus cells
Class IV (Grade D)	:	Denuded oocytes
Only COCs of grades A, B and C were utilized for *in vitro* maturation.		

Evaluation of developmental competence of OPU oocytes

***In vitro* maturation**

On the day of use, the already prepared TCM-199 medium was supplemented with 10 per cent FBS (Gibco, USA), one µg/ml estradiol-17β (Sigma Aldrich, USA), gonadotropins (FSH- 1 µg/ml, LH- 10 µg/ml-Sigma Aldrich, USA) and pencillin-streptomycin (Sigma Aldrich, USA) in which 50 µl droplets were laid under mineral oil in 35mm sterile polystyrene culture dish (Nunc, USA) and equilibrated in the culture environment for two to three h in the CO_2 incubator at 38.3 °C, 95 per cent relative humidity and five per cent CO_2 and five per cent O_2 for maturation. The immature oocytes were washed thrice in maturation medium and eight to 10 COCs were placed in 50µl droplets of maturation medium for

24 h *in vitro* culture.

***In vitro* fertilization**

Matured oocytes were removed from IVM droplets and were washed three to five times in IVF medium. Eight to 10 matured oocytes were transferred to 50 µl of fertilization droplets and inseminated with 2 x 10^6 million sperm per ml. The oocytes and sperms were co-incubated for 18-24 h at 38.3 ^{0}C under five per cent CO_2, five per cent O_2 and 95 per cent RH.

***In vitro* culture of embryos**

The presumptive zygotes were denuded of their cumulus cells and surrounding sperms by vortex agitation after 15 to 18h of fertilization and they were washed thrice in culture medium. Prior to use, the culture medium and wash media dishes were pre-equilibrated for 2h CO2 incubator. The denuded zygotes were then transferred to 50 µl droplets of culture medium and co-cultured with OEC followed by the cleavage which was assessed at 24 - 48hr of *in vitro* culture.

Cumulus cell expansion

Oocytes were observed after 24 h of *in vitro* culture under stereozoom microscope for assessment of cumulus cell expansion and extrusion of first polar body. Mucification and loosening of the cumulus layers were also considered to have undergone cumulus expansion. Cumulus expansion was scored as per the method of Sreenivas *et al.* (2013) [42],

Degree 2	:	Cumulus cells were homogenously spread and clustered cells were no longer present (Full cumulus cell expansion).
Degree 1	:	Cumulus cells were slightly expanded and clustered cells were still observed (Moderate cumulus cell expansion).
Degree 0	:	No morphological change compared with fresh COCs (Slight or no expansion).

Assessment of cleavage rate

Percentage of total embryos produced *in vitro* under each developmental stage was recorded and were assessed for quality and viability. The cleavage rate was assessed after formation of two cell embryos.

Assessment of viability

3', 6'- diacetylfluorescein (FDA) staining

A stock solution of 5 mg /ml FDA (Sigma Aldrich, USA) of acetone was prepared and stored at -20 ºC. On the day of use 0.5 µl of stock was diluted with 1 ml of DPBS containing 4 mg/ml BSA (Sigma Aldrich, USA) and used immediately (final concentration 2.5 µg FDA/ml). The embryos to be stained were washed thrice in DPBS and incubated in DPBS (Sigma Aldrich, USA) containing FDA for exactly 1 min and washed again in DPBS without FDA for at least 1 minute to remove intercellular FDA. The embryos were subsequently exposed to UV light to observe the fluorescence. The embryos which fluoresced brightly were considered as live indicating intact cell membrane permeability and an integrity of the intracytoplasmic esterase enzyme activity. Embryos with no fluorescence were considered as dead. These were subjected for DAPI (Sigma Aldrich, USA) staining to assess the number of (dead blastomeres) fluorescent nuclei. (Noto *et al.*, 1991) [36]

4', 6'- diamidino-2-phenylindole (DAPI) staining

Embryos were washed twice in PBS (Sigma Aldrich, USA), stained with 0.1 µg/ml 4', 6'- diamidino-2-phenylindole (DAPI) (Sigma Aldrich, USA) in PBS for 15 min (Beker *et al.*, 2002) [10]. The slides were mounted with a mixture of vaseline and paraffin wax (9:1 w/w) which was used to support the coverslip in contact with oocytes without excessive pressure. Fluorescence microscope was used to evaluate the nuclear status. DAPI's principle was based on fluorescent dye exclusion in which the nuclei of living blastomere will not take up the stain and instead the nuclei of non-viable embryos or of dead blastomeres fluoresce after incubation with DAPI. This fluorescence was characterized by the blue coloration of the nuclei upon exposure to UV light while the cytoplasm of the blastomere did not fluoresce.

Statistical analysis

Statistical analysis of the data with Chi square test and Karl Pearson correlation coefficient analysis were done in this study. The statistical analysis was performed by IBM® SPSS® version 20.0 for windows, a statistical package for social sciences.

Results

The study involved semen from six Jersey crossbred bulls were collected by artificial vagina. The mean volume of semen samples ranged from 3.85±0.08 ml to 4.73±0.08 ml. The colour and consistency of the semen samples was creamy white and thick (Bull No: 1, 2, 3 and 5) and thin (Bull No: 4 and 6). The percentage of motility for Bull No: 3 was 80 and Bull No: 1, 5, 2, 4 and 6 were 75, 75, 70, 60 and 60 per cent respectively.

The percentage of normal and abnormal sperm count ranged from 80.08 to 95.50 and 4.50 to 19.92 respectively. Chi-square test showed that there was a highly significant difference between bulls with regard to normal and abnormal sperm count.

The percentage of HOST reacted sperms was 78.00, 75.00, 73.00, 66.83, 60.50 and 18.33 for Bull No: 1, 5, 2, 6, 4 and 3 respectively. The percentage of HOST not reacted was 39.50, 33.17, 27.00, 25.00, 22.00 and 18.67 was Bull No: 4, 6, 2, 5, 1 and 3 respectively. Chi-square test revealed that there was a highly significant difference between bulls with regard to hypo-osmotic swelling test.

The percentage of osteopontin expressed in sperm was higher in Bull No: 3 (92.69) followed by Bull No: 1 (91.45), Bull No: 5 (85.54), Bull No: 2 (70.48), Bull No: 6 (68.67) and Bull No: 4 (67.18). Statistical analysis revealed that there was a highly significant difference between bulls with respect to expression of osteopontin in bull semen.

Osteopontin expression by flow cytometer

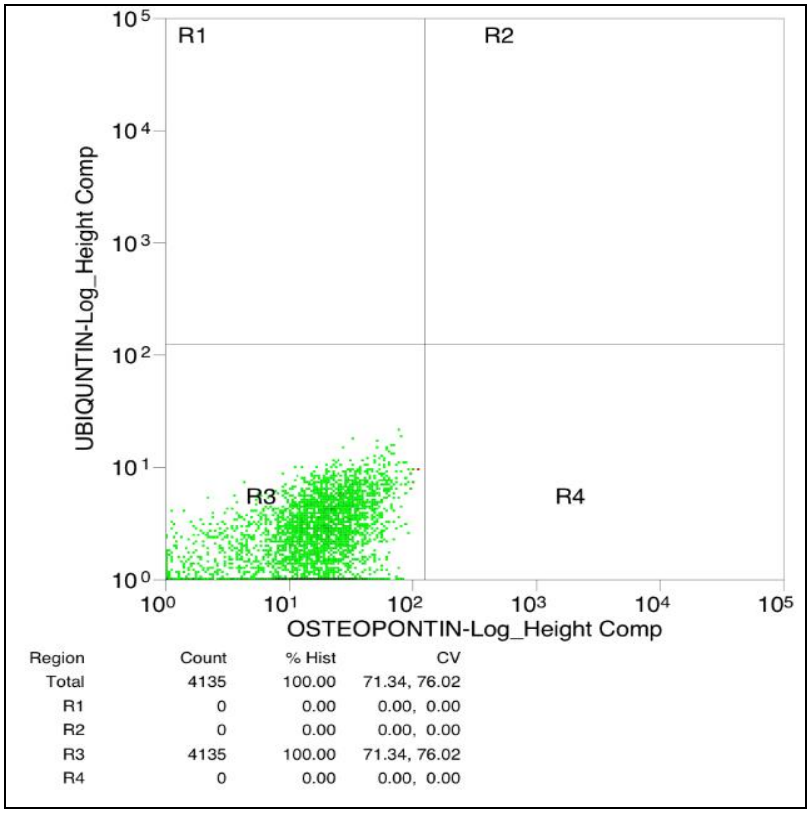


Fig 1: Unstained semen

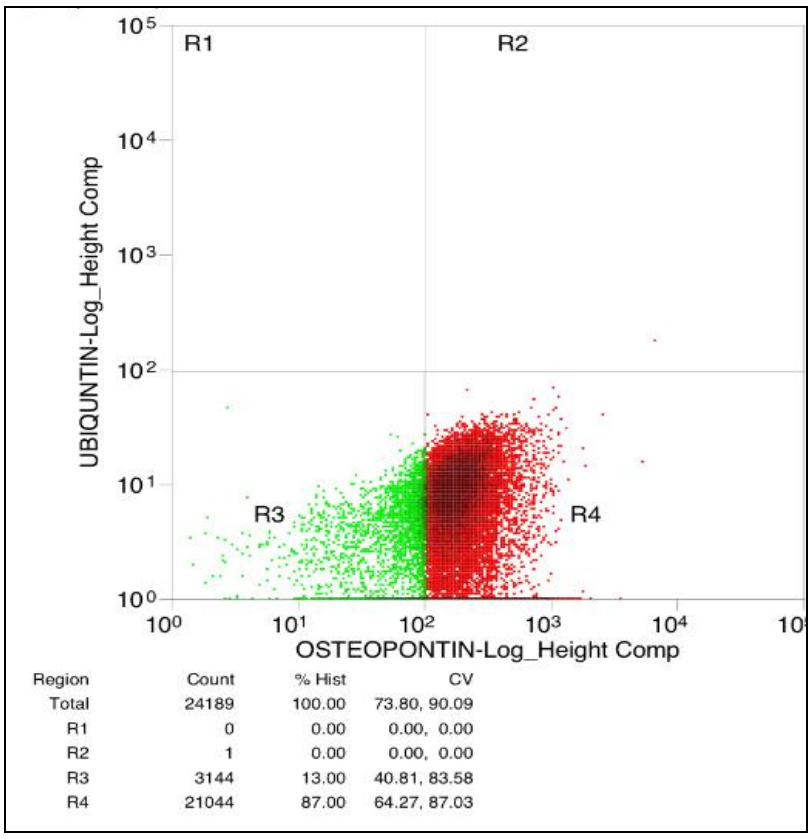
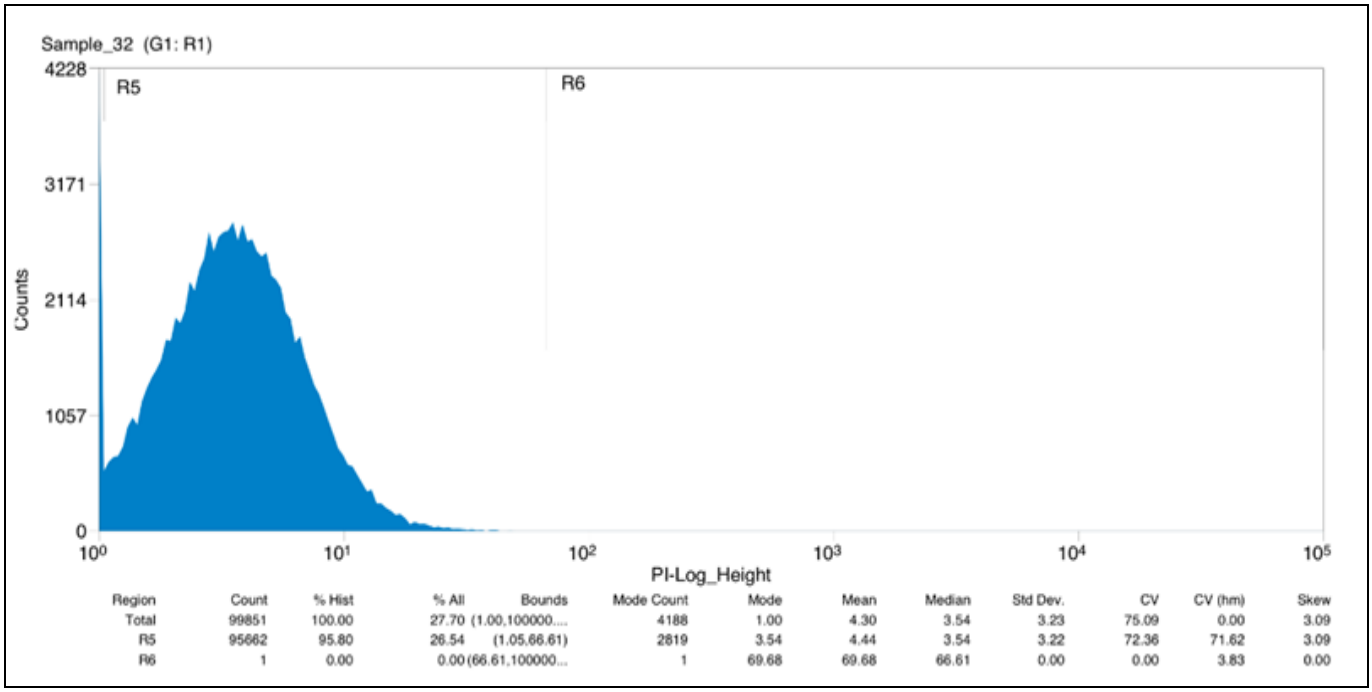


Fig 2: Stained semen showing osteopontin expressed sperms in R4

The percentage of live sperm was 82.89, 82.18, 80.26, 79.43, 75.20 and 70.73 for Bull No: 3, 1, 5, 2, 6 and 4 respectively. The dead sperm percentage was 29.27, 24.80, 20.57, 19.74, 17.82 and 17.11 for Bull No: 4, 6, 2, 5, 1 and 3 respectively.

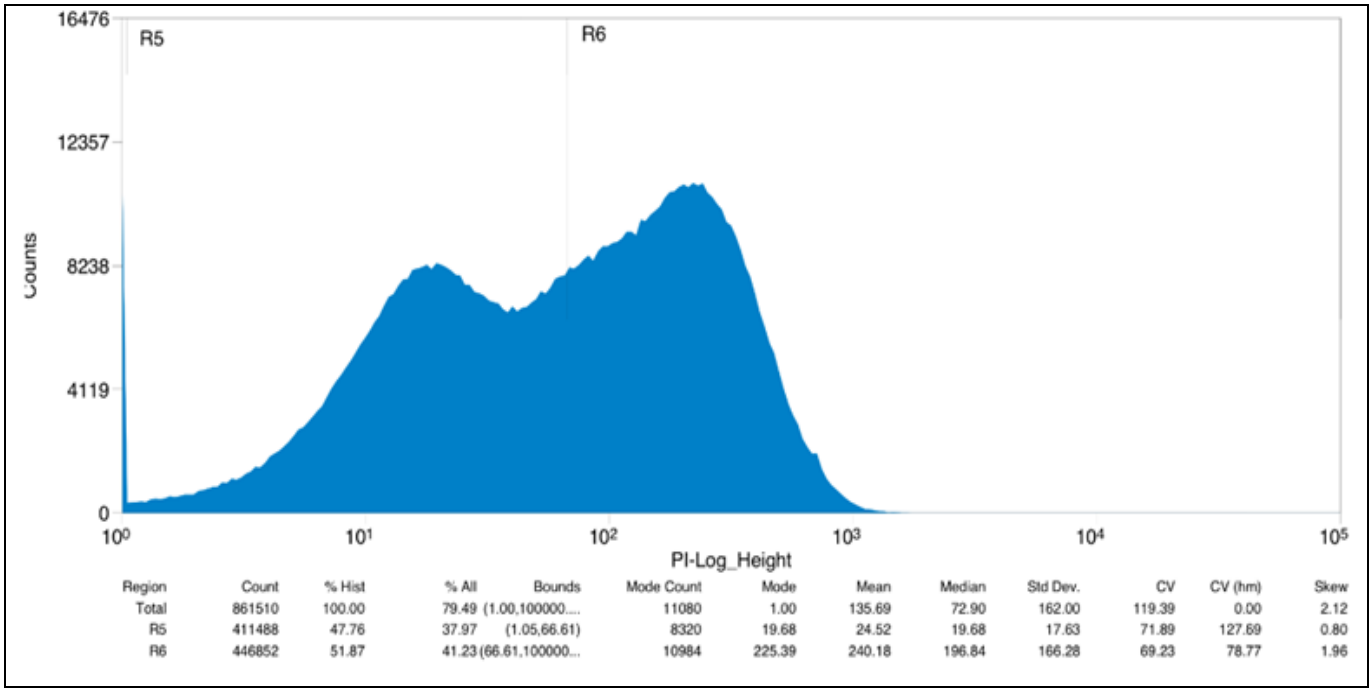
The percentage of viable sperm was 82.28, 81.83, 79.68, 79.15, 75.12 and 69.34 for Bull No: 3, 1, 2, 5, 6 and 4 respectively and non-viable sperm was 30.66, 24.88, 20.85, 20.32, 18.17 and 17.72 for Bull No: 4, 6, 5, 2, 1 and 3

respectively. The percentage of viable intact sperm was 78.48, 75.28, 72.05, 70.38, 65.45 and 58.55 for Bull No: 3, 1, 5, 2, 6 and 4 and viable damage sperm was 10.80, 9.68, 9.29, 7.10, 6.40 and 3.80 for Bull No: 4, 6, 2, 5, 1 and 3 respectively. The percentage of viable damage and non-viable sperm was 41.45, 34.44, 29.62, 27.94, 24.57 and 21.52 respectively. Chi square test revealed that there was a highly significant difference between bulls.



Region	Count	% Hist	% All	Bounds	Mode Count	Mode	Mean	Median	Std Dev.	CV	CV (hm)	Skew
Total	99851	100.00	27.70	(1.00,100000....	4188	1.00	4.30	3.54	3.23	75.09	0.00	3.09
R5	95662	95.80	26.54	(1.05,66.61)	2819	3.54	4.44	3.54	3.22	72.36	71.62	3.09
R6	1	0.00	0.00	(66.61,100000...	1	69.68	69.68	66.61	0.00	0.00	3.83	0.00

Fig 3a: Unstained semen



Region	Count	% Hist	% All	Bounds	Mode Count	Mode	Mean	Median	Std Dev.	CV	CV (hm)	Skew
Total	861510	100.00	79.49	(1.00,100000....	11080	1.00	135.69	72.90	162.00	119.39	0.00	2.12
R5	411488	47.76	37.97	(1.05,66.61)	8320	19.68	24.52	19.68	17.63	71.89	127.69	0.80
R6	446852	51.87	41.23	(66.61,100000...	10984	225.39	240.18	196.84	166.28	69.23	78.77	1.96

Fig 3b: Stained semen

Fig 3: Sperm viability analysis using propidium iodide

The percentage of double stranded DNA was 87.33, 86.42, 80.63, 79.20, 75.45 and 68.50 for Bull No: 3, 1, 5, 2, 6 and 4 respectively and single stranded DNA was 31.50, 24.55, 20.80, 19.37, 13.58 and 12.67 for Bull No: 4, 6, 2, 5, 1 and 3 respectively. The percentage of DFI was 31.50, 24.55, 20.80, 19.37, 13.58 and 12.67 for Bull No: 4, 6, 2, 5, 1 and 3 respectively. Chi-square test revealed that there was a highly significant difference between bulls with regard to SCSA.

The percentage of ROS reacted to sperm was 16.47, 15.14, 14.13, 12.53, 11.46 and 10.42 for Bull No: 4, 6, 2, 5, 1 and 3 respectively and ROS not reacted sperm was 89.58, 88.5, 87.47, 85.87, 84.86 and 83.53 respectively. Chi-square test revealed that there was a highly significant difference between bulls with regard to oxidative stress.

Twenty four OPU sessions were carried out in 12 cows. A total of 202 follicles were observed by ultrasonography. Out of 202 follicles, 135 follicles were aspirated and 108 oocytes were retrieved. The percentage of oocyte recovery rate was

80.00. Out of 108 oocytes, 30, 39, 32 and 7 oocytes were graded as A, B, C and D respectively.

Out of 101 oocytes which were cultured under *in vitro* condition, 73.27 oocytes showed degree 2 cumulus expansion, 15.84 oocytes showed degree 1 cumulus expansion and 10.89 oocytes showed degree 0 maturation.

The percentage of cleavage rate was 53.33, 46.67, 46.67, 26.67, 20.00 and 13.33 for Bull No: 3, 1, 5, 2, 6 and 4 respectively.

Bovine performance score (BPS) was calculated by summing up the proportion of all the semen parameters like viability, acrosomal integrity, inverse of DFI, inverse of ROS, HOST and expression of osteopontin. Among all the semen sample of bulls, Bull No: 3 had the highest score of 6.079 followed by Bull No: 1 (5.95), 5 (5.67), 2 (5.43), 6 (5.18) and 4 (4.89). It was evident that the bovine performance score had high correlation with the cleavage rate (r=0.969).

The present study also assessed the influence of osteopontin expression on cleavage rate. From the results, it was revealed that the percentage of osteopontin expression was 92.67, 91.45, 85.54, 70.48, 68.67 and 67.18 for Bull No: 3, 1, 5, 2, 6 and 4 respectively and also high correlation with cleavage rate (r=0.97) was 53.33, 46.67, 46.67, 26.67, 20.00 and 13.33 for Bull No: 3, 1, 5, 2, 6 and 4 respectively.

Discussion

The present study has been conducted to study the influence of OPN on *in vitro* sperm characteristics of Jersey crossbred bull semen. Hence the role of fertility protein on fertilization and cleavage rate correlating with other semen parameters has been discussed below for better understanding.

Macroscopical and microscopical evaluation of fresh semen

The present study revealed that the volume, colour, consistency and gross motility showed significant role in fertilization. The finding of this study is in accordance with the observation of Laing *et al.* (1988) [29] who had reported that a bull with high fertility produced greater semen volume than that of lower fertility bull. Thus, volume of an ejaculate may be a good indicator of fertility.

In the present study, the percentage of motility ranged from 60 to 80. These finding are similar to Mortimer (2000) [34] who stated that motility was one of the most important factor of fertile spermatozoa. It was the first, and continues to be the most widely used indicator of sperm function. Sperm motility is an important attribute, because it is readily identifiable and reflects several structural and functional competence, as well as essential aspects of spermatozoa metabolism. Pena Martinez (2004) [39] reported that sperm motility was expressed as the percentage of total motile or progressively motile spermatozoa. Light microscopic evaluation of sperm is a simple, inexpensive, accurate and rapid method for assessment of sperm quality.

Moura*et al.* (2006) [35] had stated that spermatozoa displaying increased OPN gene expression had high motility than spermatozoa with lower gene expression. This could be due to the fact that OPN increases intracellular calcium and thereby increases sperm motility. On contrary, Hall (1981) [24] stated that the main disadvantage with the evaluation of motility was that it was not an accurate measure of the fertility of the sperm. Many semen samples which were highly motile failed to produce successful conception and the sperm motility was not always correlated with the fertility.

Sperm evaluation for abnormalities

In this study, the percentage of normal sperm count ranged from 80.08 to 95.50 and abnormal sperm count ranged from 4.50 to 19.92 as have been reported earlier (Hallap *et al*., 2004) [25]. They observed that 80 per cent of spermatozoa were morphologically normal in bulls. In fertile bulls, the incidence of abnormal morphology of spermatozoa was found to be 10 to 18 per cent. The role of morphologically normal sperm in fertility has been widely accepted. Normal sperm morphology may be an indicator of the fertility potential of a given male (Esteso *et al*., 2006) [18]. The association of increased morphological abnormalities of spermatozoa with reduced reproductive efficiency has been reported in bulls (Walters *et al*., 2005) [45]. In bulls, higher proportion of abnormal spermatozoa could be of genetically heritable character (Hafez, 1987) [23]. Morphometric analysis of sperm heads has been shown to be an indicator of *in vitro* fertility (Kruger *et al.*, 1993) [28]. These morphological abnormalities might have a genetic origin. Sperm DNA damage could be considered as the physiological basis of some morphological defects. Some studies have reported that certain aspects of bull fertility, including morphological abnormalities are probably under genetic control

Plasma membrane integrity

In the present study, the percentage of HOST reacted and not reacted sperm ranged from 60.50 to 81.33 and 18.67 to 33.17 respectively. These results were in agreement with Esteves *et al*. (2007). They reported that the percentage of spermatozoa exhibiting tail swelling after exposure to the hypo-osmotic solution was 73.5 per cent in fresh and 50.0 per cent in frozen semen. HOST gives information about the intactness of the plasma membrane and the osmotic activeness of the sperm. When the sperm cells are exposed to hypo-osmotic solution, the plasma membrane of the tail swells to maintain osmotic equilibrium. This is based on the fact that the semi-permeable nature of the sperm cell membrane which allows water to move inside to maintain the osmotic equilibrium. Due to swelling of the membrane, curling of tail takes place (Jeyendran *et al*., 1984) [27]. Zubair *et al*. (2013) [47] reported that the mean percentage of motility, morphology, livability and HOS coiling of Friesian bulls was 62.5, 57.5, 65.0 and 40.0 respectively. There was a significant correlation between HOS coiling, motility, normal spermatozoa and livability. The mean values of semen evaluation parameters including HOS coiling, motility, morphology and livability of Sahiwal bulls was 47, 60, 56 and 70 per cent respectively. The correlation between HOS coiling, motility, normal spermatozoa and livability was significant. The mean value of the HOS coiling was 27 per cent in crossbred bull. Whereas the motility, normal morphology and livability were 50, 40 and 55.8 per cent respectively and these last three values were significantly correlated with HOS swelling.

The use of HOS test in semen evaluation is much applicable than eosin and nigrosine staining. The fertilizing capacity of sperm plasma membrane can be estimated through functional activity by HOS test (Madeja *et al*., 2003) [31]. This test has the ability to sign whether the plasma membrane of sperm is biochemically active or dead. The osmolarity of the hypo-osmolar solution must have the capacity for creation of best result without the death of sperm. In the present study, the best swellings in three bulls was observed in 150 mOsm/ L with sodium citrate and fructose and similar kind of findings were observed by Jeyendran *et al*. (1984) [27].

Expression of osteopontin in sperm by flow cytometry

In the present study, the percentage of expression of osteopontin in sperm by flow cytometry ranged from 67.18 to 92.67 for six bulls. The present findings are in agreement with the studies of Moura*et al*. (2006) [35] who found that highly fertile Holstein bulls had higher concentration of OPN in their accessory gland fluids than in low fertile bulls. Exposure of the accessory gland fluids of highly fertile bulls improves the sperm fertilizing ability of low fertility bulls. The amount of OPN in the seminal plasma of highly fertile bulls is 4-fold higher when compared to that in low fertile bull. Aida *et al*. (1999) [1] reported that the relationship between OPN in seminal plasma and male fertility may be direct. Brown *et al*. (1992) [12] stated that OPN could influence male fertility indirectly by protecting epithelial surfaces of accessory sex glands from bacterial infections, thus having a positive influence on male fertility. OPN had known activities in anti-apoptosis and cell survival through activation of integrins and CD44 membrane receptors and signal transduction mechanism, including the activation of MAP kinases and phosphoinositide (PI) 3- kinase pathways.

Evaluation of sperm viability by flow cytometry

In the present study, the percentage of viable and non-viable sperm by flow cytometry ranged from 70.73 to 82.89 and 17.11 to 24.80 respectively. These results were in accordance with Graham *et al*. (1990) [22] who studied sperm cell viability, acrosomal integrity and mitochondrial function using flow cytometry and found that PI stained cells (red, non-viable) ranged from 7 to 41 per cent. Propidium iodide cannot pass through an intact plasma membrane, but passes into and stains the nuclei of degenerated spermatozoa. Assays using PI and eosin/nigrosin stains for intact plasma membrane produced nearly equivalent results indicating that PI is an accurate supravital stain for analyzing sperm by flow cytometry.

The reasons for better viability of spermatozoa from OPN expressed bull was evident from the fact that OPN is a cell survival factor and protect cells from undergoing apoptosis. OPN is suggested to enhance the protective effect on spermatozoa in the male reproductive tract as well as in the female reproductive tract after insemination. It can influence host defense mechanism during inflammatory conditions. Erickson (2006) [17] observed that OPN significantly increased the percentage of viable sperm on *in vitro* treatment. He also suggested that this effect was not a dose dependent one and very small amount of OPN was required to increase sperm viability.

Evaluation of acrosomal integrity by flow cytometry

In the present study, the percentage of viable sperm, non-viable sperm, viable intact sperm, viable damaged sperm and viable damaged- non-viable sperm ranged from 75.12 to 82.28, 17.72 to 30.66, 58.55 to 78.48, 3.80 to 10.80 and 21.52 to 41.45 respectively. These results were in agreement with Garner *et al.* (1986) [20] who reported that the percentage of intact acrosomes ranged from 70 to 90 in cryopreserved bovine semen from 14 bulls. The percentages of viable, acrosome intact spermatozoa as assessed by dual staining and triple staining immediately after thawing (0 h) was 63.57 and 59.36 respectively.

Spermatozoa must maintain an intact acrosome to which the zonapellucida of oocytes binds. Upon binding with oocyte, acrosomal enzymes are activated and they digest the zonapellucida thereby allowing the sperm cells to access the oolemma (Yanagimachi, 1981) [46]. Staining of the acrosome was done to evaluate the acrosomal status. When the acrosomal status of larger population of sperm has to be evaluated, fluorometery (Salazar *et al*., 2000) [40] and flow cytometry (Purdy and Graham, 2004) [39] methods have been used. Sperm with damaged acrosome emits green fluorescence whereas sperm with intact acrosome will not emit fluorescence. Visualization of the acrosomal status of the sperm is also an important factor in semen quality evaluation. At the time of ejaculation, sperm without intact acrosome may lead to infertility. Cancel *et al*. (1999) [14] conducted a study on acrosomal integrity of spermatozoa and stated that the amount of OPN was more in acrosomal intact spermatozoa and vice versa. They also stated that either OPN or its receptors were lacking in case of acrosomal non-intact spermatozoa. OPN was localized in acrosomal cap of spermatozoa. Integrin and CD44 receptors are also found in acrosomal cap of spermatozoa and OPN secreted from accessory sex glands binds with receptors (Marroquin *et al*., 2004) [32].

Sperm chromatin structure assay by flow cytometry

In the present study, the percentage of double stranded DNA, single stranded DNA and DFI ranged from 75.45 to 86.42, 12.67 to 31.50 and 12.67 to 24.55 respectively. These findings were similar to Viswam (2015) [44] who reported that the percentage of buffalo semen was 89.51.

The chromatin of sperm cells are highly condensed than in the nucleus of a somatic or a spermatogenic cell and it has been proposed that this condensation is a mechanism to protect the DNA from environmental stress and mutagenesis. It has also been proposed that alterations in chromatin structure may affect the rate of decondensation, a pre-requisite for male pronucleus formation during fertilization, and thereby disrupt embryo development (Virro *et al*., 2004) [43].

One benefit of SCSA over other methods is that evaluation of the DNA integrity is relatively easy, quick and thousands of sperms can be evaluated and also can be used to check the gamete quality after cell manipulation. The most important parameter revealed by SCSA is the DNA fragmentation index, which assesses the percentage of spermatozoa showing susceptibility of DNA to the acid induced denaturation *in situ*. Strong correlations have been demonstrated between sperm chromatin denaturation and fertility rankings in bulls (r = 0.94). A high DFI was related to decreased fertility rate, delayed pregnancy (Evenson and Wixon, 2006). OPN supplementation in *in vitro* culture media prevent oxidative stress to spermatozoa. Hence, DNA integrity of spermatozoa was better upon treatment with OPN (Viswam, 2015) [44].

Evaluation of Oxidative stress by flow cytometry

In the present study, the percentage of ROS reacted and not reacted sperm ranged from 10.42 to 16.47 and 83.53 to 89.58 respectively. These findings were similar to Baumber *et al*. (2000) [9] who studied the effect of reactive oxygen species on equine sperm viability, acrosomal integrity and mitochondrial membrane potential. CellROX® fluorescent probe is able to detect ROS production in sperm by *in vitro* and *in vivo* induction is a new technique to detect sperm oxidative stress. Increased generation of ROS resulted in a marked decline in sperm motility; there was no detectable decrease in either sperm viability or acrosomal integrity. Mitochondrion is a major site of intracellular ROS formation, which results in the

disruption of electron transport (Halliwell and Gutteridge. 1999) [26].

Oxidative stress is generated as a result of an imbalance between production ROS and antioxidants scavenging. Certain level of ROS is essential for sperm functions such as capacitaion, acrosome reaction, sperm-oocyte fusion and phosphorylation of protein tyrosine. 20 per cent – 40 per cent of infertility was due to the high levels of ROS. There are several proposed mechanisms for the decline in sperm motility associated with oxidative stress. One of the frequently cited mechanisms is the lipid peroxidation in the sperm membrane (Aitken *et al*., 1993) [2]. As a consequence of the peroxidation, loss of membrane integrity and increase in membrane permeability, lead to a loss of capacity which regulate the intracellular concentrations of ions involved in the control of sperm movement.

Recovery of oocytes from cow by OPU technique

A total of 108 oocytes were recovered by aspiration of 135 follicles. The percentages of A, B, C and D graded oocytes aspirated by OPU technique were 27.78, 36.11, 29.62 and 6.48 respectively in the present study. These finding were in agreement with Pellerano *et al*. (2007) [37] who reported that a total of 78 COC's were collected following aspiration of 271 follicles in cows. The percentage of A, B, C and D grade oocytes were 31.6, 22.4, 53.9 and 15.8 respectively. The percentage of A and B grade oocytes were similar to our studies but C and D grades oocytes were higher than in the present study. On contrary, Arun (2003) [6] who reported that a total of 42 COC's were aspirated from 84 follicles from the Jersey crossbred cows. Out of 42 COC's, 4 (9.52), 17 (40.48), 16 (38.09) and 5 (11.90) were graded as A, B, C and D respectively. Manik *et al*. (2003) reported that the percentage of A, B, C and D grade oocytes were 8, 24, 35 and 34 respectively in Karan Fries cattle. This might be due to age of the cow, breed, reproductive status, individual response, needle gauge size, vacuum pressure and operator's experience. The climate and temperature also plays a key role on bovine follicle formation and development. Heat stress suppresses follicular dominance, resulting in a number of changes in follicular growth.

The original OPU procedure includes no hormone stimulation. It is routinely performed twice a week, which allows the maximum recovery of oocytes with suitable quality for embryo production in a given time interval when compared to once-a-week OPU, where no dominant follicle develops when all visible follicles are aspirated in the OPU process. In most once-a-week collections, a dominant follicle develops at the successive collection, which causes the regression and degeneration of the subordinate follicles.

Evaluation of maturation process

The percentages of *in vitro* maturation of oocytes were 73.27, 15.84 and 10.85 for degree 2, degree 1 and degree 0 respectively in the present study. These results were in agreement with Arun (2003) [6] and they reported that 75.00 per cent of maturation was noticed in A grade oocytes and for the B and C graded oocytes maturation rate was 64.71 per cent and 62.50 per cent respectively. The proportion of percentage of maturation of B and C grade oocytes were 15.84 and 10.85 respectively in the present study which is low when compared to the earlier reports of Arun (2003) [6]. On contrary to the present work, Manik *et al*. (2003) observed that the percentage of *in vitro* maturation of oocytes were 8, 24, 35 and 34 for A, B, C and D grade oocytes respectively.

The percentage of cleavage rate of *in vitro* produced embryos

In the present study, overall cleavage rate of two cell embryos was 34.44 per cent. This result was in agreement with Manik *et al*. (2003) who reported that 73 oocytes were cultured *in vitro* and inseminated with good quality semen and 33.00 per cent of two cell embryos were developed. On contrary, Bols *et al*. (1995) [11] reported that the cleavage percentage was 69 per cent on three day post-insemination. Santl *et al*. (1998) [41] stated that the percentage of embryos cleaved were 58.1 which was higher than in the present study.

In the present study, 53.33 per cent of cleavage rate was observed in bull No. 3 which expressed higher percentage of osteopontin in semen when compared to other bulls. The relative amount of osteopontin in bovine seminal plasma is correlated positively with the fertility of bulls.

Conclusions

Osteopontin in bull semen had significant influences on sperm motility, viability, plasma membrane integrity, acrosomal integrity, maintenance of sperm chromatin structure and reducing the oxidative stress. Higher percentage of expression of osteopontin in bull semen was positively correlated with cleavage rate of *in vitro* produced embryos.

Acknowledgements

The authors are sincerely thanks to Department of Biotechnology, Ministry of Science and Technology, Government of India for funding the project on "Establishment of Buffalo embryonic stem cell line". Facilities provided by the Tamil Nadu Veterinary and Animal Sciences University are duly acknowledged. The Senior Research Fellow, Laboratory Assistant and Animal Attendants are acknowledged for their timely help.

References

1. Aida M, Cancel D, Chapman A, *et al*., Osteopontin is the 55-kDa fertility-associated protein in Holstein bull seminal plasma. Biol Reprod 1999;57:1293-301.
2. Aitken RJ, Harkiss D, Buckingham D. Relationship between iron-catalysed lipid peroxidation potential and human sperm function. J Reprod Fertil 1993;98:257-65.
3. Alessandra G, Michele LG, Angela F *et al*., Assessment of viability, chromatin structure stability, mitochondrial function and motility of stallion fresh sperm by using objective methodologies. J Cell Anim Biol 2010;4:34-41.
4. Alves MBR, Andrade AFCD, Arruda RPD *et al*., An efficient technique to detect sperm reactive oxygen species: The CellRox deep red Fluorescent probe. Biochem Physiol 2015;4(2)
5. Amann RP, Hammerstedt RH. *In vitro* evaluation of sperm quality: an opinion. J Androl 1993;14:397-406.
6. Arun V, Ultrasound guided transvaginal follicular aspiration and *in vitro* maturation of oocytes in cows. M.V.Sc. thesis submitted to Tamil Nadu Veterinary and Animal Sciences University. Chennai-51 2003.
7. Bansal AK, Bilaspuri GS. Effects of manganese on bovine sperm motility, viability and lipid peroxidation *in vitro*. Anim Reprod 2008;5:90-6.
8. Barth AD, Oko RJ. Abnormal morphology of bovine spermatozoa.1st ed. Ames, lowa state university press, USA 1989, 130-92.

9. Baumber J, BallB, Gravance C *et al*., The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. J Androl 2000;21:895-902.
10. Beker ARCL, Colenbrander B, Bevers MM. Effect of 17β estradiol on the *in vitro* maturation of bovine oocytes. Theriogenology 2002;58:1663-73.
11. Bols PEJ, Vandenheede JMM, Vansoom A *et al*., Transvaginal ovum pick-up (OPU) in the cow: A new disposable needle guidance system. Theriogenology 1995;43:677-87.
12. Brown LF, Berse B, Van de Water L *et al*., Expression and distribution of osteopontin in human tissues: widespread association with luminal epithelial surface. Mol Biol Cell 1992;3:1169-80.
13. Cancel AM, Chapman DA, Killian GJ. Osteopontin is the 55- kilodalton fertility-associated protein in Holstein bull seminal plasma. Biol Reprod 1997;57:1293-301.
14. Cancel AM, Chapman DA, Killian GJ, Osteopontin localization in the Holstein bull reproductive tract. Biol Reprod 1999;60:454-60.
15. Cavalieri J, Van Camp SD, Bovine seminal vesiculitis. A review and update. Vet Clin North Am Food Anim Pract 1997;13:233-41.
16. Enciso M, Cisaleb H, Johnstonc SD *et al*., Major morphological sperm abnormalities in the bull are related to sperm DNA damage. Theriogenology 2011;76:23-32.
17. Erickson DW. Role of osteopontin in bovine sperm capacitation and fertilization. Thesis submitted to Pennsylvania State University 2006, 80-1.
18. Esteso MC, Soler AJ, Fernandez-Santos MR *et al*., Functional Significance of the Sperm Head Morphometric Size and Shape for Determining Freezability in Iberian Red Deer (*Cervus elaphus hispanicus*) Epididymal Sperm Samples. J Andro 2006;27:662-70.
19. Evenson DP, Wixon R, Clinical aspects of sperm DNA fragmentation detection and male fertility. Theriogenology 2006;65:979-91.
20. Garner DL, Pinkel D, Johnson LA *et al*. Assessment of spermatozoal function using dual fluorescent staining and flow cytometric analyses. Biol Reprod 1986;34:127-38.
21. Graham EF, Schmehl MKL, Nelson DS. Problems with laboratory assays. Proceedings of the eighth technical conference on artificial insemination and reproduction 1980, 59-66.
22. Graham JK, Kunze E, Hammerstedt RH Analysis of sperm cell viability, acrosomal integrity and mitochondrial function using flow cytometry. Biol Reprod 1990;43:55-64.
23. Hafez ESE, Reproduction in Farm Animals. 5th Ed. Lea & Febiger, Philadelphia 1987.
24. Hall JL. Relationship between semen quality and human sperm penetration of zona-free hamster ova. Fertil Steril 1981;35:457-63.
25. Hallap T, Nagy S, Haard M *et al*., Variations in quality of frozen-thawed semen from Swedish Red and White sires at 1 and 4 years of age. J Androl 2004;27:166-71.
26. Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine, 3rd edn. Oxford: Clarendon Press 1999.
27. Jeyendran RS, Vander-Ven HH, Perez-Pelaez M *et al*., Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characters. J Repord Fertil 1984;70:219-28.
28. Kruger TF, DuToitTC, Franken DR, A new computerized method of reading sperm morphology (strict criteria) is as efficient as technician reading. Fertil Steril 1993;59:202-9.
29. Laing JA, Morgan WJB Wagner WC. Fertility and infertility in the domestic animal, 4th edn. Baillere, Tindall, London, 1988, 91-112.
30. Lee JL, Wang MJ, Sudhir PR *et al*., Osteopontin promotes integrin activation through outside-in and inside-out mechanisms: OPN-CD44V interaction enhances survival in gastrointestinal cancer cells. Cancer Res 2007;67:2089-97.
31. Madeja Z, Waroczyk M, Strabel T *et al*., Use of the hypo-osmotic swelling test for evaluating bull and boar semen quality.Med Weter 2003;59:1115- 8.
32. Marroquin CE, Downey L, Guo H *et al*., Osteopontin increases CD44 expression and cell adhesion in RAW 264.7 murine leukemia cells. Immunology 2004;95:109-12.
33. Mazzali M, Kipari T, Ophascharoensuk V *et al*., Osteopontin: A molecule for all seasons. Q J Med 2002. 95:3-13.
34. Mortimer. Casa – Practical aspect. J Androl 2000;21(4):515-24.
35. Moura AA, KocH, Chapman DA *et al*., Identification of proteins in the accessory sex gland fluid associated with fertility indexes of dairy bulls: a proteomic approach. J Androl 2006;27(2):201-11.
36. Noto MD, Campo MD, Roziers BS *et al*., Fluorescein diacetate assessment of embryos viability after ultrarapid freezing of human multipronucleate embryos. Fertil Steril 1991;55:1171-5.
37. Pellerano G, Gasparrini B, Crudeli G *et al*., Ovum pick-up and *in vitro* production technology in field conditions in the North East of Argentina. Ital J Anim Sci 2007;6(2):743-6.
38. Pena Martínez AI, Canine fresh and cryopreserved semen evaluation. Anim Reprod Sci 2004;82-83:209-24.
39. Purdy PH, Graham JK Effect of adding cholesterol to bull sperm membranes on sperm capacitation, the acrosome reaction and fertility. Biol Reprod 2004;71:522-7.
40. Salazar P, Graham JK, Parrish JJ, *et al*., Indirect determination of stallion sperm capacitation based on esterase release from spermatozoa challenged with lysophosphatidylcholine. J Reprod Fertil Suppl 2000;56:407–14.
41. Santl B, Weingerkind H, Schernthaner W *et al*., Comparison of ultrasound-guided vs laparoscopic transvaginal ovum pick-up in simmental heifers. Theriogenology 1998;50:89-100.
42. Sreenivas D, Yarla VM, Thomas, Palnisamy A, Effect of protein supplementation on *in vitro* maturation of sheep oocytes and *in vitro* culture of preimplantation with α-tocopherol supplementation in cr1aa medium on sheep embryos to the blastocyst stage.J AllerTher 2013;4:2155-6121.
43. Virro MR, Larson-Cook KL, Evenson DP. Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy *in vitro* fertilization and intracytoplasmic sperm injection cycles. FertilSteril 2004;81:1289-95.

44. Viswam V, Influence of osteopontin on *in vitro* sperm characteristics of frozen buffalo semen. Thesis submitted to TANUVAS 2015.
45. Walters A, Eyestone W, Saacke R *et al*., Bovine Embryo Development After IVF with Spermatozoa Having Abnormal Morphology. Theriogenology 2005;63(7):1925-37.
46. Yanagimachi R. Mechanisms of fertilization in mammals. In: Mastroianni L, Biggers JD, editors. Fertilization and embryonic development *in vitro*. Plenum Pub 1981;1:81-182.
47. Zubair M, Lodhi LA, Ahmad E *et al*., Hypo osmotic swelling test as screening for evaluation of semen of bull. J EntomolZool Stud 2013;6:124-8.