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Quality and Biochemical Characteristics of Frozen Semen of Purebred and Crossbred Bulls

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ABSTRACT

In the present study, frozen semen straw were collected from the bull semen bank and performed routine semen analysis. We also studied different biochemical parameters of seminal fluid, including ROS generation and DNA fragmentation. Average viability and motility of sperm showed within 77% and 27%, respectively, which is not satisfactory. Biochemical constituents like glucose, fructose, total protein, cholesterol, and others varied among the different breeds, and there was no superiority among the breeds. The results of different electrolyte concentrations were closely associated among the different breeds that protected the sperm from osmotic lysis. Aspartate transaminase, alanine transaminase, alkaline phosphatase, and acid phosphatase activity were mostly better in exotic and crossbred breeds than in pure indigenous breeds. Antioxidant profile and oxidative stress (OS) were also studied. The results indicated that antioxidant enzyme (catalase, GPx) activity and reduced glutathione content were high in the crossbred variety. However, lipid peroxidation rate was high in the Gir breed. Flowcytometry study revealed that the ROS generation level was very high, and the rate of DNA fragmentation was around 50%, which indicated the loss of 50% of sperm due to ROS. In conclusion, it could be said that cryopreservation decreases sperm viability, motility, and increases ROS that is detrimental to sperm survival.

Key words: Semen, seminal fluid, Biochemical constituents, ROS, Antioxidants, DNA fragmentation

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INTRODUCTION

Cryopreservation (-196°C in liquid nitrogen) of sperm reduces motility, acrosomal membrane integrity, DNA stability and decreases mitochondrial functions (Celeghini *et al.,* 2008). A certain rise in temperature during thawing decreases the rate of fertilizing capacity of the semen and there can be a chance of failure of fertilization (Kumar *et al.,* 2016). The rise in temperature after cryopreservation affects the plasma membrane, acrosome structure, DNA stability, and mitochondrial integrity of the spermatozoa. Another important part of semen is seminal plasma, which is secreted by testis, epididymis, and male accessory glands (prostate, seminal vesicles). Seminal plasma contains different sugars and enzymes that provide protection, and nutrition to the spermatozoa (Juyena and Stelletta, 2012). It also provides energy for sperm motility, and fertility (Juyena and Stelletta, 2012). The major biochemical constituents of semen are glucose, fructose, protein, triglycerides, cholesterol, minerals (Na^+, K^+, Mg^{++}, Ca^{++}, Cl^-, Pi^{2-}), enzymes [aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), acid phosphatase (ACP)], antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GP_X)], and free radical scavenging antioxidants [reduced gluthione, (GSH)] (Juyena and Stelletta, 2012; Fernández *et al.,* 2013). Minerals and antioxidants protect the spermatozoa from free-radical-mediated damage (Marzec-Wŕoblewska *et al.,* 2012; Massanyi *et al.* 2008).

Inorganic ions (Na^+, K^+, Ca^{++}, Mg^{++}, Cl^-) in the seminal plasma maintain the osmotic balance and helps in buffering action, utilization of energy, and sperm motility (Gür and Demirci, 2000; Massanyi *et al*., 2003; Wong *et al*., 2001; Tvrdá *et al*., 2013b; Purohit *et al*., 1999; Machal *et al*., (2002; Çevik, *et al*., 2007). Inorganic ions are essential for the fertilization capacity of spermatozoa. The fructose in the seminal plasma is the primary source of energy. Bovine seminal plasma proteins protect the plasma membrane of the sperm and maintain sperm integrity. Seminal plasma proteins facilitate sperm penetration into oocytes. Albumin in seminal plasma shows nonenzymatic protein antioxidant properties (Tvrdá *et al.,* 2012; Bourdon and Blache, 2001). AST, ALT, and ALP are useful markers of good semen quality as they indicate membrane stability and sperm integrity. ACP primarily is a prostatic enzyme, which is the indicator of semen quality and helps in sperm activation and sperm motility. Sperms suffer from oxidative stress, which is detrimental to mitochondrial functions and DNA stability. SOD, CAT, GPx, and GSH decrease oxidative stress, prevent lipid peroxidation and protect DNA from fragmentation (Khan *et al.,* 2015; Tvrdá *et al.,* 2013b; Juyena and Stelletta, 2012).

Cryofreezing stress includes osmotic changes, pH fluctuations, energy depletion, cold shock, and cryo-damages. Estimation of enzymes, antioxidants, and minerals are considered as the markers for assessment of semen quality. Cryopreservation and temperature exposure during thawing change the biochemical characteristics of seminal fluid. Thus, the major objectives of the present work were to evaluate the quality and fertilization capacity of the frozen bovine semen at the post-thawing stage.

MATERIALS AND METHODS:

Sample Collection and thawing: The study was conducted on the sample of frozen semen straw (FSS) of different bovine bulls: Gir, Jersey (JY), crossbred Jersey (CBJ), and crossbred Holstein (CBH). FSS were procured from District Semen Bank of Paschim Banga Go-Sampad Bikas Sanastha, Midnapore, West Bengal, India. FSS were kept at 37 °C for 30 seconds in a water bath for thawing, and 200 µL of semen were collected from each FSS. Previously, it was claimed that the normal thawing process was done at 37 °C for 30 seconds (Amare and Reda, 2012; Goshme *et al.* 2021). Liquid semen was used for routine analysis of semen and seminal fluid from frozen semen was prepared through microcentrifugation.

Spermatogram: Sperm motility (%) was assessed through microscopic examination. Total sperm count (millions/mL) was done by hemocytometer method. Sperm viability (%) was measured by using eosin-nigrosine stain as per routine protocol.

Biochemical constituents: Estimation of glucose, fructose, total protein (TP), albumin, triglyceride, cholesterol, urea, uric acid and creatinine in the seminal fluid was done by using assay kits from Ranbaxy (India). The procedure of estimation was performed as described by the company in the description of the kits.

Estimation of electrolytes: Different electrolytes, including sodium, potassium, chloride, calcium, magnesium, and inorganic phosphate in seminal fluid were estimated by using gold standard assay kits of Sigma (USA). The protocol was followed as described by the company.

Estimation of different enzymes: The concentration of glutamate oxaloacetate transaminases (AST), serum glutamate pyruvate transaminase (ALT), alkaline phosphatase (ALP), acid phosphatase ACP), acid phosphatase (prostatic ACP), and lactate dehydrogenase (LDH) in seminal fluid were measured through clinical assay kit from ARKRAY Healthcare Pvt. Ltd. (India). The procedure was performed as per the instructions given in the kit box.

Assay of oxidative stress profile:

LPO

To evaluate the degree of membrane damage of spermatozoa, lipid peroxidation (LPO) was measured by estimating the formation of malondialdehyde (MDA) by the method of Ohakawa *et al*. (1979) in which thiobarbituric acid (TBA) was used as a coloring agent. The MDA content was calculated by using the molar extinction coefficient $1.43 \times 10^{-3} M^{-1}cm^{-1}$ and expressed as nmol of MDA formed/mg protein.

GSH

GSH was assayed by the method of Moron *et al*., (1979) using DTNB. The levels of GSH were expressed as µg of GSH/mg protein.

SOD, CAT, GPx

Activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were assayed as the antioxidant enzyme profile. SOD activity was determined by the method of Del Mestro and McDonald (1986) where the ability to inhibit the auto-oxidation of pyrogallol was considered. SOD activity was expressed as unit/mg of protein. CAT activity was measured by the method of Luck (1963). Catalase activity was calculated by using the molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$ for H_2O_2. The activity of GPx was determined by the method of Paglia and Valentine (1967). The activity was expressed in nmol of NADPH oxidized to NADP per min/mg of protein and the extinction coefficient value $6.2 \times 103\ M^{-1}cm^{-1}$ at 340 nm was used in this calculation.

Mitochondrial membrane potential, ROS generation and DNA fragmentation: Flowcytometry technique (Instrument: Becton Dickinson Sanj Jose, USA, Model BD Accuri C6) was applied to measure the ROS production, mitochondrial membrane potential (MMP) and DNA fragmentation. ROS generation and MMP were measured by using DCF-DA (22-72-dichlorodihydrofluorescein diacetate) and JC-1 (5, 52, 6, 62-tetrachloro 1, 12, 3, 32-tetraethylbenzimidazolyl carbocyanine iodide) dyes, respectively. The result of ROS generation was detected and expressed by the mean value of fluorescence of DCF-DA. The MMP was measured in terms of percentage (%) of cells with polarized mitochondria. DNA fragmentation was studied by the TUNNEL method in which an anti-BRDU (5-bromo-22-deoxyuridine) antibody conjugated to FITC (fluorescein isothiocyanate) was used for detection. The result was expressed in percentage (%) of cells showing DNA fragmentation.

Statistical analysis: Dispersion of individual scores arises around the mean, the statistics of the sample vary from sample to sample. To nullify the dispersion of individual scores and sapling variations, we only calculated standard deviation (SD) and standard error of mean (SE) by using the Microsoft Excel package. The results were presented as Mean ± SE.

Next, we carried out chi-square (χ^2) analysis on the data of DNA fragmentation, mitochondrial membrane potential (MMP), motility, and viability. We also performed Kruskal-Wallis chi-squared (χ^2) tests to determine the level of significance of each parameter among the test parameters. Later, we performed Dunn›s Kruskal-Wallis multiple comparison test, with p-values adjusted by using the Holm method for multiple comparisons to estimate the level of significance of each parameter within each pair of breeds (CBH-CBJ, CBH-GIR, CBJ-GIR, CBH-JY, CBJ-JY,GIR-JY) (Dunn, 1964). The significance level for all tests was set at 0.05 level.

RESULTS AND DISCUSSION

Routine analysis of semen (sperm count, viability, and motility tests) was performed to determine the semen quality. Seminal fluid was used for different biochemical tests (estimation of different enzymes, minerals, and antioxidant profiles). Flow cytometry test was performed for determination of reactive oxygen species (ROS) generation, mitochondrial membrane potential, and DNA fragmentation.

Spermiogram attributes

Fertilization capacity depends on a sufficient number of viable motile sperm present in the semen. Sperm concentration is a vital parameter for semen analysis and the highest count had been observed in CBH, but the percentage (%) of viability (64.80%) and motility (14.82%) of sperms were low in this breed in comparison to other breeds (Table 1).

Besides this, the result indicated that the number of motile sperm is not sufficient in the other two breeds (Gir and CBJ). Most of the breeds showed <30% motile sperm, while the breed Jersey gives better results in aspects of viability and motility. This breed provides 27.73% and 77.56% motile and viable sperm, respectively (Table 1). A greater number of sperms were present in Cholistani bull semen where the motility and viability were also high (Farooq *et al.,* 2013, 2015). Several authors reported that the motility

of sperm in fresh semen was 60-80% (Farooq *et al.,* 2013; Bhakat *et al.,* 2014). Goshme *et al.* (2021) also reported that the average motility of fresh semen of different breeds was greater than 60%. Recently, Dâ€™Andre *et al.* (2017) observed that the mass motility of frozen bull semen ranged from 31 to 41.6 percent. Lone *et al.* (2016) showed that the viability and motility of spermatozoa drastically fall at the post-thaw level. The semen quality of bulls of various breeds is affected by seasonal variation, diet, and age of the bull (Farooq *et al.,* 2013, 2015; Vince *et al.,* 2018).

Estimation of biochemical constituents

Seminal fluid is a nutritive and protective medium. The amount of different constituents was independently varied among the different animals and was not related to body weight and size. Sugars are the essential components in the seminal plasma. Fructose and glucose are utilized for ATP production, which is essential for capacitation, sperm motility, and fertilization (Jobim *et al.,* 2004; Assumpcao *et al.,* 2005). Cholesterol has a special relevance since it is the most abundant lipid in the spermatozoa of all mammalian species. The concentration of glucose, total proteins, albumin, triglyceride, urea, urea acid, creatinine, and cholesterol were presented in Table 2.

In the present study, the concentrations of glucose were varied among the different breeds. The highest and lowest glucose concentrations were estimated in CBJ and Gir, respectively. Jersey, both pure (224.56 mg/dL) and crossbred (227.54 mg/dL) showed more concentration of glucose than other breeds. Similar results were also reported by Khan *et al.* (2015). Seminal fluid contains a sufficient amount of fructose, which is essential for the activity of spermatozoa. The highest fructose content (471.31 mg/dL) was observed in CBH, and breed Gir showed the lowest value (352.23 mg/dL). Fructose is synthesized in the accessory gland of the male reproductive system through the polyol pathway. Spermatozoa anaerobically utilize fructose and convert it to lactic acid (Storey, 2008). Sufficient glucose and fructose concentration in seminal plasma indicates more capacity for energy production and motility (Jobim *et al.,* 2004; Assumpcao *et al.,* 2005). Moreover, the

Table 1: Post thaw sperm motility and viability in frozen semen of different breeds.

Breed variety	Sperm count (Mean ± SE) Millions/mL (10^6/mL)	Viability (%)	Motility (%)
GIR	159.67 ± 1.33	77.25	20.74
Jersey	171.66 ±2.06	77.56	27.73
Crossbred Jersey	164.5 ± 1.92	73.90	25.03
Crossbred Holstein	178.32 ±2.98	64.80	14.82
χ^2 test of 4-samples for equality of proportions (df = 3)		χ^2 = 5.4434, p-value = 0.1421 (P>0.05)	χ^2 = 5.5292, p-value = 0.1369 (P>0.05)

Table 2: Comparison of biochemical constituents in seminal fluid of different breeds. The values were presented as Mean ± SE (n-6).

Parameter	Gir	Jersey	Crossbred Jersey	Crossbred Holstein	Kruskal-Wallis (K-W) rank sum test (df =3)
Glucose (mg/dL)	185.96 ± 3.54	224.56 ± 2.03	227.54 ± 3.32	208.77 ± 2.24	(K-W) χ^2 = 27.473, p-value = 0.00004686* (P<0.001)
Fructose (mg/dL)	352.23 ± 3.21	429.25 ± 2.42	379.62 ± 4.38	471.31 ± 6.37	K-W χ^2 = 27.853, p-value = 0.000039* (P<0.001)
Total protein (gm/dL)	5.51 ± 0.26	6.12 ± 0.58	6.66 ± 0.86	6.59 ± 0.71	K-W χ^2 = 13.799, p-value = 0.003192* (P<0.001)
Albumin (gm/dL)	3.91 ± 0.14	4.91 ± 0.28	4.67 ± 0.73	4.29 ± 0.58	K-W χ^2 = 7.5978, p-value = 0.0551 (P>0.05)
Total cholesterol (mg/dL) 226.55 ± 2.89		259.88 ± 4.76	252.54 ± 3.26	263.27 ± 4.34	K-W χ^2 = 21.885, p-value = 0.0006893* (P<0.001)
Triglyceride (mg/dL)	31.11 ± 1.84	44.44 ± 2.58	43.21 ± 1.79	52.55 ± 1.62	K-W χ^2 = 32.28, p-value = 0.000004569* (P<0.001)
Urea (mg/dL)	5.27 ± 0.52	5.21 ± 0.76	5.26 ± 0.52	5.34 ± 0.85	K-W χ^2 = 2.3388, p-value = 0.5051
Uric acid (mg/dL)	2.59 ± 1.32	2.76 ± 1.14	2.36 ± 2.46	2.17 ± 0.82	K-W χ^2 = 1.7521, p-value = 0.6254 (P>0.05)
Creatinine (mg/dL)	1.47 ± 0.46	1.11 ± 0.25	1.13 ± .38	2.00 ± 0.68	K-W χ^2 = 14.578, p-value = 0.002215* (P<0.001)

concentration of glucose was affected by different conditions, including breed variety, age, feeding standard and seasonal variation.

Protein is the essential component of the biological fluids. The present study showed that the highest concentration of protein was present in CBJ (6.66 gm/dL) followed by CBH>JY>Gir (Table 2). Many studies had shown that low content of seminal proteins is associated with poor semen quality (Bergeron *et al.,* 2004). Total protein concentrations were very similar in all the four breeds. The values of total protein concentrations were very similar to other reports (Assumpção *et al.,* 2005; Tribulo *et al.,* 2015) and lower than the observations of Khan *et al.* (2015). This difference is perhaps due to higher genetic variability between the species of bulls. It was also reported that total protein content and albumin were affected by factors such as feeding regimes and seasonal variability other than the age of the bull (Vince *et al.,* 2018).

The beneficial effects of seminal plasma proteins, particularly albumin, are associated with the improvement of sperm motility. Initially, spermatozoa in epididymis can not achieve progressive motility and acquire it upon the addition of either seminal plasma (motility factor) or albumin (Słowioska *et al.,* 2014). There were no major differences in seminal albumin concentrations. The highest concentration of albumin was calculated in the JY bull (4.91 gm/dL) followed by other bulls (CBJ>CBH>Gir) (Table 2). Generally, albumin is not considered as a dominant protein of the bovine ejaculate, but the presence of this protein in seminal plasma is directly correlated with the motility of spermatozoa and antioxidant activity. It is suggested that seminal plasma albumin acts as an antioxidant protein, acting through its multiple-binding sites and free radical-trapping properties (Tvrdá *et al.,* 2012; Bourdon and Blache, 2001).

The concentrations of total cholestero1 and triglycerides are given in Table 2. The highest concentration of total cholestero1 and triglycerides had been observed in CBH and JY, respectively, while the lowest concentration had been found in Gir. The possible source of lipids in seminal plasma are the epididymis, seminal vesical, as well as spermatozoa. The cholesterol concentration was slightly higher in the Cholistani bulls as reported by Farooq *et al.* (2013). Seminal lipids specifically phospholipids and cholesterol have special relevance in the structure and functions of the plasma membrane of spermatozoa and may play significant roles in the sperm structure, metabolism, capacitation, and fertilization of female gametes. Moreover, cholesterol acts as a protective agent against environmental stress (Jacyno *et al.,* 2009). The other constituents like urea, uric acid, and creatinine were also measured. Physiologically, these are treated as the markers of protein and nucleic acid metabolism. However, these constituents were also present in seminal fluid and the values varied in different strains (Table 2). The urea and creatinine content were maximum in CBH, while the lowest value was observed in JY. In the case of uric acid, JY showed the maximum result and lowest value found in CBH. Kruskal-Wallis test for the multiple comparisons of p-values adjusted with the Holm method had been performed to estimate the p-values of different biochemical parameters. Result showed that there was no uniform pattern of significant differences between the breeds among the biochemical constituents (Fig. 1). However, comparison between purebreds (GIR-JY) exhibited significant differences (P<0.05)) for all the biochemical constituents (glucose, fructose, total protein, cholesterol, triglyceride, and creatinine) (Fig. 1). Albumin did not show any significant difference.

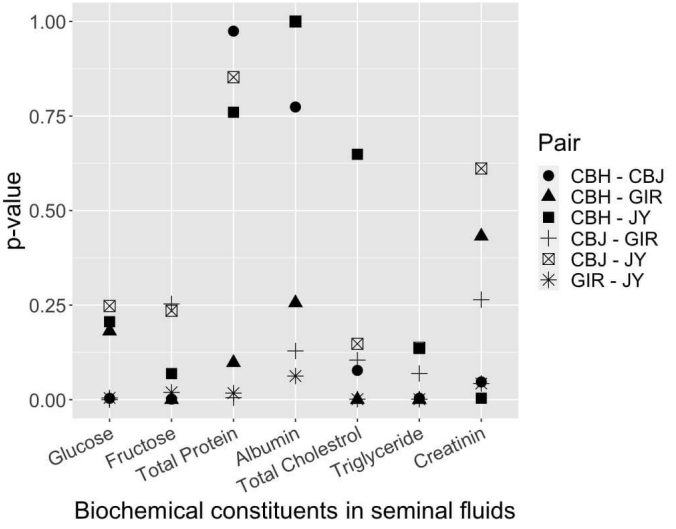

Fig. 1: Multiple comparisons of p-values adjusted with the Holm method for different biochemical constituents across various breeds, were analyzed using Dunn (1964) Kruskal-Wallis test. The Y-axis represents adjusted p-values (p.adj), while the X-axis illustrates the diverse biochemical constituents in each breed. Significance level ($p < 0.05$) is indicated for comparison. (JY; Jersey, CBJ: crossbred Jersey, CBH: crossbred Holstein)

Estimation of electrolytes

Sodium and potassium are associated with the maintenance of seminal osmolarity and activity (Massanyi *et al.,* 2008). Among the breeds, Na concentration was the highest (154.27 mmol/L) in Gir bulls than the other breeds, whereas Jersey bull represented the lowest (141.26 mmol/L) value of Na concentration (Table 3). Moreover,

sodium exerts favorable effects on semen quality and is an important electrolyte for the activity of the spermatozoa (Machal *et al.,* 2002; Mosaferi *et al.,* 2005).

Potassium concentration was measured in the seminal fluid of different strains of bovine bull and represented in Table 3. The highest (44.19 mmol/L) and lowest (34.77 mmol/L) K^+ ion concentrations were observed in JY and Gir, respectively. The probable reason for the variation of potassium concentrations in different breeds is due to the age of the bulls. A similar concentration of K^+ ion was also observed in Cholistani bull semen (Farooq *et al.,* 2013 Potassium ion is a natural metabolic inhibitor, and higher K^+ ion concentration in seminal plasma decreases the metabolic activity of spermatozoa (Gür and Demirci, 2000; Massanyi *et al*., 2003). The metabolic activity like oxygen uptake, glycolysis, and fructolysis could be inhibited by potassium ions, indicating that this element may adversely affect spermatozoa activity. Thus, there is a negative correlation between K^+ concentration and sperm motility (Joseph *et al.,* 2013).

Calcium is one of the important constituents of the seminal plasma. Calcium is essential for the activation of enzymes, penetration of zona pellucida, cortical reaction, and final maturation of the ovum. In the presented study, calcium concentrations varied among the different breeds and CBJ showed the highest value, while the lowest value was observed in Gir (Table 3).

According to Wong *et al.* (2001), Mg is regarded as a marker of seminal vesicle secretions. Magnesium plays a vital role in the utilization of ATP, which is important for sperm transport as well as fertilization. The highest Mg^{2+} concentration (9.11 mg/dL) was observed in CBH, while Gir showed the lowest value (6.25 mg/dL) (Table 3). Previously, Chandra *et al.* (2013) reported that Mg decreases LPO and increases SOD activity as well as CAT activity in rat testicular tissue. Tvrdá *et al.* (2013b) had shown that Mg^{2+} is positively associated with motility and antioxidant markers. Moreover, it has been revealed that GSH contained in semen is Mg-dependent, as the glutathione synthetase needs Mg^{2+} for its activity (Townsend *et al.,* 2003).

It is known that modulation of a variety of ion channels, like Cl^- of spermatozoa plays a characteristic event in the capacitation and acrosome reaction in the mammalian system (Purohit *et a*l., 1999). Among the different breeds, CBH showed the highest, and CBJ exhibited the lowest Cl^- ion concentration (Table 3). Inorganic phosphate (Pi) is also an important factor for the seminal fluid, and it is involved in various biochemical reactions. The concentration of Pi was the highest in Gir and the lowest in CBH (Table 3). Machal *et al.* (2002) also reported that Pi levels in bulls were positively correlated with sperm concentration. The variations of different ions in the seminal plasma of several breeds of bull were reported by Çevik *et al.* (2007). These variations are probably due to genetic dissimilarities between the bulls in addition to the climatic effects. The association of individual electrolytes among the breeds was calculated by Kruskal-Wallis test. The multiple comparisons of p-values adjusted with the Holm method were also done. Result showed that sodium, potassium, and magnesium varied significantly ($P<0.05$) between two pure breeds (GIR-JY) but other results exhibited different picture (Fig. 2). Significant difference ($P<0.05$) had been observed for calcium ion in CBJ-GIR and CBH-GIR, and chloride ion varied significantly ($P<0.05$) within cross bred bulls (CBH-CBJ) (Fig. 2).

Table 3: Comparative value of different electrolytes in seminal fluid of different breeds.

Parameter	GIR	Jersey	Crossbred Jersey	Crossbred Holstein	Kruskal-Wallis (K-W) rank sum test (df =3)
Sodium (mmol/L)	154.27 ± 5.21	141.26 ± 2.24	146.20 ± 11.42	152.57 ± 6.32	K-W χ^2 = 18.695, p-value = 0.0003161* (P<0.001)
Potassium (mmol/L)	34.77 ± 0.21	44.19± 0.47	38.28 ± 0.12	41.86± 0.68	K-W χ^2 = 29, p-value = 0.00002239* (P<0.001)
Calcium (mg/dL)	35.61 ± 0.43	37.79 ± 1.46	48.47 ± 1.23	43.05 ± 3.54	K-W χ^2 = 28.553, p-value = 0.00009634* (P<0.001)
Magnesium (mg/dL)	6.25 ± 1.40	7.94 ± 1.34	7.38 ± 1.24	9.11 ± 2.12	K-W χ^2 = 17.656, p-value = 0.001441* (P<0.001)
Chloride (mmol/L)	131.00 ± 8.56	123.78 ± 4.82	119.28 ± 5.32	139.64 ± 3.26	K-W χ^2 = 26.298, p-value = 0.0002755* (P<0.001)
Inorganic Phosphate (mg/dL)	9.20 ± 1.37	8.56 ± 2.16	8.88 ± 2.35	7.66 ± 2.7	K-W χ^2 = 15.246, p-value = 0.004218* (P<0.001)

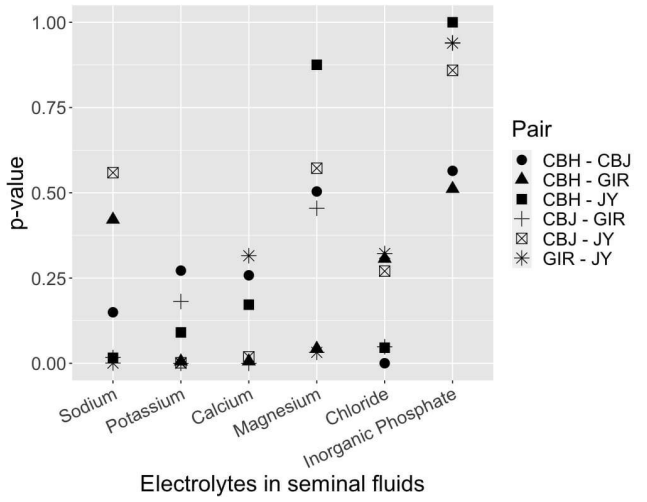

Fig. 2: Multiple comparisons of p-values adjusted with the Holm method for different electrolytes across various breeds were analyzed by using Dunn (1964) Kruskal-Wallis test. The Y-axis represents adjusted p-values (p.adj), while the X-axis illustrates the diverse electrolytes in each breed. Significance level ($p < 0.05$) is indicated for comparison. (JY; Jersey, CBJ: crossbred Jersey, CBH: crossbred Holstein)

Estimation of different enzymes in bovine seminal fluid

Table 4 showed the activity of different enzymes like AST, ALT, ALP and ACP of different bovine bulls. The highest value of AST (219.61 U/L) and ALP (143.41 U/L) were measured in the breed JY. CBH showed maximum value of ALT (23.05 U/L) and ACP (718.0 U/L), respectively. The lowest activity of all the enzymes except ALP had been found in the breed Gir.

Measurement of transaminase enzymes (AST and ALT) in semen is considered an indicator of semen quality because the appearance of these enzymes depends on the membrane stability of spermatozoa. The activity of transaminase enzymes in the seminal plasma depends on the damage to the sperm membrane and also due to the leakage of enzymes from spermatozoa (Gündoğan, 2006). The ALP and ACP are the two most active dephosphorylating enzymes present in semen, which is directly related to fertility. The major sources of ALP in seminal plasma are prostatic and epididymal origin, while a very small amount comes from the sperm cell itself in normal condition. ACP is mostly of prostatic origin, and its concentrations also reflect the functional status of the accessory sex glands and the metabolic activity of bull spermatozoa (Veerabramhaiah *et al.,* 2011). Kruskal-Wallis test for the multiple comparisons of p-values adjusted with the Holm method was performed to estimate the p-values of different intracellular enzymes. Results did not show any uniform pattern of significant differences. AST, ALT, ALP, and ACP varied significantly ($P<0.05$) in different breeds (AST: GIR-JY; ALT: CBH-GIR; ALP: CBH-JY, CBJ-JY, CBJ-GIR; ACP: GIR-JY; pACP: CBH-CBJ) (Fig. 3).

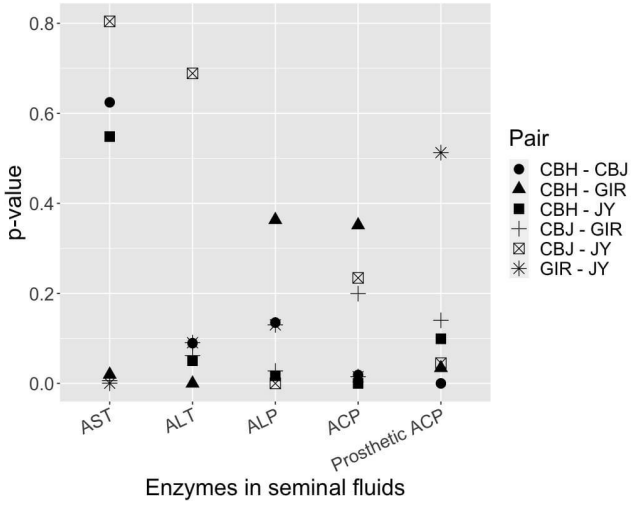

Fig. 3. Multiple comparisons of p-values adjusted with the Holm method for different intracellular enzymes across various breeds were analyzed by using Dunn (1964) Kruskal-Wallis test. The Y-axis represents adjusted p-values (p.adj), while the X-axis illustrates enzyme constituents in each breed. Significance level ($p < 0.05$) is indicated for comparison. (AST: aspartate transaminase, ALT: alanine transaminase, ALP: alkaline phosphatase and ACP: acid phosphatase, JY; Jersey, CBJ: crossbred Jersey, CBH: crossbred Holstein)

Table 4: Activity of different enzymes of seminal fluid of different breeds. The values were presented as Mean ± SE (n-6).

Parameter	GIR	Jersey	Crossbred Jersey	Crossbred Holstein	Kruskal-Wallis (K-W) rank sum test (df =3)
AST (U/L)	190.53 ± 5.64	219.61 ± 7.34	213.33 ± 7.23	215.98 ± 6.98	K-W χ^2 = 19.646, p-value = 0.000201* (P<0.001)
ALT (U/L)	11.35 ± 0.88	17.76 ± 1.64	18.47 ± 1.25	23.05 ± 3.46	χ^2 = 20.48, p-value = 0.0004014* (P<0.001)
ALP (U/L)	129.15 ± 4.72	143.41 ± 8.54	110.16 ± 2.31	124.27 ± 3.68	K-W χ^2 = 25.51, p-value = 0.0003972* (P<0.001)
ACP (U/L)	662.25 ± 14.78	565.00 ± 16.52	628.75 ± 12.54	718.00 ± 18.24	K-W χ^2 = 28.753, p-value = 0.00008774* (P<0.001)
Prosthetic ACP (U/L)	658.42 ± 8.21	662.05 ± 12.38	626.86 ± 14.58	713.59 ± 8.32	K-W χ^2 = 27.541, p-value = 0.0001545* (P<0.001)

Study of oxidative stress profile:

The ability of spermatozoa to undergo capacitation, acrosome reaction, and fusion to oocyte depends on the antioxidant and mineral profile of the semen. The antioxidant enzymes and others constituents of seminal fluid are involved in the protection against oxidative stress (OS) and OS-induced damage in the spermatozoa (Marzec-Wróblewska *et al.,* 2012).

The results of Table 5 represent the antioxidant profile of the seminal fluid of different breeds. The highest activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GP_X) were observed in Gir variety (410.13 mmol of H_2O_2 consumption/mL of sample/min), JY (261.35 mmol of H_2O_2 consumption/mL of sample/min) and CBJ (1298.01 mg of protein/dL), respectively (Table 5), while, the lowest value was found in CBH, and Gir (GPx), respectively (Table 5). These anti-oxidant enzymes have positive effects on sperm motility and semen quality. Several studies revealed that decreased concentrations and activities of free radical scavenging molecules increased the risk of oxidative stress, which is associated with decreased quality, viability, and fertilization capacity of semen (Tvrdá *et al.,* 2013a; Tvrdá *et al.,* 2012). Reduced glutathione (GSH) is an important free radical trapping molecule proving the nonenzymatic antioxidant system in the semen (Agarwal *et al.,* 2004). It has a positive effect on sperm viability and motility (Eskiocak *et al.,* 2005). The seminal fluid of the JY variety showed the highest level of GSH content, and the lowest value was observed in breed CBH (Table 5). The functions of different antioxidant enzymes are inter-correlated (Fig. 5). Lone *et al.* (2016) observed that SOD, CAT, and GPx activity drastically fell at post-thaw levels. Vince *et al.* (2018) reported that younger bulls showed higher activity of SOD and GPx during the summer season. However, younger bulls also showed better CAT activity and GSH content than in older bulls in the winter period.

Malondialdehyde (MDA) is the specific marker of LPO. The experimental results of MDA content showed that the JY variety carried the lowest value (16.30 µmol/mL of sample) followed by CBJ and others (Table 5). SOD and GPx were negatively associated with LPO (Gürler *et al.,* 2015). Tavilani *et al.* (2008) reported that MDA content is negatively correlated with the activity of antioxidant enzymes. Similarly, Kruskal-Wallis test had been performed to estimate the p-values of different antioxidant enzymes, GSH and MDA content. Results did not show uniform picture. Except SOD, other parameters (CAT, GPx, GSH, and MDA) varied significantly ($P<0.05$) in crossbred (CBH-CBJ) and purebred (GIR-JY) pair (Fig. 4). Moreover, significant differences ($P<0.05$) had also been observed in other pairs of breeds (Fig. 4).

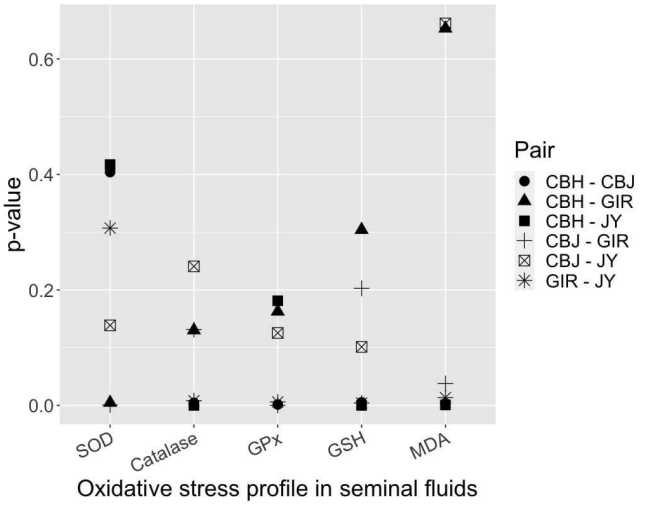

Fig. 4: Multiple comparisons of p-values adjusted with the Holm method for antioxidant enzymes, GSH, and MDA content across various breeds, were analyzed using Dunn (1964) Kruskal-Wallis test. The Y-axis represents adjusted p-values (p.adj), while the X-axis illustrates enzymes, GSH, and MDA content in each breed. Significance level ($p < 0.05$) is indicated for comparison. (SOD: superoxide dismutase, GPx: glutathione peroxidase, GSH: reduced glutathione, MDA: malondialdehyde, JY; Jersey, CBJ: crossbred Jersey, CBH: crossbred Holstein)

Table 5: Activity of SOD, catalase, GP_X and GSH and MDA content in seminal fluid of different breeds.

Parameter	Gir	Jersey	Crossbred Jersey	Crossbred Holstein	Kruskal-Wallis (K-W) rank sum test (df =3)
SOD (mmol of H_2O_2 consumption/mL of sample/min)	410.13 ± 12.36	396.06 ± 12.50	378.41 ± 13.35	360.82 ± 48.54	K-W χ^2 = 18.009, p-value = 0.0004381* (P<0.001)
Catalase (mmol of H_2O_2 consumption/mL of sample/min)	165.12 ± 3.46	261.35 ± 4.37	169.69 ± 4.54	153.12 ± 4.87	K-W χ^2 = 28.494, p-value = 0.00009903* (P<0.001)
GPx (U/mg of protein/dL)	998.59 ± 12.56	1184.47 ± 22.56	1298.01 ± 32.26	1088.94 ± 38.48	K-W χ^2 = 16.013, p-value = 0.001127* (P<0.001)
GSH (µmol/mL of sample)	0.11 ± 0.004	0.35 ± 0.001	0.13 ± 0.002	0.09 ± 0.001	K-W χ^2 = 30.876, p-value = 0.00003245* (P<0.001)
MDA (µmol/mL of sample)	23.03 ± 2.86	16.30 ± 2.34	17.73 ± 2.37	26.50 ± 2.56	K-W χ^2 = 27.159, p-value = 0.0001846* (P<0.001)

Spermatozoa contains high amount of polyunsaturated fatty acid, and they are highly susceptible to LPO by ROS. The primary source of ROS in bovine semen is dead spermatozoa via oxidation of aromatic amino acids (Juyena and Stelletta, 2012). ROS shows detrimental effects on spermatozoa, but it has some beneficial effects. Gonçalves *et al.* (2010) reported that in the bovine system, ROS plays an important role in sperm capacitation, acrosome reaction, and stabilization of the mitochondrial capsule in the midpiece. The beneficial effect of ROS depends on the concentration and nature of ROS. A fine balance is maintained between ROS generation and antioxidant activity. Any imbalance between ROS production and antioxidant activity leads to detrimental effects of ROS on spermatozoal function. Cryopreservation affects sperm viability and antioxidant capacity and increases oxidative stress (Aitken and Baker, 2004). Poor antioxidant capacity and high levels of oxidative stress promote the risk of LPO. Andrabi (2007) made a comparative study with fresh semen and cryoprserved semen and concluded that cryoprserved spermatozoa showed a shorter lifespan and poor fertility rate.

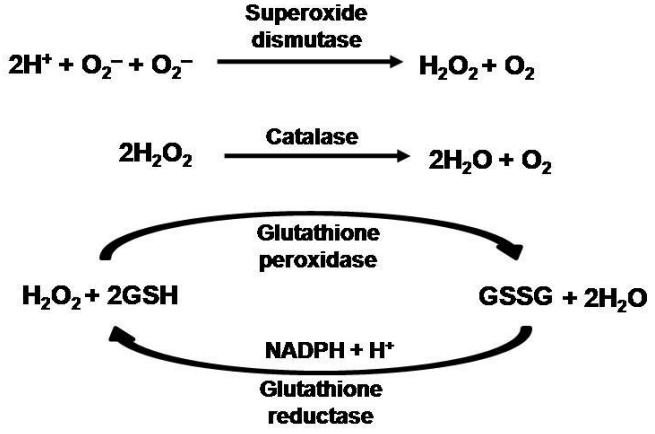

Fig. 5: Role of different antioxidant enzymes and reduced glutathione to scavenge ROS

Study of the production of reactive oxygen species (ROS), mitochondrial membrane potential (MMP) and DNA fragmentation:

The results of ROS, MMP and DNA fragmentation are given in Table 6 and Figures 6, 7 and 8 respectively. The Highest amount of ROS (1632.80 fluorescence count) was observed in the breed JY, followed by Gir, CBJ, and CBH (Table 6, Fig. 6). ROS is the byproduct of mitochondrial oxidative phosphorylation. The production of O_2^- is triggered by leakage electrons from the mitochondrial electron transport chain.

In the present study, the highest MMP was observed in the breed JY (30.57%) followed by CBH (25.45%) >Gir (15.73%) >CBJ (14.81%) (Table 6, Fig. 7). Mitochondrial membrane potential is positively associated with ROS production. Generally, low MMP had been observed when mitochondrial ATP production is in appropriate condition. Higher MMP indicates metabolic disorder and mitochondrial disintegration that induces greater amount of ROS production. Although the ROS production was the lowest (483.50 fluorescence count) in CBH, the MMP was slightly higher than other breeds like Gir and CBJ. This may be due to the poor activity of the antioxidant enzymes like SOD, CAT and GP_X (Table 5). Mitochondrial dysfunction hampers the functions of mitochondrial electron transport chain and decreases energy output, which helps to drop the MMP. Decreased activity of the mitochondrial respiratory chain triggers ROS production (Suski *et al.,* 2012). Additionally, the effect of freezing and thawing of bovine semen revealed that cryopreservation significantly reduced antioxidant capacity by 50% or more (Bilodeau *et al.,* 2000). Thus, it is assumed that the freezing/thawing process promotes oxidative stress and increases ROS generation, which exerts detrimental effects on spermatozoa.

Table 6: Represents the results of flocytometry analysis of bovine semen of different breeds collected from frozen semen straw. The data were presented as mean value.

Breed variety	ROS production (fluorescence count)	MMP (% of cells with polarized mitochondria)	DNA fragmentation (% of cells with DNA fragment)
Gir	1212.05	15.73	48.82
Jersey	1632.80	30.57	53.06
Crossbred Jersey	766.17	14.81	49.94
Crossbred Holstein	483.50	25.45	52.43
χ^2 test of 4-samples for equality of proportions (df = 3)		χ^2 = 10.37, p-value = 0.01567* (P>0.05)	^{2} = 0.48617, p-value = 0.9219 (P>0.05)

DNA fragmentation is a vital study to measure chromosomal integrity. The rate of DNA fragmentation is very close to each other among the different breeds (Table 6 and Fig. 8). The highest level of DNA fragmentation was observed in JY (53.06%), while the lowest value was found in breed Gir (48.82%). It indicated that above 50% of sperms contain intact DNA. Previously, several authors reported on low DNA fragmentation and higher value of intact DNA in different breeds of bovine bulls (Farooq *et al.*, 2015; Mukhopadhyay *et al.*, 2011; Batool *et al.*, 2012). Generally, DNA fragmentation depends on the level of ROS production and the activity of antioxidant enzymes (SOD, CAT and GP_X) and GSH content. ROS value was the highest in the JY breed, and its DNA fragmentation level was also higher than other breeds. The lowest value of DNA fragmentation was observed in the breed Gir, although its ROS production level is higher than CBJ and CBH. This result indicated that the antioxidant enzymes (Table 5) were not sufficient to scavenge ROS. The protective measures may be exerted by the antioxidant enzymes, which maintain the redox homeostasis within the cell. Table 5 shows the pattern of antioxidant profile among the breeds. Dissimilarities in the activity of antioxidant enzymes and GSH content are not capable of providing significant protection against ROS generation and DNA fragmentation. Moreover, there are some differences in genetic stability in different breeds. Thus, multiple factors are associated with DNA fragmentation and cell damage.

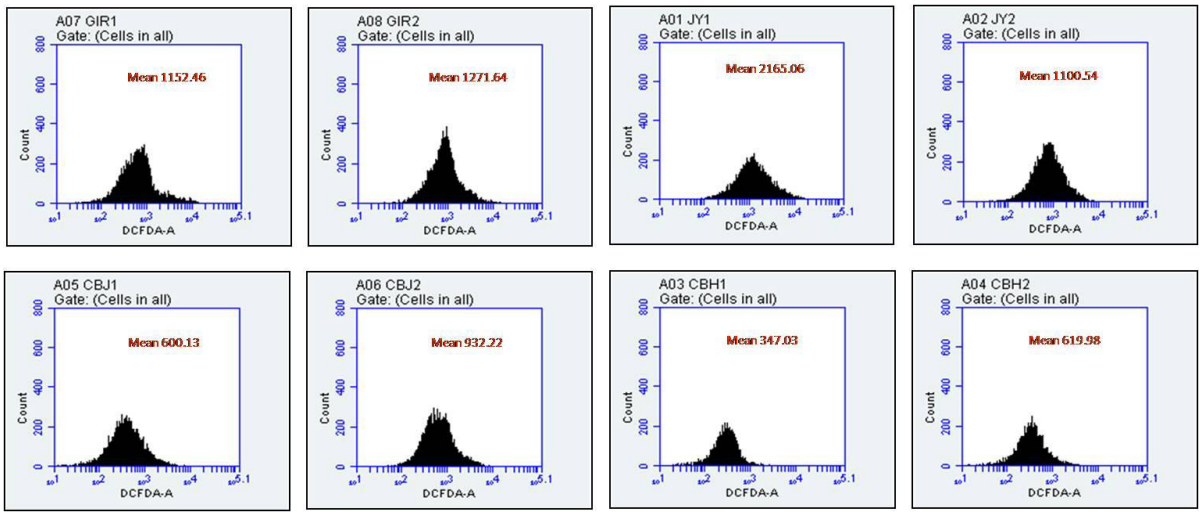

Fig. 6: Detection of ROS production by flocytometer. Mean value was given on the basis of expression of fluorescence of DCF-DA.

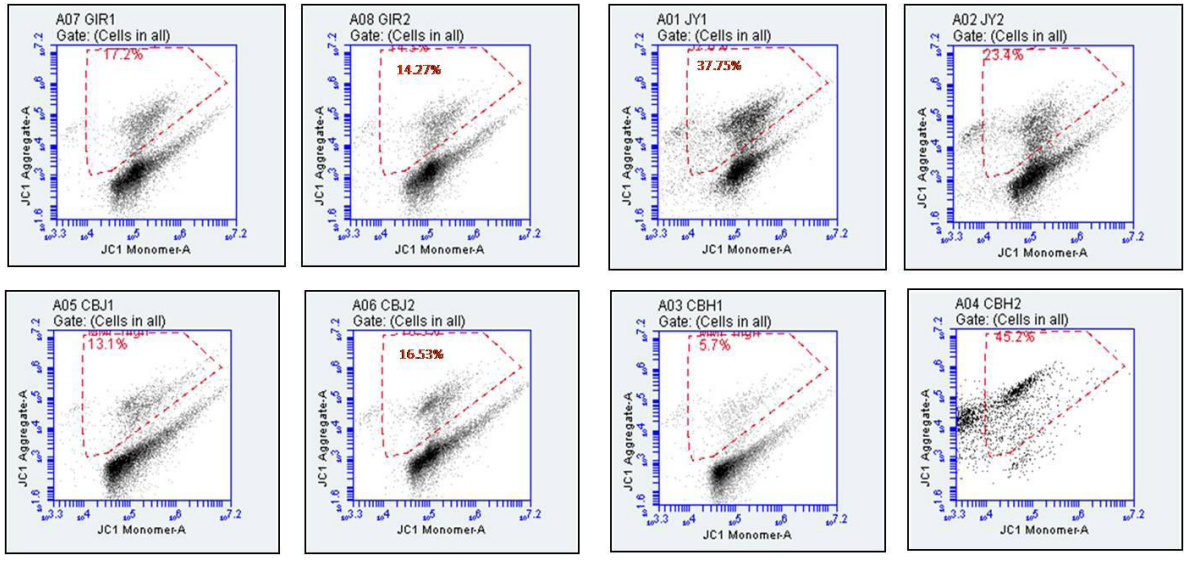

Fig. 7: Measurement of mitochondrial membrane potential (MMP) by flocytometry. The MMP was measured in terms of percentage (%) of cells having polarized mitochondria.

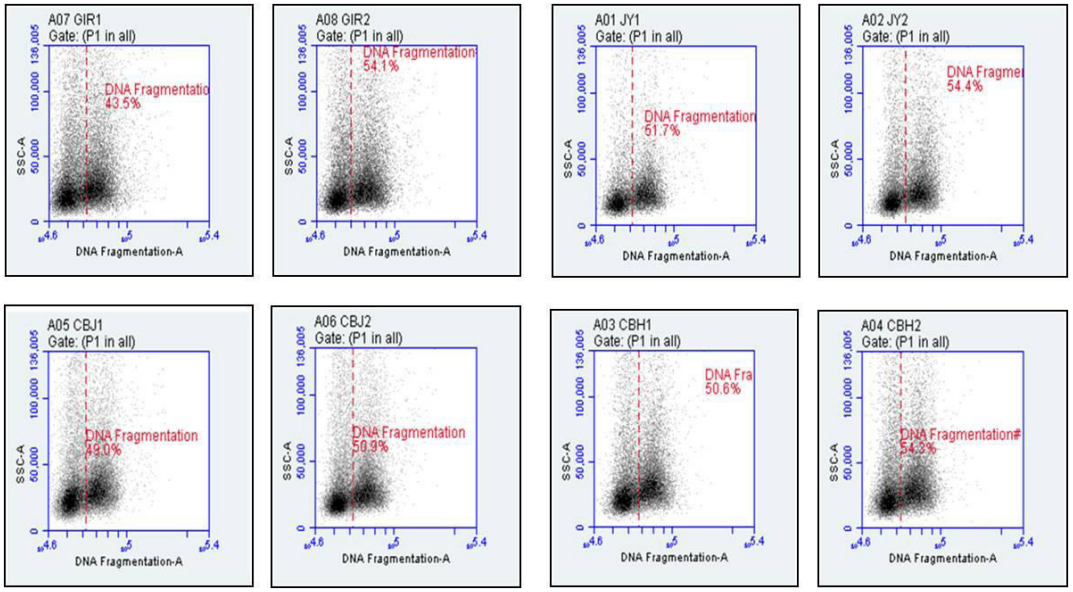

Fig. 8: Study of DNA fragmentation by flocytometry. Result was expressed by the percentage (%) cells showed DNA fragmentation.

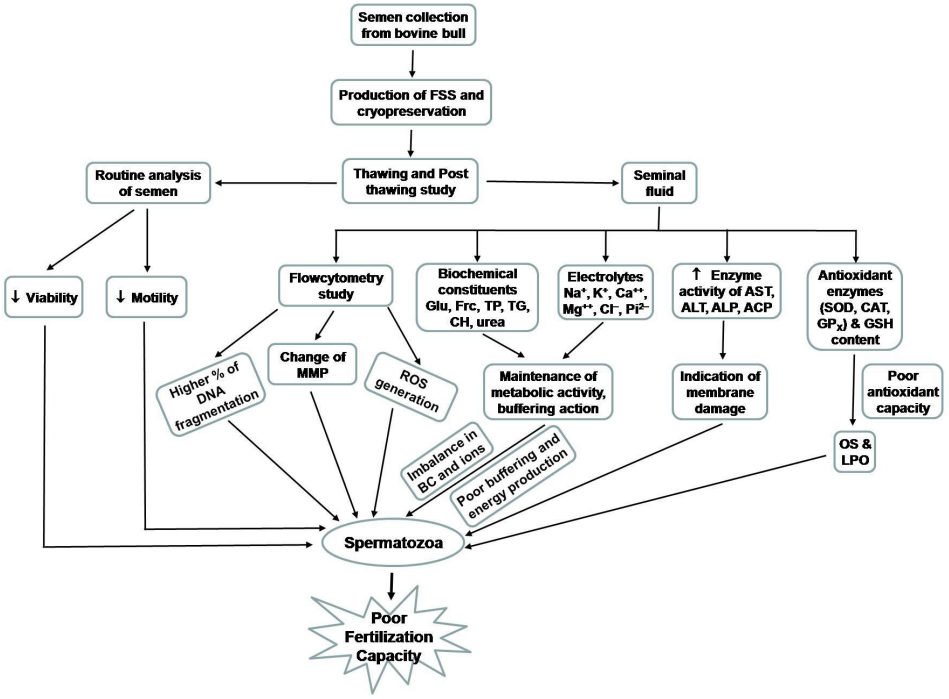

Fig. 9: Summary of role of different factors on spermatozoa after cryopreservation. ☐: Increase; ☐: Decrease; BC: Biochemical constituents; Glu: Glucose; Frc: Fructose; TP: Total protein; TG: triglyceride; CH: Cholesterol; LPO: Lipid peroxidation; MMP: Mitochondrial membrane potential; OS: Oxidative stress; ROS: Reactive oxygen species.

Semen can be stored up to 48 hours at 5-8 °C without a significant decline in sperm activity. Chilling of semen provides short-term storage with some adverse effects on the spermatozoa, including poor viability, motility, and integrity (Medeiros *et al.*, 2002). Cryopreservation is now an acceptable technique for sperm storage; however, this process is known to be detrimental to sperm functions. Lessard *et al.* (2000) reported that cryopreservation decreased sperm viability up to 50%, along with seven-fold low fertilizing capacity. Several authors reported that cryopreservation affects acrosomal activity, mitochondrial functions, sperm viability, motility, and DNA integrity

(Chaveiro *et al*., 2006; Coote e*rt al*., 2005; Wongtawan *et al*., 2006). Rapid cooling acts as a stressor that affects membrane structure and permeability (Lessard *et al*., 2000). Cold shock alters metabolic activity and increases oxidative stress, cellular damage, and sperm death. There are different types of cryoprotective agents such as glycerol, egg yolk, milk, bovine serum albumin, and polyvinyl alcohol. These are used as the extenders to protect the spermatozoa from detrimental effects (Raheja *et al.,* 2018). The effects of cold shock are irreversible, and they are not repaired during post-thawing conditions (Pommer *et al*., 2002). Finally, the role of different factors is summarized in Figure 9. Thus, this study would greatly be helpful in identifying the drawbacks of cryopreservation of semen and also be helpful to improve the success of the AI.

CONCLUSIONS

Sperm viability, motility and fertilization capacity depend on the number of viable sperm and the functions of seminal fluid. The present data indicated that the average value of sperm viability and motility was around 70% and 20%, respectively. Among the breeds, Jersey and crossbred Jersey showed good result. Jersey and crossbred Jersey also exhibited better results in case of biochemical constituents and antioxidant enzymes. There was a high level of ROS within the semen sample. DNA fragmentation rate was around 50%. Crossbred Jersey showed quite better result in this part.

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CONFLICT OF INTEREST

The authors have no competing interest to disclose.

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