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Rathod PG,
M.V.Sc. Scholar, Department of Veterinary Physiology & Biochemistry, Veterinary College, Kamdhenu University, Junagadh, Gujarat, India

Singh VK
Assistant Professor, Department of Veterinary Physiology & Biochemistry, Veterinary College, Kamdhenu University, Junagadh, Gujarat, India

Choravada DR
M.V.Sc. Scholar, Department of Veterinary Physiology & Biochemistry, Veterinary College, Kamdhenu University, Junagadh, Gujarat, India

Padodara RJ
Assistant Professor, Department of Veterinary Physiology & Biochemistry, Veterinary College, Kamdhenu University, Junagadh, Gujarat, India

Odedara AB
Assistant Professor, Department of Veterinary Physiology & Biochemistry, Veterinary College, Kamdhenu University, Junagadh, Gujarat, India

Solanki GB
Assistant Professor, Department of Veterinary Gynaecology & Obstetrics, Veterinary College, Kamdhenu University, Junagadh, Gujarat, India

Javia BB
Associate Professor, Department of Veterinary Microbiology, Veterinary College, Kamdhenu University, Junagadh, Gujarat, India

Corresponding Author:
Singh VK
Assistant Professor, Department of Veterinary Physiology & Biochemistry, Veterinary College, Kamdhenu University, Junagadh, Gujarat, India

Effect of white blood cells on physico-morphological parameters and lipid peroxidation of bovine spermatozoa during *in-vitro* culture

Rathod PG, Singh VK, Choravada DR, Padodara RJ, Odedara AB, Solanki GB and Javia BB

Abstract
Leukocytospermia is a condition in which a high count of white blood cells (WBCs) is present in semen. In this study, the cattle spermatozoa were *in-vitro* cultured in presence of cattle white blood cells to evaluate the effect of WBCs on physico-morphological (motility, viability, membrane integrity and acrosomal integrity) parameters and lipid peroxidation of bovine spermatozoa. A total of eighteen ejaculates were used to co-culture one million spermatozoa with, 0, 50, 500 or 5000 white blood cells (WBCs) per millilitre, respectively in Sperm-Tyrode's Albumin Lactate Pyruvate (Sp-TALP) media for 4 hours at 37 °C. The results showed that the presence of 5000 leukocytes/ million spermatozoa significantly ($p<0.05$) decrease physico-morphological parameters like viability, motility, membrane integrity and acrosome integrity and significantly ($p<0.05$) increase lipid peroxidation of Gir bull spermatozoa when cultured *in-vitro* for 4 hours. Hence, we conclude that 5000 or more leukocytes per million spermatozoa adversely affect physico-morphology and lipid peroxidation of bovine spermatozoa resulting in reduction in functional competence of bull semen.

Keywords: White blood cells, physico-morphological parameters, lipid peroxidation, bovine spermatozoa

1. Introduction

Leukocytospermia (LCS) or Pyospermia is a condition in which there is an abnormally high concentration (>1 x 10^6 /mL) of white blood cells (WBCs) in the semen (WHO, 2010). The role of leukocytospermia in the pathogenesis of male infertility remains controversial despite its relatively high incidence (10% to 20%) among infertile men. Different studies have correlated leukocytospermia with poor sperm quality and defective sperm function (Alvarez *et al.*, 2002) [4]. Leukocytospermia is considered an inflammatory disease. In most cases, the inflammatory syndrome is secondary to a urogenital bacterial disorder. However, other conditions may also lead to leukocytospermia, including viral infections, varicocele and trauma such as spinal cord injury (Lemkecher *et al.*, 2005) [13]. Leukocytes are the main source of reactive oxygen species (ROS) in semen, producing up to 1,000 times ROS than normal spermatozoa (Plante *et al.*, 1994) [21]. Globally, about 10-20% of infertile men have elevated seminal leucocyte concentrations caused by infections or inflammatory responses and other factors (Henkel *et al.*, 2007) [12].

Most leukocytes are suggested to originate from the epididymis and are present in very low count in semen of animals or human and probably play important physiological role in immune-surveillance and phagocytosis of abnormal spermatozoa. Among the various leukocytes, granulocytes are the most prevalent followed by macrophages and T-Lymphocytes (Aziz *et al.*, 2004) [5]. It is also reported that an abnormal high count of WBCs (leukocytospermia) affects quality and fertilizing potential of spermatozoa (O'Connell *et al.*, 2002) [17]. Although, it has been linked to increase rate of infertility but, there is uncertainty about its clinical significance (Brunner *et al.*, 2019) [8]. Leukocytospermia is a marker for underlying inflammation or possible infection, which negatively impacts spermatogenesis or maturation. While the origin of leukocytes in semen is not well understood, it is believed that macrophages and lymphocytes originate from the testicular in terstitium and epididymis, and neutrophils in the lower reproductive tract particularly the seminal vesicles and the prostate (Wolff, 1995) [25]. Physiological levels of ROS are required for normal sperm functions such as hyperactivation, capacitation and acrosome reaction.

Oxidative stress occurs in spermatozoa when levels of ROS (both extra and intracellular ROS level) exceed the available total antioxidant capacity Sperm have a limited amount of cellular cytoplasm in which scavenging enzymes are found, making sperm highly susceptible to ROS damage (Bansal and Bilaspuri, 2008) [6]. The reactive oxygen species can readily permeate the membranes and cause damage to macromolecules within the cell (Coyle *et al.*, 2006) [10].

Although, some foreign pathogens may generate ROS themselves, leukocytes are the most important source of seminal ROS (Pasqualotto *et al.*, 2000) [19]. These oxidants can damage cells and tissues and consequently have the potential to damage spermatozoa and alter sperm functions (Aitken, 1995) [2]. Neutrophil and macrophage oxidants may also damage sperm DNA with implications for fertility (Lewis *et al.*, 2013) [14]. Lipid peroxidation is the oxidative degradation of lipids by ROS. Since lipids form a major component of cell membranes, lipid peroxidation leads to breakdown of the membrane structure and leakage of cellular components. During oxidation, reactive oxygen species degrade cellular lipids into lipid peroxides that serve as indicators of oxidative stress in cells and tissues. However, these lipid peroxides are extremely unstable, have very short half-lives and cannot be measured directly (Ajina *et al.*, 2016) [3]. Ordinary antioxidants in semen include vitamin E, vitamin C, superoxide dismutase, glutathione and thioredoxin. These antioxidants neutralize free radical activity and protect sperm from ROS that already produced (Tremellen, 2008) [23]. Reports of leukocytospermia are scanty across the species in animals. Even in humans, leukocytospermia is an ill-defined and poorly understood condition affecting up to 30% of male factor infertility. Recently, a case report of leukocytospermia associated with seminal vesiculitis in stallions has been published (Oliveira *et al.*, 2020) [18]. Hence, the present study was designed to evaluate the effect of WBCs on physico-morphological parameters like viability, motility, membrane integrity, and acrosome integrity as well as lipid peroxidation of bovine spermatozoa during *in vitro* culture.

2. Materials and Methods

All the glassware, plastic ware and chemicals used in this study were from Broil, Tar sons and Hi media respectively, unless otherwise stated. The kits used in the study were procured from Hi Media, India. The semen samples used in this study was collected form apparently healthy Gir cattle bulls reared at Cattle Breeding Farm, Junagadh. All the bulls were reared following standard nutritional and manage mental practices. A total of eighteen semen samples (3 ejaculates form 6 bulls) with 3.5 or more mass motility score and above 80 percent individual motility were used in this study.

2.1 Washing of semen samples

Neat semen samples were washed two times with freshly prepared Sp-TALP (Sperm-Tyrone's Albumin Lactate Pyruvate) media containing 1% BSA. Briefly, 900 µL Sp-TALP was added to 100 µL semen sample in an Eppendorf tube and centrifuged at 280 g for 5 min. The supernatant was discarded and the pellet was re-suspended in sp-TALP media.

2.2 Swim up technique and adjustment of spermatozoa count

Swim up technique was used to isolate live motile spermatozoa from dead and immotile cells. Briefly, 1 mL washed semen sample was carefully placed beneath the rewarmed freshly prepared Spa-TALP media (2 mL) in a round bottom centrifuge tube. The tubes were then placed inclined at 45º in an incubator set at 37 °C for 60 minutes. After the incubation, the tubes were returned to vertical position and 1 mL of supernatant was collected from each tube using sterile pipette. By this method, motile and healthy sperm was separated from dead and unwanted cells (Henkel *et al.*, 2003) [11]. The concentration of sperm was determined using haemocytometer and diluted with Sp-TALP media to adjusted final concentration of 1 x 10^6 cells/mL for *In-vitro* culture.

2.3 Isolation of WBCs from whole blood

5 mL blood sample of cattle was collected from teaching veterinary clinical complex in a EDTA containing blood collection vial and mixed gently. In a 15 mL conical centrifuge tube 5 mL His opaque 1077 was added and 5 mL of blood was layered over it gently. The tubes were centrifuged at 400g for 30 minutes at room temperature. After centrifugation, layer above opaque interface was discarded and opaque interface having leukocytes was collected into collection tube and examined in Neuberger's chamber under microscope for counting.

2.4 *In vitro* culture of Spermatozoa with 0, 50, 500 or 5000 WBCs per millilitre

One million spermatozoa were cultured in Sp-TALP media containing 1% BSA with 0, 50, 500 or 5000 white blood cells per mL as control (C), Treatment 1 (T1), Treatment 2 (T2), and Treatment 3 (T3), respectively. The cultured cells were incubated for 4 hours at 37 °C and 95% relative humidity in a CO_2 incubator (LEEC Culture Safe CO_2 UK). Aliquots were prepared for the assessment of physico-morphological (viability, motility, mitochondrial membrane potential, plasma membrane integrity and acrosome integrity) and oxidative (lipid peroxidation, Glutathione S- Transferase and total antioxidant activity) parameters.

2.5 Individual Motility

At zero and four hours the spermatozoa motility was observed under microscope at 200X magnification in a 10 µL drop of sperm suspension on a pre-warmed glass slide duly covered with a cover slip. Short videos were recorded per field and the number of motile and non-motile sperm cells were counted to calculate percent motility.

2.6 Viability

The viability of cultured spermatozoa was assessed using Eosin-Nigrosin staining method. Briefly, 10 µL of diluted semen was mixed with 5 µL of 5% Eosin and 5 µL of 10% Nigrosin on a microscopic slide and incubated for 2 min at 37 °C. The Eosin-Nigrosin stained sperm were smeared on clean grease free glass slide and allowed to dry for 10-15 min. Prepared slides were examined under 400X of microscope.

2.7 Membrane Integrity

The hypo-osmotic swelling test was used to assess membrane integrity. In brief, 100 µL of diluted semen was added to 900 µL of hypo-osmotic solution (150 mOsm/L sodium citrate and fructose solution in distilled water) and incubated for half an hour at 37 °C After incubation, a small drop of semen suspensions was placed on clean, dry and grease-free glass slides and covered with cover-slip. The slides were examined under 400X magnification.

2.8 Acrosome integrity
Working Giemsa solution was prepared freshly before use by diluting stock solution (Hi Media, India) to a 1:20 dilution. Thin smears of semen were made on clean grease free glass slides, air dried, fixed and stained with 1:20 Giemsa solution for 60 minutes. Slides were washed under running tap water gently, air dried and examined under 1000X of light microscope.

2.9 Lipid peroxidation
Thiobarbituric acid reactive substances (TBARS) produced from cultured sperm with or without WBCs was measured with TBARS estimation kit (Hi Media) for lipid peroxidation following the instruction manual. The colour developed was measured spectrophotometrically for the standard and samples and lipid peroxidation of the samples were estimated.

3. Results and Discussion
3.1 Effect of white blood cells on physico-morphological parameters of Gir bull spermatozoa during *in-vitro* culture
3.1.1 Mean percent Viability
The Eosin Nigrosine (EN) staining (Image 1) of cultured Gir bull spermatozoa in control (C), treatment 1(T1), treatment 2 (T2) and treatment 3 (T3) groups at 0 hour and 4 hours are shown in table 1. The mean percent viability of cultured spermatozoa did not differ significantly (p>0.05) between the groups at 0 hour. However, a highly significant (p<0.01) decrease in mean percent viability was observed at 4 hours compared to 0 hour in respective groups. Furthermore, a highly significant (p<0.01) decrease in mean percent viability was observed in spermatozoa cultured with 5000 WBCs/mL as compared to all the groups at 4 hours. Our result is in accordance with Djordjevic *et al.* (2018) [27] and Castellini *et al.* (2019) [28]. Who also observed decreased viability in case of leukocytospermia? Similarly, Shi *et al.* (2011) [34] reported that, treatment of sperm cells with phorbol microstate acetate (PMA) and polymorph nuclear cells (PMN), after 4 hours of incubation, viability of cultured spermatozoa decreases. Their results showed that the respiratory burst of PMN accelerates the impairment of viability and plasma membrane integrity of human spermatozoa due to production of reactive oxygen species (ROS). The observed decrease in viability is probably due to the damaging effect of leukocytes on spermatozoa. Leukocytes are known to produce reactive oxygen species (ROS) that may cause change in plasma membrane permeability, lipid peroxidation, DNA damage and ultimately cell death.

3.1.2 Mean percent Motility
The mean percent motility of cultured Gir bull spermatozoa in C, T1, T2 and T3 groups at 0 and 4 hours are shown in table 1. The mean percent motility of cultured Gir bull spermatozoa did not differ significantly (p>0.05) between the groups at 0 hour. However, a highly significant (p<0.01) reduction in percent motility was observed following 4 hours *in vitro* culture in respective groups. At 4 hours, among the groups, a significant (p<0.01) decrease in percent motility of treatment 2 was observed compared to control. Furthermore, a highly significant (p<0.01) decrease in mean percent motility was observed in spermatozoa cultured with 5000 WBCs/mL as compared to all the groups at 4 hours. Our results are in accordance with Moubasher *et al.* (2018) [29] and Castellini *et al.* (2019) [28]. The damage of sperm motility by activated granulocytes was mainly mediated by the reactive oxygen species (ROS) like hydrogen peroxide (H_20_2) and superoxide (OH^-). In another study it was shown that the non-leukocytospermic patients had significantly higher sperm concentrations and a percentage motility than the groups of infertile patients with leukocytospermia (Aziz *et al.*, 2004) [5]. Inflammatory reactions enhance rigidity of sperm flagella membrane by reducing the lipid component of the membrane thereby sperm motility is decreased causing sperm agglutination and asthenozoospermias (Tremellen, 2008) [23]. Lackner *et al.*, (2010) [33] reported that the percentages of sperm with normal morphology and progressive motility were higher in semen samples with a leukocyte concentration between 0 and 1 X 10^6/mL than in samples with a leukocyte concentration> 1 X 10^6/mL. They also stated that all types of sperm deformities increased progressively with increasing leukocyte counts. In another study it was shown that Phorbol my ristate acetate (PMA) and polymorph nuclear cells (PMN) significantly inhibited sperm motility in a time-dependent manner that was similar to negative control groups (Shi *et al.*, 2011) [34]. Infertile men with leukocytospermia had observed a significantly lower progressive and total sperm motility percentage compared to the control group. Also, the sperm dynamic motility parameters were significantly lower in patients with leukocytospermia. These changes in sperm motility parameters correlated with the number of peroxidase positive leukocytes. Hence, it can be concluded that leukocytospermia has a significant impact on sperm dynamic motility patterns, in infertile men (Moubasher *et al.*, 2018) [29].

3.1.3 Mean percent Plasma membrane integrity
The mean percent plasma membrane integrity of cultured Gir bull spermatozoa was estimated by Hyposomotic swelling test (HOST; Image 3) and the means at 0 and 4 hours are shown in table 1. The mean percent plasma membrane integrity of cultured spermatozoa did not differ significantly (p>0.05) at 0 hour. However, there was significant (p<0.01) decrease in percent Hypoosmotic swell reacted spermatozoa (HOS reacted spermatozoa) at 4 hours in T1 and T3 groups following 4 hours of *in-vitro* culture. The mean membrane integrity was significantly (p<0.01) lower than all other groups in T3. Similar finding was observed by Kaleli *et al.* (2000) [30], in which they observed decreased hypoosmotic swelling test in leukocytospermic samples (P=0.015). Leukocyte count of 1-2 and 2-3 X 10^6/mL was linked with HOS test score 37.26±24.12% and 28.10±21.29% (p<0.0133, to key HSD). As already discussed in earlier sections that the leukocytes are responsible for generation of ROS that ultimately result in membrane damage is responsible for decreased membrane integrity.

3.1.4 Mean percent Acrosome integrity
The mean percent acrosome integrity of cultured Gir bull spermatozoa was estimated by 1:20 Giemsa staining (Image 2) at 0 and 4 hours and shown in table 1. The mean percent acrosome integrity of cultured Gir bull spermatozoa did not differ significantly (p>0.05) between the groups at 0 hour. But, at 4 hours, treatment 3 and treatment 2 group showed highly significant (p<0.01) difference as compared to control and treatment 1 group. Our results are in accordance with Aziz *et al.* (2004) [5] and Kaleli *et al.* (2000) [30]. The acrosome reaction was increased in leukocytospermic samples (p<0.015). Leukocyte count of 1 - 2 and 2 - 3 x 10^6/mL was linked with enhanced acrosome reaction (p<0.01, Tukey

HSD) 13.82±7.92 and 27.57±11.16, respectively (Kaleli *et al.*, 2000) [30]. Result of decreased acrosome integrity may be due to dual effect of leukocytes on spermatozoa. Firstly, they affect membrane integrity via a ROS mediated membrane damage and secondly, they may induce ROS mediated capacitation like changes and acrosome reaction in the spermatozoa.

3.2 Effect of white blood cells on lipid peroxidation of Gir bull spermatozoa

3.2.1 Lipid peroxidation (µM)

The table 1 shows the mean lipid peroxidation of cultured Gir bull spermatozoa of C, T1, T2 and T3 groups at 0 and 4 hours respectively. The mean lipid peroxidation of cultured Gir bull spermatozoa of T2 and T3 groups differ significantly ($p<0.05$) as compared to control and T1 groups at 0 hours. The lipid peroxidation increased significantly ($p<0.01$) at 4 hours compared to 0 hour in all the respective groups. The lipid peroxidation in T3 was significantly ($p<0.01$) higher in T3 group compared to all the groups at 4 hours. However, T2 showed significantly ($p<0.01$) higher lipid peroxidation than control and T1 at 4 hours. Similar observation was found by Trevizan *et al.* (2018) [31] and Ajina *et al.* (2016) [3]. Malondialdehyde (MDA), a stable product, is produced during lipid peroxidation (LPO) in spermatozoa. Formation of MDA can be assayed by the thiobarbituric acid (TBA) reaction which is a simple and useful diagnostic tool for the measurement of LPO for *in-vitro* and *in-vivo* systems (Sheweita *et al.*, 2005) [32]. The amount of lipid peroxidation take place in living cell can be estimated with the help of a TBARS estimation kit. Lipid peroxidation in the spermatozoa plasma membrane of Nellore bulls was higher ($p<0.05$) in the sperm of aged bulls compared to the young bulls. The percentage of sperm with oxidative DNA damage was higher in sperm of aged bulls, compared to young bulls ($p<0.05$) (Trevizan *et al.*, 2018) [31]. In another study, 10 x 10^6 spermatozoa with 1 x 10^6 polymorph nuclear WBCs incubated for 2 hours resulted in a significant increase of TBARS production over that in controls incubated without WBCs ($p<0.001$, paired T-test). TBARS production by spermatozoa also increased significantly after incubation with the xanthine-xanthine oxidase system ($p<0.01$, paired T-test), with ferrous sulphate and sodium ascorbate as a promoter or peroxidation ($p<0.01$). There was a concurrent decrease of polyunsaturated fatty acid (PUFA) in the phospholipids of spermatozoa under these conditions (P = 0.07, $p<0.01$, and $p<0.001$, respectively) (Zalata *et al.*, 1998) [35]. In infertile men, there was increase production of MDA (µmol/L) 1.58±0.43 as compared to fertile men 1.38±0.49. Production of malondialdehyde (MDA) and total antioxidant activity (TAA) in fertile and infertile men was significantly different as compared with each other (Ajina *et al.*, 2016) [3]. Leukocytes are responsible for production of ROS that result in oxidative peroxidation of lipids present on the plasma membrane resulting in membrane damage and lipid peroxidation. This ultimately increases membrane permeability and decrease functional competence of spermatozoa.

Table 1: Mean percent viability, motility, plasma membrane integrity, acrosome integrity and lipid peroxidation of *in-vitro* cultured Gir bull semen with 0 (C; Control), 50 (T1; Treatment 1), 500 (T2; Treatment 2) or 5000 (T3; Treatment 3) WBCs, respectively at 0 and 4 hours. Small letter super script indicates significant ($p<0.05$) difference between the column# Super scripts indicates significant ($p<0.05$) difference between the rows

Time	Treatment				P Value
	C	T1	T2	T3	
% Viability					
0 h	92.94±0.62$^{\#}$	92.17±0.70$^{\#}$	91.22±0.67$^{\#}$	90.17±0.65$^{\#}$	P=0.052
4 h	89.28±0.82^{b}	88.22±1.10^{b}	86.50±1.06^{b}	80.83±1.05^{a}	$p<0.01$
	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$	
% Motility					
0	88.61±1.33$^{\#}$	87.22±0.92$^{\#}$	85.83±1.09$^{\#}$	84.44±1.13$^{\#}$	P=0.06
4	77.22±1.23^{c}	71.67±1.07^{bc}	66.39±1.84^{b}	56.94±2.22^{a}	$p<0.01$
	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$	
% Plasma membrane integrity					
0	73.89±1.25$^{\#}$	71.72±1.04$^{\#}$	70.89±1.01$^{\#}$	70.17±0.86$^{\#}$	P=0.08
4	68.00±1.62^{b}	67.72±1.69^{b}	68.22±2.28$^{\#b}$	58.44±1.45^{a}	$p<0.01$
	$p<0.01$	P = 0.025	P = 0.146	$p<0.01$	
% Acrosome integrity					
0	95.50±0.57$^{\#}$	96.23±0.30$^{\#}$	95.50±0.34$^{\#}$	95.00±0.36$^{\#}$	P=0.47
4	88.67±0.86^{c}	86.30±1.03^{bc}	82.67±0.9^{ab}	79.89±1.25^{a}	$p<0.01$
	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$	
% Lipid peroxidation					
0	0.31±0.01$^{a\#}$	0.32±0.01$^{a\#}$	0.34±0.01$^{b\#}$	0.35±0.01$^{b\#}$	P=0.028
4	0.48±0.02^{a}	0.50±0.02^{a}	0.70±0.02^{b}	1.06±0.08^{c}	$p<0.01$
	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$	

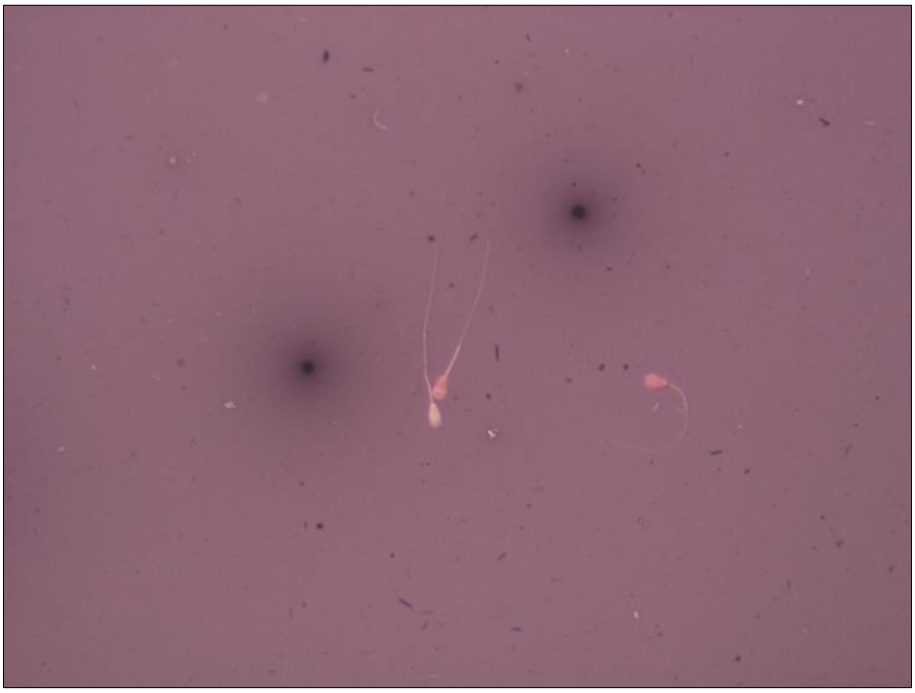

Fig 1: Eosin Nigrosin (EN) staining of spermatozoa, White cells are Live (L) and pink cells are Dead cell (D). (400X)

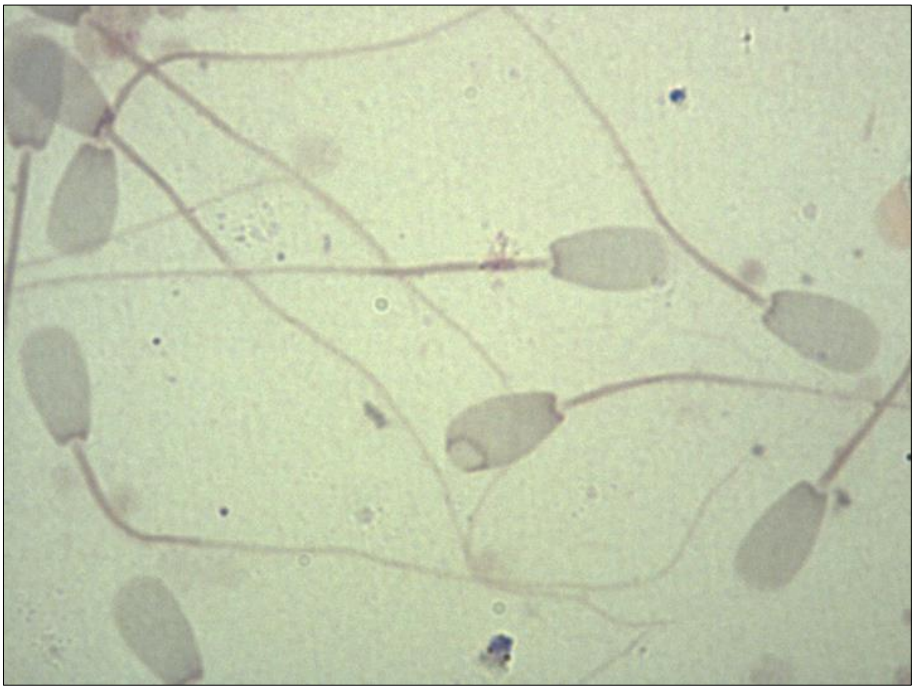

Fig 2: Semen smear, stained with Giemsa stain, Arrow indicates damaged acrosome. (1000x)

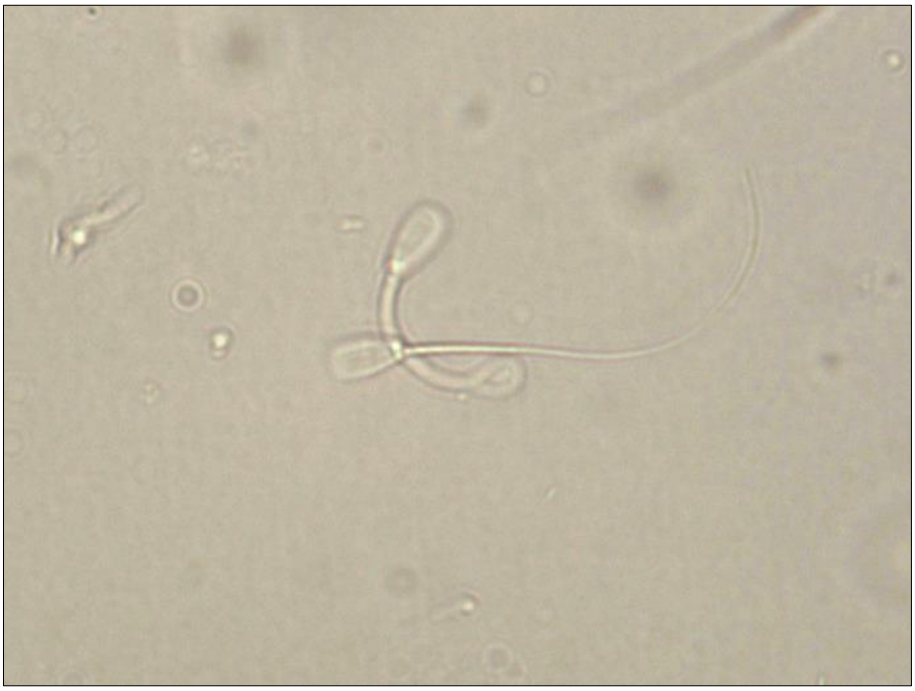

Fig 3: Smear of spermatozoa in hypoosmotic swelling test (HOST), curled tail indicates intact plasma membrane (IPM) and straight tail indicates damaged plasma membrane (DPM). (400X)

Conclusions

The presence of 5000 or more leukocytes /Million Gir bull spermatozoa affects physico-morphological parameters and significantly decreases motility, viability, plasma membrane integrity, and acrosome integrity and increase lipid peroxidation within 4 hours. Thus, leukocyte count 5000 or more/ Million spermatozoa affects physico-morphological parameters and cause oxidative damage resulting in reduction of functional competence of bull semen.

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Conflict of Interest: None.

References

1. Agarwal A, Rana M, Qiu E, AlBunni H, Bui AD, Henkel R. Role of oxidative stress, infection and inflammation in male infertility. Andrologia. 2018;50(11):1-13.
2. Aitken RJ. Free radicals, lipid peroxidation and sperm function. Reproduction, Fertility and Development. 1995;7(4):659-668.
3. Ajina T, Sallem A, Haouas Z, Mehdi M. Total antioxidant status and lipid peroxidation with and without *in vitro* zinc supplementation in infertile men. Andrologia. 2016;49(7):1-5.
4. Alvarez JG, Sharma RK, Ollero M, Saleh RA, Lopez M, Thomas A, *et al*. Increased DNA damage in sperm from leukocytospermic semen samples as determined by the sperm chromatin structure assay. Fertility and Sterility. 2002;78(2):319-329.
5. Aziz N, Agarwal A, Lewis-Jones I, Sharma RK, Thomas JAJ. Novel associations between specific sperm morphological defects and leukocytospermia. Fertility and Sterility. 2004;82(3):621-627.
6. Bansal AK, Bilaspuri GS. Oxidative stress alters membrane sulfhydryl status, lipid and phospholipid contents of crossbred cattle bull spermatozoa. Animal Reproduction Science. 2008;104:398-404.
7. Barati E, Nikzad H, Karimian M. Oxidative stress and male infertility: Current knowledge of pathophysiology and role of antioxidant therapy in disease management. Cellular and Molecular Life Sciences. 2020;77(1):93-113.
8. Brunner RJ, Demeter JH, Sindhwani P. Review of guidelines for the evaluation and treatment of leukocytospermia in male infertility. The World Journal of Men's Health. 2019;37(2):128-137.
9. Chen SS, Chang LS, Chen HW, Wei YH. Polymorphisms of glutathione S-transferase M1 and male infertility in Taiwanese patients with varicocele. Human Reproduction. 2002;17(3):718-725.
10. Coyle CH, Martinez LJ, Coleman MC, Spitz DR, Weintraub NL, Kader KN. Mechanisms of H2O2-induced oxidative stress in endothelial cells. Free Radical Biology and Medicine. 2006;40(12):2206-2213.
11. Henkel RR, Schill WB. Sperm preparation for ART. Reproductive Biology Endocrinology. 2003;1(1):1-22.
12. Henkel R, Maaß G, Jung A, Haidl G, Schill WB, Schuppe HC. Age-related changes in seminal polymorphonuclear elastase in men with asymptomatic inflammation of the genital tract. Asian Journal of Andrology. 2007;9(3):299-304.
13. Lemkecher T, Dartigues S, Vaysse J, Kulski O, Barraud-Lange V, Gattegno L, *et al*. Leucocytospermia, oxidative stress and male fertility: facts and hypotheses. Gynécologie Obstétrique Fertilité & Sénologie. 2005;33:2-10.
14. Lewis SE, Aitken RJ, Conner SJ, Iuliis GD, Evenson DP, Henkel R, *et al*. The impact of sperm DNA damage in assisted conception and beyond: Recent advances in diagnosis and treatment. Reproductive Biomedicine Online. 2013;27(4):325-337.

15. Lysiak JJ, Zheng S, Woodson R, Turner TT. Caspase-9-dependent pathway to murine germ cell apoptosis: mediation by oxidative stress, BAX, and caspase 2. Cell and Tissue Research. 2007;328(2):411-419.
16. Marí M, Cederbaum AI. Induction of catalase, alpha, and microsomal glutathione S-transferase in CYP2E1 overexpressing HepG2 cells and protection against short-term oxidative stress. Hepatology. 2001;33(3):652-661.
17. O'connell M, McClure N, Lewis SEM. The effects of cryopreservation on sperm morphology, motility and mitochondrial function. Human Reproduction. 2002;17(3):704-709.
18. Oliveira SN, Andrade JrLRP, Silva FMC, Araujo EAB, Rayashi RM, Segabinazzi LGTM, *et al*. Fractionated semen collection as a tool to rescue fertility in stallions with seminal vesiculitis. Theriogenology. 2020;157:110-120.
19. Pasqualotto FF, Sharma RK, Nelson DR, Thomas JrAJ, Agarwal A. Relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing infertility investigation. Fertility and Sterility. 2000;73(3):459-464.
20. Pisoschi AM, Negulescu GP. Methods for total antioxidant activity determination: a review. Analytical Chemistry. 2011;1(1):1-10.
21. Plante Mde, Lamirande E, Gagnon C. Reactive oxygen species released by activated neutrophils, but not by deficient spermatozoa, are sufficient to affect normal sperm motility. Fertility and Sterility. 1994;62(2):387-393.
22. Sarkar O, Bahrainwala J, Chandrasekaran S, Kothari S, Mathur PP, Agarwal A. Impact of inflammation on male fertility. Frontiers in Bioscience-Landmark. 2011;3:89-95.
23. Tremellen K. Oxidative stress and male infertility-a clinical perspective. Human Reproduction Update. 2008;14(3):243-258.
24. Wagner H, Cheng JW, Ko EY. Role of reactive oxygen species in male infertility: An updated review of literature. Arab Journal of Urology. 2018;16(1):35-43.
25. Wolff H. The biologic significance of white blood cells in semen. Fertility and Sterility. 1995;63:1143-1157.
26. World Health Organization, Laboratory manual for the examination and processing of human semen. 5th ed. World Health Organization: Geneva, Switzerland; c2010.
27. Djordjevic D, Rondovic G, Surbatovic M, Stanojevic I, Udovicic I, Andjelic T, *et al*. Neutrophil-to-lymphocyte ratio, monocyte-to-lymphocyte ratio, platelet-to-lymphocyte ratio, and mean platelet volume-to-platelet count ratio as biomarkers in critically ill and injured patients: which ratio to choose to predict outcome and nature of bacteremia?. Mediators of inflammation. 2018 Oct;2018.
28. Chiariotti P, Martarelli M, Castellini P. Acoustic beamforming for noise source localization–Reviews, methodology and applications. Mechanical Systems and Signal Processing. 2019;120:422-448.
29. Moubasher AA, Abdel-Sater MA, Soliman ZS. Yeasts and filamentous fungi associated with some dairy products in Egypt. Journal de mycologie medicale. 2018 Mar 1;28(1):76-86.
30. Kaleli, Semih, Fahri Öçer, Tülay Irez, Erdal Budak, Feridun Aksu M. Does leukocytospermia associate with poor semen parameters and sperm functions in male infertility?: The role of different seminal leukocyte concentrations. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2000; 89(2):185-191.
31. Toyer S, Trevizan F, Thiébaux S, Xie L. Action schema networks: Generalised policies with deep learning. In Thirty-Second AAAI Conference on Artificial Intelligence; c2018 Apr 26.
32. Sheweita SA, Tilmisany AM, Al-Sawaf H. Mechanisms of male infertility: Role of antioxidants. Current drug metabolism. 2005 Oct 1;6(5):495-501.
33. Zeller T, Wild P, Szymczak S, Rotival M, Schillert A, Castagne R, *et al*. Genetics and beyond–the transcriptome of human monocytes and disease susceptibility. PloS one. 2010 May 18;5(5):e10693.
34. Shi S, Richardson AE, O'Callaghan M, DeAngelis KM, Jones EE, Stewart A, *et al*. Effects of selected root exudate components on soil bacterial communities. FEMS microbiology ecology. 2011 Sep 1;77(3):600-10.
35. Zalata AA, Christophe AB, Depuydt CE, Schoonjans F, Comhaire FH. The fatty acid composition of phospholipids of spermatozoa from infertile patients. Molecular human reproduction. 1998 Feb 1;4(2):111-8.