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Determination of the seminal leucocytary profile in cattle and buffalo semen using the Leishman, and Papanicolaou staining protocols

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Abstract
Despite indications that seminal leucocytes can alter spermatozoa quality, investigations on the qualitative and quantitative parameters of such cells in bull semen, as well as methods for determining them are scarce. Therefore, the objective of this study was to assess the concentration of leucocytes in different breeds and age groups using the staining methods viz. Leishman and Papanicolaou stain. Semen ejaculates from two indigenous breeds (Sahiwal, Murrah) and one cross breed named Vrindavani were randomly collected. For the studies on effects of age, the ejaculates were divided into 2 groups, namely Group I, ejaculates from bulls less than 8 years of age (5 bulls x 6 ejaculates) and Group II, ejaculates from bulls more than 8 years of age (5 x 6 x 2 groups = 60 samples). All the samples are stained with Leishman and Papanicolaou stain for leucocytary counts. For the studies on effect of breed data was divided into 6 ejaculates x 3 bulls x 3 breeds (n=54). The concentration of leucocytes comprising mostly macrophages were found and was shown to be significantly higher in the Sahiwal breed (3.05±0.18) than other breeds and in Group II, more than 8 years old bulls (3.16±0.16) with Leishman stain. The study finds Leishman staining procedure better in terms of ease of doing and results obtained for leucocyte count than the Papanicolaou staining protocol.

Keywords: Leucocytary profile, Leishman stain, Papanicolaou stain

Introduction
Semen is a complex liquid suspension containing spermatozoa, secretions from the accessory sex glands, epithelial cells and varied concentration of leucocytes from the male reproductive tract. Thus, an ejaculate may contain one or more non-sperm cells such as round cells, leucocytes, epithelial cells, and immature germ cells in various developmental phases in addition to spermatozoa (Garner & Hafez 2000) [15]. In a typical semen report, they are referred to as "round cells", and thus are not differentiated into leucocytes or immature germ cells. According to the differential round cell counts, leucocytes make up 10-20% of the total round cells while immature germ cells making up 80-90% of the total round cells. Occurrence of large number of such non-spermatozoa cells are indicative of pathological conditions. For example, leukospermia, often referred to as pyospermia, is an anomaly that occurs when there are a large amount of leukocytes in the semen. According to the WHO recommendation 1999, the average normal human ejaculates may contain $<5x10^6$ round cell/mL while the number of leukocytes should be $<1x10^6$ round cell/mL. In the male reproductive system, leucocytes are produced at the epididymis and testicular parenchyma (Barratt *et al.* 1990) [7]. As previously shown (Kiesslings *et al.*, 1995) [20], a positive correlation exists between seminal leukocyte concentration and semen quality. This finding is supported by the fact that leukocytes aid in the removal of defective spermatozoa from the semen by phagocytosis (Tomlinson *et al.* 1992) [34]. In agreement, it was suggested that spermatozoa motility could be enhanced by increased seminal leukocyte concentration Ziyyat *et al.* (2008) [36].
Moreover, it was observed that spermatozoa motility increased in semen samples when the seminal leukocyte content was less than 1×10^6 /mL, but paradoxically, sperm motility decreased when the threshold of 1×10^6 /mL leucocyte was exceeded (Ziyyat *et al.* 2008) [36]. Such observations could be attributed to the fact that leucocytes are involved in the elimination of defective spermatozoa from the ejaculate, boosting the proportion of spermatozoa with normal morphology (Tomlinson *et al.*, 1992) [34].

Nevertheless, leukocytospermia was found to be positively correlated with spermatozoa tail abnormalities, acrosome damage, and high sperm deformity index scores (Aziz *et al.*, 2004) [6]. It was revealed that leucocytes show negative impact on the semen quality by the production of the free radicals which are toxic to the spermatozoa. This further leads to loss of integrity and stability of the cell membrane as well as in sperm chromatin ((Alvarez *et al.*, 2002) [3]. Due to changes in the immunological environment, seminal leucocytes in bulls are frequently linked to inflammatory, infectious, or degenerative events in the testis, epididymis, or accessory glands (Mcentee, 1990) [23]. However, the prevalence of these disorders often remains unreported in standard breeding soundness evaluations (Kennedy *et al.*, 2002; Fernandes and Moraes, 2009) [19, 11]. Excessive production of reactive oxygen species (ROS) by activated granulocytes is one possible method by which leucocytospermia may cause changes in sperm function. There has been discussion on how oxidative stress may cause changes in the way spermatogenesis is regulated, leading to structural flaws in the sperm (Gil-Guzman *et al.*, 2001; Ollero *et al.*, 2001) [16, 25]. Leucocytospermia and high levels of ROS have been demonstrated to induce chromatin modifications and DNA damage in sperm as measured by the sperm chromatin structure assay (Alvarez *et al.*, 2002) [3].

In bulls, average concentration of leucocytes was 4.7 x 10^6 /mL, with values ranging from 1.2 to 13.7 x 10^6 /mL (Zart *et al.*, 2014) [35]. Because sperm concentration in bulls is higher than other animals, a higher value for leucocyte concentration was expected. Such data could be interpreted more effectively when information about the concentration of leucocytes in the different breeds and age groups of bulls, as well as protocols for their evaluation are available, which unfortunately is lacking.

There are various methods that are frequently used to determine the leucocyte concentration in semen samples. Leishman stain is a fundamental, affordable, and non-invasive test for screening a large number of samples (Johanission *et al.*, 2000). In recent times, it is customary to apply papanicolaou staining (Johanission *et al*., 2000) to distinguish leucocytes from spermatids and spermatocytes. Other attempts employing the peroxidase ortho-Toluidine Blue method to distinguish neutrophils from degenerating spermatids have been made (Nahoum and Cardozo, 1980). Use of panleukocyte (CD45) immunocytochemical labelling to elaborate leucocyte differentiation (Homyk *et al*., 1990) has also been reported. The evaluation of the number of cells on a bi-dimensional scale makes leukocyte counting in Papanicolaou-stained smears merely a semi-quantitative approach. Therefore, the objective of this study was to explore the relationship between breeds and age groups of bulls' leucocytary profile using the Leishman and the Papanicolaou staining procedures.

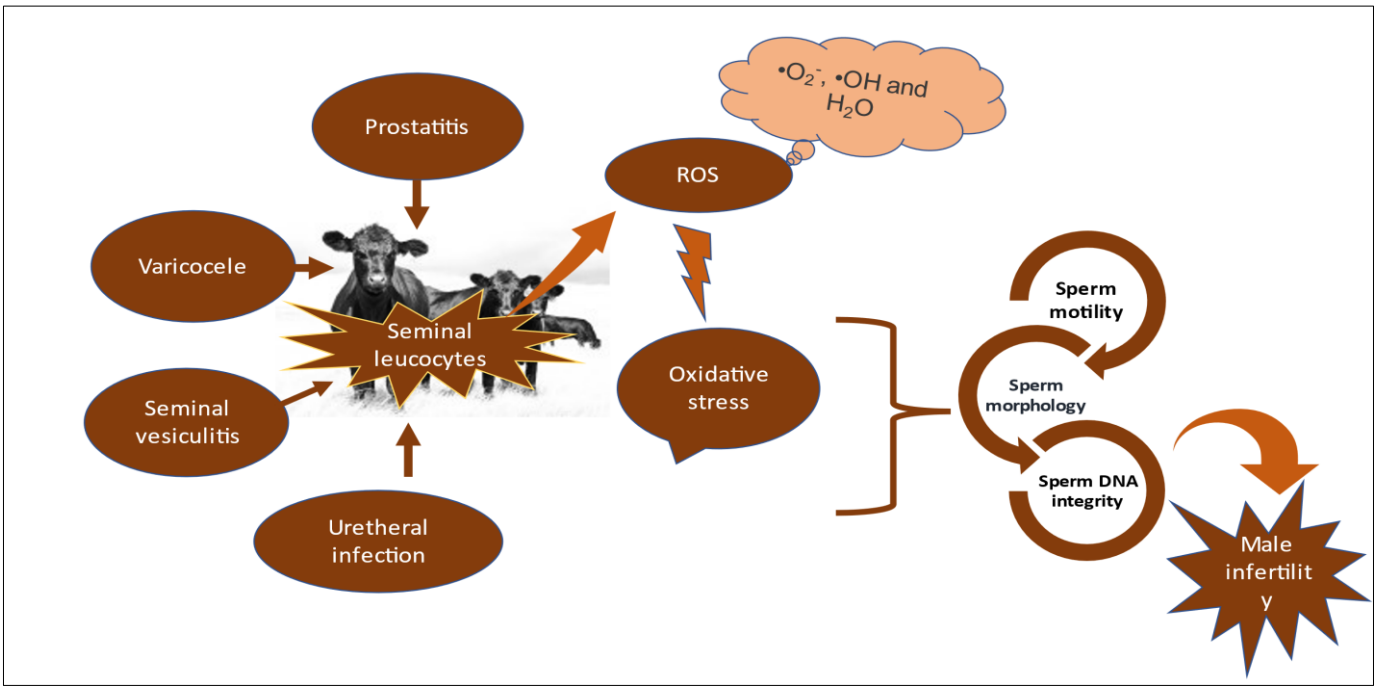

Fig 1: The major source of ROS in spermatozoa is leucocytes, which are activated mostly by inflammatory condition of the accessory gland, or urinary tract infection. Such leucocutes generate 1000 times more ROS than spermatozoa, resulting in male infertility.

Material and Methods

Experiment

The study aimed at the leucocytary profile of different breeds and age groups which were stained by Leishman and Papanicolaou stain. The studies were conducted on bulls kept at the Germ Plasm Centre, ICAR-Indian Veterinary Research Institute, Bareilly, India. The ejaculates were collected through artificial vagina method, and its mass motility (0–5) and initial progressive motility (0–100%) were assessed right away. The semen sample having mass motility >3 and initial progressive motility >70% were selected for further staining. A total 6 ejaculates x 3 bulls x 3 breeds from two indigenous breeds (Sahiwal, Murrah) and one cross breed (Vrindavani) were collected. For the studies with respective to age, the ejaculates were split into two groups, with Group I consisting of ejaculates from bulls younger than 8 years of age (5 bulls x 6 ejaculates) and Group II consisting of ejaculates from bulls older than 8 years of age (5x 6 x 2 groups = 60 samples). The obtained ejaculates were diluted in a 1:1 PBS solution before one small drop put on to the slides followed by staining with the Papanicalaou and Leishman stains.

Papanicolaou staining

A drop of diluted semen was placed on a slide and fixed in an equal mixture of ethanol 95% and ether for 15 minutes. Smear was then fixed for 30 seconds in ethanol 80%, which resulted in cell dehydration. The fixed sperm slide was immersed in Harris Haematoxylin for 4 min to impart the blue stain to the nucleus after being dipped in purified water for 30 seconds to rehydrate it. Nuclear haemotoxylin that was in excess was removed by washing with purified water. For destaining and to deplete non-specifically bound probe from the cytoplasm, 4-8 dips in Acidic ethanol was made. To reduce acidity and blue nuclear stain smear was washed with running cold tap water. The smear was immersed in Scott's solution for 4 min to return blue nuclear stain. For excess removal of stain, slide was dipped in running water. To dehydrate smear to permit ethanol-soluble orange G stain, smear was kept in ethanol 50%, Ethanol 80% for 30 s each and in final ethanol 95% for more than 15 min. Further, to stain the cytoplasm pink, smear was immersed in G-6 orange stain for 2min. For slow dehydration the stained smear dipped into ethanol 95% for 30 s for 15 times to allow the use of ethanol soluble mountants. The smear was then immersed in EA-50 green stain to stain cytoplasm pink. For slow dehydration, the stained smears dipped into ethanol 95% and 100% for 30 sec x 10 times to allow the use of ethanol soluble mountant. Finally, to allow the use of ethanol–insoluble moutants, slide was dipped for 2 min each in the 3 jars of xylene. After drying the smear was examined through a microscope's oil immersion lens.

Under microscopic view, the lymphoctes appears as multinucleated cells of diameter 8-12 micrometer, having bluish-green cytoplasmic borders after Papanicolaou staining. In contrast the spermatids present in the semen samples are usually degenerated and the cytoplasm takes pink colour after staining.

Leishman staining

Leishman stain was used to stain smears using the traditional staining technique (Johanisson *et al*., 2000) [18]. The smear was air dried for 10 min, followed by application of Leishman stain drop by drop and allowed to sit for 2 min. After that, double the amount of distilled water was added, blown with a pipette and allowed to sit at 37 ºC for 2 min. Finally, all of the slides were examined through a microscope's oil immersion lens.

Under microscopic view, leucocytes appears as round cells with bluish appearance after being stained with Leishman stain.

Stastical Analysis

The results were analysed by the graph Pad Prism 8.4.2 software. Data was collected and mean values were compared by the Oneway ANOVA.

Results and Discussion

In this study, the leucocyte count was performed on 20 random microscopic fields. This methodology, which has been previously recommended in routine semen evaluations in dogs and rams, proved to be efficient, practical and accessible in bulls as well. The lymphocytes mostly macrophages found in the Papanicolaou stain had a small cytoplasmic border with bluish green colour (Fig. 2) but were blue in Leishman stain (Fig. 3). The average numbers of leucocytes in human semen stained with Leishman stain was 0.4 to 1.2 million/mL (Patil *et al*., (2013) [27]. Macrophages make up 25% of all testicular stromal cells and are derived from the interstitial tissue of the testis and epididymis (Pelliccione *et al.,* 2009) [28]. In order to maintain the quality of the spermatozoa, semen contain a tiny amount of macrophages (Solis *et al*., 2003) [32]. The number of macrophages in the semen will dramatically rise in the presence of an infection in the male reproductive system or when the "blood-testis" barrier is compromised. According to a review study on the morphological distinctions between "round cells" in sperm (Johanisson *et al.*2000) [18], no difference in the size and shape of neutrophils as detected on peripheral blood smears and coloured sperm smears was found. The advantage being that the Leishman stain is routinely used in the laboratories for staining blood smears and the pathologists are well versed with the methodology.

Papanicolaou stain is used in the leucocyte counts for blood smear, however, no morphological difference in leucocytes was found in semen (Johanisson *et al.,* 2000) [18]. Papanicolaou staining uses over 12 distinct chemicals, some of them can result in significant hypoosmotic conditions causing shrinkage in cells from a variety of species, including humans (Baszewska *et al*., 2015; Andraszek *et al*., 2018) [8, 5]. The Papanicolaou staining methods to identify White Blood cells (WBC) in semen is labour-intensive, demand a skilled reader, and are not always accurate because some types of immature germ cells still resemble WBC even after staining.

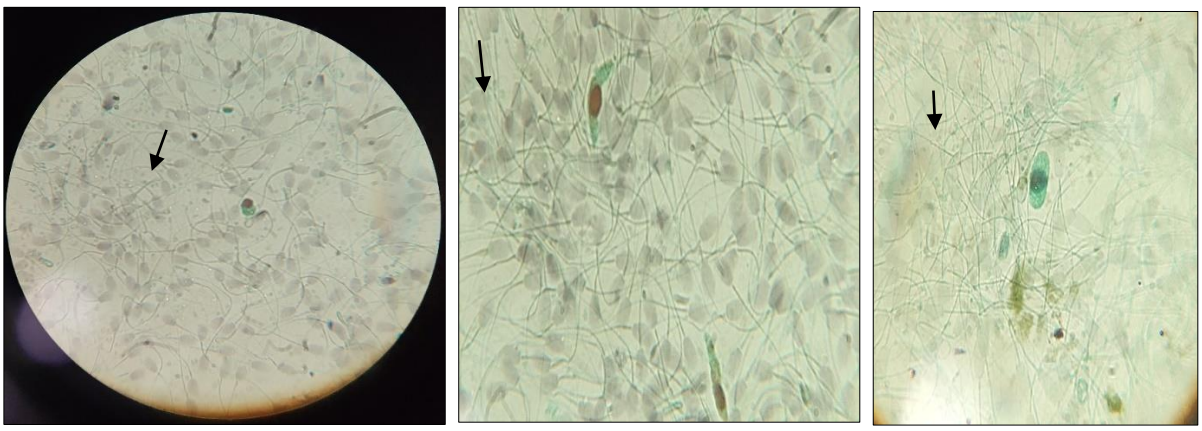

Fig 2: Microscopic view of leucocytes (macrophages) under 100x magnification using PAP stain. Leucocytes appear as bluish -green round cell in stained semen smear

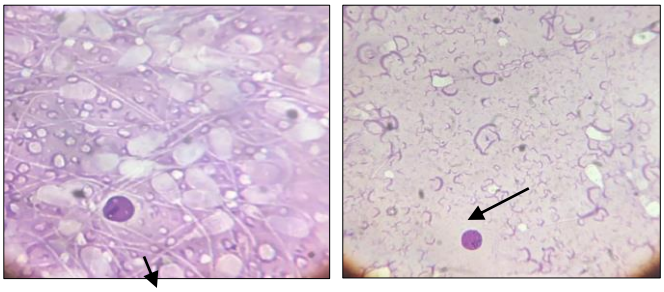

Fig 3: Microscopic view of leucocytes under 100x magnification using Leishman stain. Leucocytes (macrophages) appears as bluish round cell in stained semen smear

Table 1 shows significantly higher leucocyte count in Sahiwal (3.05±0.18) with Leishman stain than Murrah (2.88±0.21) and Vrindavani (2.38±0.16). Similarly, in papinicolaou stain the Sahiwal shows significant higher leucocytes count (2.61±0.14) than Murrah (2.33±0.14) and Vrindavani (2.16±0.16).

The mean concentration of leucocytes in cross breed bulls was 4.7 x 10^6 /ml with the value ranging from 1.2-13.7x 10^6/ml which is in agreement (Zart *et al*., 2014) [35]. Sperm concentration was the only characteristic that was higher in zebu bulls compared with the taurine and crossbreed, which is in agreement with the findings of Silva *et al.* (2009) [31]. It could be attributed to higher spermatozoa concentration in these bulls than in males of other species.

Table 1: Leucocyte count in different breeds with different stains

	Breed		
	Sahiwal	**Murrah**	**Vrindavani**
Leishman	3.05±0.18	2.88±0.21	2.38±0.16
Papanicolaou	2.61±0.14	2.33±0.14	2.16±0.16

In the Leishman stain, the results demonstrates significantly (p<05) greater number of leucocytes in Group II than Group I (Table 2). Similarly, in the Papanicolaou staining procedure, Group II exhibit significantly greater leucocyte count than Group I. The Leucocytes profile was examined in the group containing 'more than 8 years old bulls' was 3-7x10^6 leucocytes/mL whereas in group containing 'less than 8 years old bulls' it was 1-2 x10^6 leucocytes/mL (Zart *et al.*, 2014) [35]. In a similar study the leucocyte count was highest in 3 year old rams which was significantly different (p<0.005) from 1, 2 and 4 year old rams (Fontbonne 2011) [12]. There was no apparent pattern in the change in leucocyte with increasing age. In contrast Egbe-Nyiwi *et al.* (2000) [10] found that the age had no effect on the leucocyte in rams.

Table 2: Leucocytes count in different age group of bulls

Age	**Stain**	
	Leishman	**Papanicolaou**
Group I(Age<8years)	3.16^b±0.16	2.44 ± 0.14^a
Group II(Age > 8 years)	3.72^b±0.21	$2.61±0.21^a$

The Sprencher *et al*. (1999) [33] observed that 75% of bull spermatozoa samples had up to 1 leucocyte per field. Although the authors did not find a link between spermatozoa quality and the amount of leucocytes in the sperm, they do recommend that samples with more than 5 leucocytes per field be sent for bacterial culture, especially if the percentage of normal spermatozoa is low. In rams, the presence of more than 5 leucocytes per field (400x magnification) is considered diagnostic of reproductive system infection or inflammation, particularly epididymitis triggered by ovine brucellosis. Previously, Kott *et al.* (1988) [21] found that 92% of rams infected with Brucella ovis had more than 10 leucocytes per field. Paolicchi *et al*., (2000) [26] reported >5 leucocytes/field in 71.4% of rams infected with B. ovis. Normal canines typically have six leucocytes per field. When leucocyte count increases than this count a bacterial culture of the ejaculate is indicated (Kustritz 2007; Fontbonne 2011) [22, 12].

The association between leucocytes and seminal parameters appears to be remarkably complex, involving a number of factors such as seminal plasma antioxidant capacity (Sharma *et al*. 1999) [30], the production of pro-inflammatory cytokines (Fraczek and Kurpisz 2007) [14], and, most importantly, the release of free radicals (De Lamirande and Gagnon 1992) [9]. Whereas spermatozoa create reactive oxygen species (ROS), macrophages and neutrophils are the primary producers of free radicals in the spermatozoa. When free radicals come into contact with a sperm cell, they cause lipid peroxidation of the plasma membrane, which not only affects membrane stability but also enters the cell, damaging mitochondrial DNA and reducing intracellular ATP generation (Aitken 2002; Pentyala *et al*., 2007) [1, 29]. It is reported that spermatogenesis and spermatozoa maturation can be hampered by leukocytospermia. Moreover, subclinical genital tract inflammation can disrupt spermatogenesis by changing cytokine levels, which in turn impairs sertoli cell activity.

Low amounts of ROS, on the other hand, are involved in controlling the activity of essential proteins required for spermatozoa cell differentiation and functionality (Andrabi 2007) [4], and thus to enhance sperm capacitation and acrosome reaction (Ford 2004; Aitken and Bennetts 2006) [13, 2]. As a result, it is obvious that leucocytes have a dual effect on spermatozoa, and the repercussions appear to be connected to the number and timing of contact between these cells and the spermatozoa. It has been demonstrated that leukocytospermia hinders the fertilization potential of spermatozoa by interfering with the acrosome reaction and the fusion of sperm and egg (Aziz *et al*., 2004) [6].

Conclusion

Since the major kind of leukocyte in spermatozoa produce reactive oxygen species that might decrease spermatozoa motility, a substantial link between leukocytospermia and spermatozoa tail function abnormalities can be anticiopated. This necessitates the evaluation of leucocyte in the semen to pin point loss of spermatozoa freezability and quality. Of the two methods for determination of leucocyte count evaluated in the study Leishman stain was found to be better than the Papanicolaou staining procedure.

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