Advancements in Semen Separation Methods for Buffaloes

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The livestock industry is crucial to the nation's economy, making substantial contributions to agriculture, rural sustenance, and ensuring food security. The native water buffalo (*Bubalus bubalis*) plays a crucial role in India's agricultural economy, with the country leading globally in both buffalo production and population. Nevertheless, reproductive techniques utilized in this sector encounter numerous challenges including issues like silent heat, inadequate oestrus detection, anoestrus, seasonal infertility, extended calving intervals etc. Addressing these limiting traits specific to the female component can be achieved through the use of high-quality semen, which also needs to be optimized for fixed-time insemination. Under in vivo conditions, potentially fertile spermatozoa undergo separation from immotile spermatozoa, debris, and seminal plasma in the female genital tract via active migration through the cervical mucus. This process not only selects progressively motile spermatozoa but also induces physiological changes known as capacitation, which are essential for the sperm's functional competence, including acrosome reaction. The advent of assisted reproduction, particularly in vitro fertilization (IVF), in the 1980s spurred the development of various sperm separation methods.

Table 1: Mean volume of the ejaculate in Indian buffalo breeds

Breed	Volume (mL)
Banni	4.09 (± 1.59)
Bhadawari	4.11 (± 1.57)
Jaffarabadi	5.10 (± 1.80)
Murrah	4.48 (± 1.87)
Pandharpuri	4.79 (± 1.80)
Surti	4.68 (± 1.73)

Buffaloes and cattle bulls, both considered large ruminants, exhibit anatomical and sperm characteristic differences. Notably, these include variations in ejaculate volume, concentration, pH, sperm density, and abnormal sperm percentages. Additionally, there are significant differences in their capacity for in vitro fertilization and subsequent embryo development. The average ejaculate volume in genetically characterized Romanian buffaloes was determined to be 4.07 (± 0.02) mL, which is lower compared to other indigenous buffalo breeds (Table1).

Sperm separation methods

In addition to various enrichment protocols, sperm separation techniques (Fig 1) have become valuable tools in the realm of reproductive biology and assisted reproduction. The optimal method for separating sperm should be swift, easy and cost-effective while maximizing the isolation of viable spermatozoa. It should avoid inducing damage or non-physiological changes to the sperm cells, while effectively removing dead spermatozoa, leukocytes, bacteria, and harmful substances such as decapacitation factors or reactive oxygen species (ROS). Additionally, it should have the capacity to process larger volumes of ejaculates.

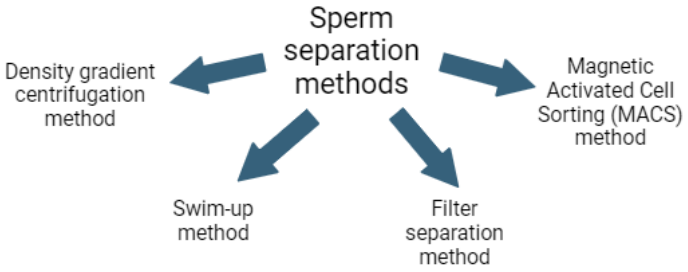


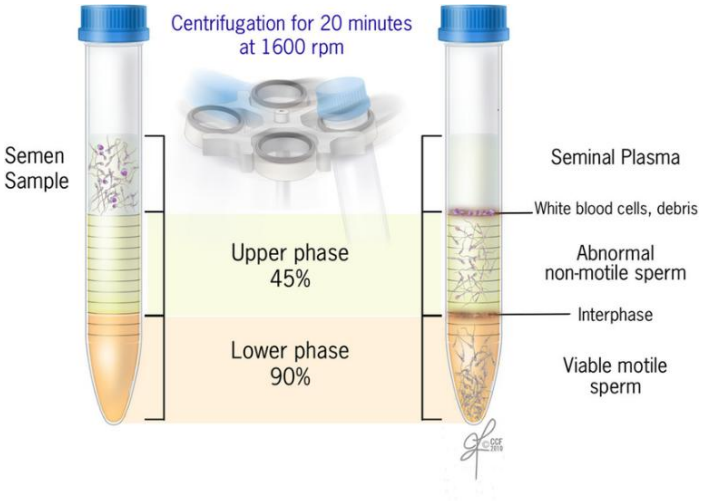
Fig. 1: Different sperm separation methods

Density gradient centrifugation method

Various density gradients have been assessed for selecting bovine sperm, aiming to obtain samples with increased motility while maintaining intact gene expression, acrosomal membrane, and DNA integrity. Percoll gradient centrifugation uses the higher density of sperm cell nuclei to make them settle in the denser part. Additionally, research in bovines demonstrated that highly motile sperm cells align more faster with centrifugal forces, resulting in rapid deposition. Percoll density gradient technique also increases the proportion of viable X-bearing sperm by as much as 70%, while maintaining the integrity of the sperm membrane and acrosome, supports its application for sexed semen in livestock reproductive studies. In buffalo, enriched semen following Percoll treatment

was also investigated for the separation of X and Y sperm, with later evaluation of its impact on sperm quality compared to filtration methods or traditional swim-up techniques. The usage of taller and denser column could make it harder for the sperm cells to move, making them stay in contact with Percoll for longer. This could lead to some changes, like more capacitation and earlier acrosome reactions. So, it's possible that using smaller volumes of Percoll gradients might cause less damage to the acrosome. Therefore, when using large volumes of density gradients in assisted reproduction, we should consider the chance of this kind of acrosomal damage.

Fig. 2: Density gradient centrifugation method (Image taken from Malvezzi et al; 2014)



The swim-up method

First described in 1984, swim-up (SU) works based on the ability of moving sperm to swim towards a medium without cells typically positioned above the sample. Researchers evaluated SU's usefulness and its impact on sperm quality in buffaloes, finding that it outperformed other separation techniques for certain important traits needed in assisted reproductive technology (ART). Previous studies found that sperm separated using the swim-up (SU) method showed better motility and significantly higher motility index compared to density gradient centrifugation (DGC), although the recovery rates were generally lower. Additionally, analysis of cleavage rates suggested that SU might be more suitable for in vitro fertilization (IVF) (with significant differences in cleavage rate and cleavage index $p < 0.05$). A modified swim-up (SU) method has been confirmed for sperm sexing, with a notably higher recovery rate for sperm carrying the X chromosome compared to those carrying the Y chromosome. Although both X and Y groups showed higher motility index and acrosome integrity rates compared to the control group, the progressive motility of the unseparated samples was higher. However, these findings were observed before freezing, and post-thawed samples subjected to the modified SU method actually showed superior progressive motility.

Fig. 3: Swim-up method (Image taken from Agarwal and Sharma; 2016)

Filter separation method

The glass wool filtration involves a meticulous protocol wherein a tuberculin syringe is cut and filled with 15 mg of glass wool, followed by flushing with medium to remove debris. The ejaculate is washed, and the pellet is resuspended and laid onto the glass wool for gravity filtration. This technique exploits the altered plasma membranes of dead or damaged spermatozoa, leading to their binding with glass fibers and enhancing filtration efficacy. Notably, the type of glass wool used significantly impacts the method's success, as evidenced by its superior results compared to colloid centrifugation in stallions and buffaloes. Moreover, glass wool filtration enables the recovery of higher proportions of motile spermatozoa compared to other methods like swim-up or density gradient centrifugation.

Sephadex gel filtration utilizing specific pore sizes of dextran gel columns, is effective in trapping spermatozoa with damaged membranes. Comparative studies highlight the superiority of

Sephadex filtration over other methods in enhancing sperm quality and fertilizing ability across various species. Notably, Sephadex filtration outperforms swim-up and glass wool filtration in terms of total and motile sperm recovery rates and post-filter quality in buffalo, bovine, and boar semen samples. Additionally, the choice of Sephadex grade and buffer type significantly influences the filtration outcomes, further emphasizing its versatility and efficacy in sperm separation and enrichment.

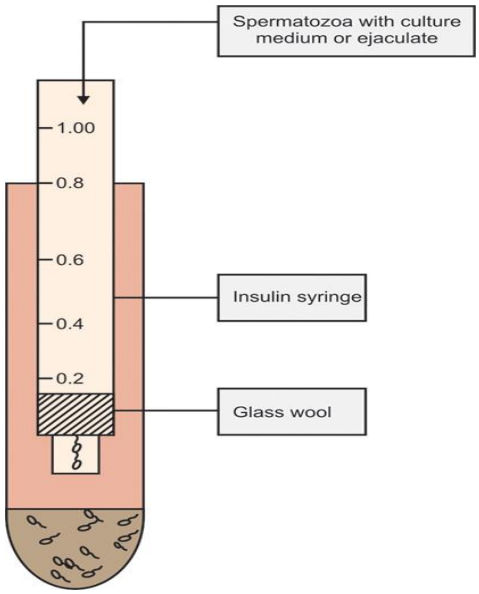


Fig. 4: Glass wool filtration method (Image taken from Malik and Ved; 2007)

The magnetic-activated cell sorting (MACS) method

This method addresses the lack of correlation between sperm density and apoptosis observed in density gradient centrifugation (DGC), potentially leading to unsuccessful fertilization. By exploiting phosphatidylserine (PS) externalization, MACS separates apoptotic and non-apoptotic spermatozoa using annexin V-conjugated microbeads. Combining MACS with DGC enhances sperm quality and function, as indicated by improved motility, viability, and reduced apoptotic markers. The annexin-negative fraction obtained through MACS + DGC exhibits minimal cell loss and expresses fewer apoptotic markers compared to DGC alone. Furthermore, MACS + DGC significantly reduces sperm DNA fragmentation, mitochondrial membrane potential disruption, and PS externalization. The increased sperm recovery rates observed with MACS + DGC underscore its superiority over DGC alone, particularly in producing motile, viable, and non-apoptotic spermatozoa. This combined technique shows promise even in cases of teratospermic, asthenozoospermic, and oligoasthenozoospermic conditions, offering improved DNA integrity and functionality and potentially enhancing the production of good quality embryos in assisted reproduction.

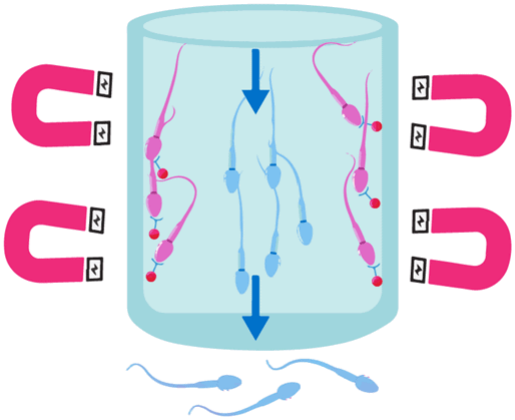


Fig. 5: The magnetic-activated cell sorting (MACS) method (Source: https://www.invitra.com/en/annexin-v-column-macs/)

Conclusion

Advancements in buffalo reproduction have led to new avenues for diagnosing and treating subfertility, particularly in sperm analysis. Traditional sperm sorting methods, often involving centrifugation, raise concerns about oxidative stress and cell damage. Quantitative assessment of sperm motility, morphology, and genetics aids in diagnosing male infertility and selecting optimal sperm for assisted reproductive technologies (ART). Various buffalo sperm selection methods have pros and cons, with conflicting results raising questions about their clinical relevance. Sperm-up (SU) shows higher motility index than density gradient centrifugation (DGC), but with lower recovery rates. Magnetic-activated cell sorting (MACS) combined with DGC yields higher recovery rates than DGC alone. Sperm separation techniques improve the freezability of low-quality buffalo ejaculates, but research in veterinary medicine, especially in buffaloes, is still limited.

Further investigation is needed to fully understand the efficacy and clinical applicability of these techniques in buffalo reproduction and male fertility enhancement. In summary, while semen separation techniques show promise, their widespread use requires thorough validation and research.

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