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Buffalo Bull Fertility: Key Factors and Predictive Markers

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ABSTRACT

Buffalo bull fertility is pivotal to reproductive success and genetic improvement in the dairy sector, especially in regions with high buffalo populations such as India. Despite their economic importance, reproductive inefficiencies, most notably low conception rates after artificial insemination, remain major challenges. Male fertility is a complex trait influenced by sperm quality, seminal plasma composition, hormonal and biochemical profiles, genetic determinants, and environmental factors. Key functional markers associated with high fertility include elevated sperm motility (total 77.6%, progressive 73.73%), membrane and acrosome integrity (74.17% and 92.83%, respectively) in fertile bulls versus significantly lower values in sub-fertile animals. Important seminal plasma biochemical markers such as follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone, insulin-like growth factor-1 (IGF-1) are all higher in fertile buffalo bulls, for example, LH at 0.28 ± 0.02 mU/mL and testosterone at 0.53 ± 0.02 ng/mL in fertile animals. Fertile bulls also show superior antioxidant capacity (TAC, catalase, glutathione peroxidase, and nitric oxide) and lower oxidative stress marker malondialdehyde (MDA). Proteomic studies have identified differentially abundant sperm proteins such as AKAP3, AKAP4, Sp17, and PDIA3 (upregulated in high fertility) while DLD is downregulated, all strongly linked to sperm motility, structural integrity, and zona pellucida binding. Transcriptomic profiling further highlights up-regulated hub genes (e.g., RPL36AL, EIF5A, RPLP0) as promising molecular markers for sperm quality and fertility assessment. Integrating conventional semen analysis with molecular technologies, including omics-based biomarker identification, offers improved accuracy in fertility prediction and supports effective selection of breeding bulls. These strategies are essential for optimizing reproductive outcomes, minimizing economic losses, and advancing sustainable buffalo productivity.

Introduction

The water buffalo (*Bubalus bubalis*) holds an indispensable

position within the global livestock sector, particularly in the Indian dairy industry, where it significantly contributes to both milk and meat production. India, possessing the largest buffalo

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population globally, accounts for approximately 63% of the world's buffalo milk and 95% of Asia's buffalo milk output. Furthermore, buffaloes contribute a substantial 51.1% to India's total milk production, underscoring their critical role in the national economy and food security (DAHD, 2012-2013). Despite their significant contribution, the efficiency of buffalo production systems is frequently hampered by reproductive challenges, notably economic losses stemming from failed pregnancies following artificial insemination (AI) (Karanwal et al., 2023). A primary determinant of successful conception in AI programs is the fertilizing potential of the breeding bull. Given that a single ejaculate from a bull can be utilized to inseminate thousands of females, the fertility status of the bull is paramount for maintaining and enhancing herd genetics and overall productivity (Parisi et al., 2014). Consequently, accurately predicting the fertility status of buffalo bulls prior to their use in AI programs is crucial for mitigating economic losses and optimizing reproductive outcomes in buffalo breeding initiatives.

Male fertility is fundamentally defined as the intrinsic capability of spermatozoa to successfully fertilize an oocyte and subsequently initiate and sustain pregnancy, a process of paramount importance for the productivity and genetic advancement of livestock populations (De Oliveira et al., 2013). This trait, particularly in breeding bulls, is not a singular characteristic but rather a complex, multifactorial phenomenon influenced by an intricate interplay of various biological and environmental elements (Assumpcao et al., 2005) and shown in Fig.1

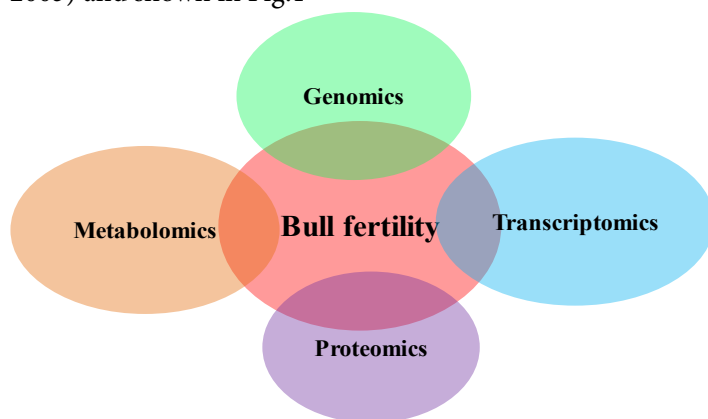


Fig. 1. Assessing the bull fertility through 'Omics' (Klein et al., 2022)

Specifically, male fertility is contingent upon several key factors:

- **Behaviour and physical fitness:** The physiological and behavioural attributes of the male, including libido and overall physical condition, contribute significantly to successful reproductive outcomes.
- **Semen quality:** This encompasses a range of parameters such as sperm motility, viability, morphology, and concentration, which are direct indicators of the spermatozoa's functional integrity and fertilizing potential.
- **Biochemical and hormonal components:** The

composition of seminal plasma (SP) and systemic hormonal profiles play a critical role. Seminal plasma contains a complex array of electrolytes, hormones, proteins, enzymes, carbohydrates, and lipids that are essential for sperm function and cryosurvival (Rodriguez-Martinez et al., 2021). Hormones such as Follicle-stimulating hormone (FSH), Luteinizing hormone (LH), and Testosterone are crucial regulators of spermatogenesis and steroidogenesis, directly impacting fertility (Hafez and Hafez, 2013).

- **Genetic factors:** Fertility is also a genetically controlled trait, with specific genes influencing various stages of male reproduction and fertilization (Kumar et al., 2025). Genetic abnormalities can contribute significantly to subfertility.
- **Environmental factors:** External influences, including nutrition and management practices, can also modulate reproductive performance.

➤ The present review article aims to synthesize the current scientific understanding regarding the multifaceted factors that influence fertility in water buffalo (*Bubalus bubalis*) bulls and to critically evaluate the various methodologies employed for its assessment and prediction. Given the significant economic implications of subfertility in the buffalo breeding industry, a comprehensive overview of these aspects is crucial for developing more effective reproductive management strategies.

Factors influencing buffalo bull fertility

Semen quality and sperm characteristics

Sperm motility:

Sperm motility is a paramount characteristic directly correlated with fertility. Both total motility (TM) and progressive motility (PM) are crucial for spermatozoa to navigate the female reproductive tract and reach the oocyte for fertilization. Studies consistently demonstrate that fertile buffalo bulls exhibit significantly higher proportions of total and progressive motility compared to sub-fertile bulls. For instance, fertile buffalo bulls have been observed with a total motility of 77.60% and progressive motility of 73.73%, significantly greater than sub-fertile bulls (68.64 and 63.87%, respectively). This superior motility is often attributed to normal testicular function in fertile animals (Almadaly et al., 2023). However, sperm motility is highly susceptible to adverse conditions, particularly oxidative stress. Reactive oxygen species (ROS) are naturally generated during sperm metabolic activities and capacitation; however, excessive ROS production can severely impair sperm quality and fertility (de Lamirande et al., 1993; Garg et al., 2009; Kumar et al., 2025). High levels of ROS adversely affect the

contractile apparatus of the sperm flagellum, leading to decreased motility (Guthrie et al., 2012). Buffalo bull sperm, in particular, are highly vulnerable to oxidative stress due to their elevated content of polyunsaturated fatty acids, which are prone to lipid peroxidation (Kumar et al., 2015). The defense mechanisms against oxidative stress, such as total antioxidant capacity, are therefore essential for sustaining both total and progressive motility (Kumar et al., 2017).

Sperm viability and membrane integrity:

The integrity of the sperm's plasma membrane and acrosome are indispensable for successful fertilization. An intact plasma membrane is vital for maintaining sperm viability and regulating the physiological processes necessary for capacitation and the acrosome reaction. Similarly, an intact acrosome is essential for the sperm to penetrate the oocyte's zona pellucida. Research indicates that fertile buffalo bulls possess significantly higher proportions of spermatozoa with intact plasma membranes and intact acrosomes compared to sub-fertile bulls (Singh et al., 2018). For example, fertile bulls showed 74.17% intact-plasma membrane and 92.83% intact-acrosome, which were markedly higher than observed in sub-fertile bulls (59.67 and 84.83%, respectively) (Almadaly et al., 2023). These higher proportions directly contribute to enhanced oocyte penetration and the overall fertilization process (Minervini et al., 2013). Functional plasma membrane integrity can be assessed using tests like the Hypo-osmotic Swelling Test (HOST) (Jeyendran et al., 1984) or Carboxy fluorescein diacetate (CFDA) in conjunction with propidium iodide (PI) (Singh et al., 2016). Acrosomal membrane integrity is typically determined using techniques such as Fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) staining, often combined with PI (Esteves et al., 2007; Almadaly et al., 2012).

Sperm morphology and concentration:

While motility and membrane integrity are critical, sperm morphology and concentration also play a role in overall semen quality. Although not detailed extensively in the connected documents for buffalo, these parameters are generally considered in conventional semen analysis to identify abnormalities that could impede fertilization. Normal morphology ensures the sperm's structural integrity for movement and interaction with the oocyte, while adequate concentration ensures a sufficient number of viable spermatozoa are available for fertilization. Enciso et al. (2011) reported that bulls with a higher proportion of morphological abnormalities in their semen exhibited reduced reproductive efficiency. Hence, evaluating spermatozoa morphology can be a useful tool for identifying and excluding bulls with poor fertility during breeding soundness examinations.

Biochemical components of seminal plasma:

Seminal plasma (SP) is a highly complex biological fluid containing a diverse array of electrolytes, hormones, proteins, enzymes, carbohydrates, and lipids, all of which significantly impact sperm cell function and cryosurvival, making it a reliable indicator of male fertility (Rodriguez-Martinez et al., 2021). Hormonal profiles in both seminal plasma and serum are crucial, with follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone (T), and insulin-like growth factor-1 (IGF-1) levels being significantly higher in fertile buffalo bulls compared to sub-fertile ones (Almadaly et al., 2023). LH and FSH regulate testosterone synthesis and are essential for male fertility, while testosterone plays a crucial role in spermatogenesis and testicular function (Macpherson et al., 2002). IGF-1 contributes to spermatogenesis and steroidogenesis, and its presence is positively associated with fertility, also enhancing the metabolic activity and progressive motility of buffalo bull spermatozoa by increasing intracellular calcium ion concentration (Macpherson et al., 2002; Kumar et al., 2021). Regarding antioxidant status and oxidative stress, fertile buffalo bulls exhibit significantly higher seminal plasma levels of total antioxidant capacity (TAC), Catalase (CAT), Glutathione peroxidase (GPx), and Nitric oxide (NO) (Ahmed et al., 2020). Conversely, Malondialdehyde (MDA), a marker of oxidative stress, is found in significantly greater concentrations in the seminal plasma of sub-fertile bulls (Tvrdá et al., 2013). This vulnerability to oxidative stress in buffalo bull sperm is attributed to their high content of polyunsaturated fatty acids (Furland et al., 2007).

Energy metabolites are also vital for sperm function. Fructose and glucose serve as the main energy sources for sperm in most mammalian species, positively impacting metabolizable energy and fertility potential (Williams et al., 2001). Seminal fructose levels are notably higher in highly fertile bulls, suggesting its potential as a seminal biomarker for fertility (Velho et al., 2018). Finally, proteins and lipids within seminal plasma are integral to sperm health. Seminal plasma proteins, including albumin and globulin, contribute to the buffering capacity of SP, and a low protein content can diminish sperm quality (Rolim Filho et al., 2013; Divyashree et al., 2018; Fu et al., 2019). Seminal plasma lipids are important for sperm membrane structure and function, influencing semen volume, motility, and concentration (Lu et al., 2016). Seminal levels of total protein, albumin, triglycerides, cholesterol, and high-density lipoproteins (HDL) are greater in fertile bulls, whereas serum levels of these components are generally comparable between fertility groups (Kumar et al., 2017; Almadaly et al., 2023).

Genetic and proteomic markers:

The investigation of male fertility has been significantly advanced by high-throughput omics technologies, which

facilitate the identification of specific genetic and proteomic markers associated with reproductive success. Comparative proteomic profiling of spermatozoa from high fertile (HF) and low fertile (LF) buffalo bulls, employing techniques such as label-free liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS), has been instrumental in establishing global proteomic landscapes and identifying protein candidates for fertility prediction (Aslam et al., 2019). These studies have revealed numerous differentially abundant proteins (DAPs) in the spermatozoa of HF and LF buffalo bulls, including AKAP3, AKAP4, Sp17, ACE3, PDIA3, and DLD, which are implicated in various critical sperm functions and contribute to differential fertility outcomes (Karanwal et al., 2023).

Beyond individual proteins, transcriptomic analyses have identified “hub genes” that serve as crucial control points within the complex gene regulatory networks governing semen quality and fertility (Doering et al., 2008; Goymer et al., 2008). For progressive motility (PM), hub genes include MAPK3 (ERK1), which is involved in tyrosine kinase pathways and stimulates PM, with cellular responses to oxygen-containing compounds being a top enriched biological process for up-regulated genes. In the context of acrosome integrity (AI), hub genes such as MCL1 (a pro-survival factor), RPL36AL, RPS27A, and EXT2 are linked to lipid metabolic processes and apoptosis pathways. For functional membrane integrity (FMI), EIF5A, RPL36AL, and RPS27A are identified as hub genes, with associated biological processes including glucose metabolism, fertilization, protein localization to the membrane, and translation pathways. Finally, hub genes such as RPLP0, RPS28, RPS18, and EXT2 are associated with the overall fertility rate (FR), primarily through their involvement in ribosomal pathways. These genetic and proteomic markers offer promising avenues for predicting buffalo semen quality and fertility by targeting key regulatory elements within the sperm transcriptome and proteome translation (Swathi et al., 2023).

Age of bull: The age of the bull significantly influences reproductive performance, particularly the age at first use (AAFU) of semen, which has a direct impact on conception rates. Research on Murrah breeding bulls has demonstrated a negative association between the conception rate (CR) and the AAFU of semen, indicating that older bulls at first use tend to yield lower fertility outcomes. Specifically, the highest predicted conception rate was observed when Murrah bulls were utilized at an AAFU of less than 3.5 years (Mir et al., 2015). This suggests that optimizing the age at which bulls are first used for artificial insemination can lead to improved reproductive efficiency within a herd.

Methods for fertility assessment and prediction

In vivo fertility classification:

Bulls are systematically classified into fertile and sub-fertile categories based on their in vivo fertility, a critical determinant of reproductive success. This classification primarily relies on the conception rate (CR) achieved through artificial insemination (AI) records, which are obtained from insemination programs involving a significant number of cows (Almadaly et al., 2023; Karanwal et al., 2023). For example, buffalo bulls have been categorized as fertile if their CR is $\geq 55\%$ and sub-fertile if their CR is $\leq 35\%$ following the insemination of estrus buffaloes with their frozen-thawed semen (Almadaly et al., 2023). Similarly, other research classifies high-fertility (HF) bulls as those with CRs above a specific threshold (e.g., $> 48.11\%$) and low-fertility (LF) bulls as those below another threshold (e.g., $< 38.01\%$), based on extensive insemination records collected over time (Verma et al., 2014; Batra et al., 2020). This rigorous classification method ensures that subsequent investigations into biochemical or genetic markers are founded upon empirically validated fertility outcomes.

Conventional semen analysis:

Computer-assisted sperm analysis (CASA) is considered a reliable and standardized tool for determining spermatozoa motility, as it provides objective measurements of kinematics across thousands of sperm cells (Contri et al., 2010). Modern CASA analysers are capable of evaluating multiple fields within viewing chambers, thereby capturing data from large numbers of spermatozoa. The automated calculation of progressive motility, achieved by analysing motion characteristics, eliminates the bias associated with subjective estimation by researchers (Amann and Waberski, 2014). Typically, post-thaw motility assessment using CASA yields lower values compared to subjective microscopic evaluation. Reported post-thaw motility measured by CASA generally ranges from 22% to 30% (Kumar et al., 2016; Ivanova et al., 2020).

Biochemical and hormonal assays:

For fertility assessment and prediction, seminal plasma (SP) and serum samples are systematically collected from buffalo bulls. Luteinizing hormone (LH) at 0.28 ± 0.02 mUI/mL in fertile and 0.11 ± 0.01 mUI/mL in sub-fertile bulls, testosterone (T) at 0.53 ± 0.02 ng/mL in fertile and 0.38 ± 0.02 ng/mL in sub-fertile bulls, and Insulin-like growth factor-1 (IGF-1) at 57.67 ± 1.71 ng/mL in fertile and 50.33 ± 1.48 ng/mL in sub-fertile bulls (Francis et al., 1988). Biochemical analyses encompass the estimation of TAC (Lone et al., 2016),

CAT (Sinha et al., 1971), GPx (Kumar et al., 2015), and NO (Wo et al., 2013) using specific assay kits, while MDA levels are also determined (Kumar et al., 2015). Seminal fructose is measured via colorimetric methods, and serum glucose levels are determined using analyser like the Cobas c 311/501. Additionally, total protein (Parvin et al., 1965), albumin (Dumas et al., 1972), triglycerides, cholesterol, and high-density lipoproteins (HDL) are quantified in both serum and seminal plasma using colorimetric methods and commercial kits. Specific proteins like Osteopontin (55 kDa) are also reported to be significantly higher in fertile buffalo bulls' seminal plasma, with a protein percentage of $5.00 \pm 0.13\%$ in fertile bulls compared to $1.93 \pm 0.16\%$ in sub-fertile bulls. These comprehensive biochemical and hormonal profiles are crucial for a reliable prediction of buffalo bull fertility.

Omics technologies for biomarker identification

Proteomics:

Proteomics involves the high-throughput global profiling of proteins within spermatozoa to identify candidates associated with fertility (Table 1). This is typically achieved using techniques such as liquid chromatography-tandem

mass spectrometry (LC-MS/MS) (Karanwal et al., 2023). This approach is particularly advantageous for mature spermatozoa because they are terminally differentiated cells, lacking significant transcriptional and translational machinery, and thus carry pre-synthesized proteins as their final products (Rahman et al., 2013; Sun et al., 2021; Hamilton et al., 2022). By comparing the proteomic profiles of spermatozoa from bulls with contrasting fertilizing abilities (e.g., high and low fertility), researchers can identify differentially abundant proteins (DAPs) and unique proteins that may regulate various sperm functions, including motility, capacitation, acrosome reaction, and zona pellucida binding. Differentially abundant proteins (DAPs) in bull sperm linked to fertility, with proteins like AKAP3, AKAP4, Sp17, and PDIA3 significantly upregulated in high fertile bulls, contributing to motility, zona pellucida binding, and sperm structure integrity. Conversely, DLD was found to be downregulated in high fertile bulls, indicating its potential role in energy metabolism and acrosome function. These expression patterns highlight the importance of these proteins in regulating sperm functions crucial for fertility differences between high and low fertile bulls. A study identified 1,385 proteins in buffalo spermatozoa, with 553 being significantly differentially abundant between high and low fertile groups, highlighting their potential roles in fertility regulation (Karanwal et al., 2023).

Table 1: Proteomics analyses of bull fertility (Ozbek et al., 2021; Klein et al., 2022)

Sl.	Functions	Proteins as biomarkers
1	Energy metabolism	ATP5B, ATP5D, ENO1, MD2, DECR1, AK1, NADHD
2	Acrosome reaction & Capacitation	CAPN7, BSP1, BSP3, BSP5, PEBP1 , PEBP4
3	Oxidative stress	ASPP2, GPx4, QSOX1, UQCRC2 , CLU
4	Motility	RIBC1, TUBB3, NPCC, AKP4
5	Cell signalling	Ropporin-1, USP12, LGALS3BP
6	Membrane integrity	GDI2, RIBC1, TMEM43
7	Immune system	AHSG, IFNRF4
8	Egg- sperm binding	SPADH1, BSP5

● Up-regulated in high fertile bulls

● Up-regulated in low fertile bulls

● Mixed response

Transcriptomics:

Transcriptomic analysis of bull sperm has identified several functionally (Table 2) important differentially expressed genes (DEGs) associated with sperm traits such as progressive motility (PM), acrosome integrity (AI), functional membrane integrity (FMI), and fertility rate (FR). Key DEGs include MAPK3, which is upregulated in bulls with good progressive motility and plays a role in signalling; RPL36AL and hub genes like RPS27A and EXT2, which are linked to apoptosis and acrosome integrity; EIF5A, involved in translation and upregulated in bulls with high functional membrane integrity; and ribosomal pathway genes such as RPLP0, RPS28, and RPS18, which are more expressed

in high fertility bulls. Overall, these genes show a distinct pattern where they are significantly upregulated in high fertility (HF) or high-functioning bulls compared to low fertility (LF) counterparts, suggesting their importance in supporting sperm functionality and fertilization competence. This differential expression highlights molecular pathways including translation, apoptosis, and ribosome biogenesis as key contributors to sperm quality and fertility outcomes, making these genes potential biomarkers for assessing bull fertility (Swathi et al., 2023). These networks help in understanding the complex molecular mechanisms underlying sperm functions and fertility status (Goymer et al., 2008; Doering et al., 2012).

Table 2: Sperm transcripts as biomarkers for fertility

S. No	mRNA Transcripts	Cell Type	References
1	Cadherin 15, EST, PABPC4, PABPN1	spermatozoa cells	Lalancette et al., 2008
2	PRM1, PRM2	spermatozoa cells	Bissonnette et al., 2009
3	PRM1, PRM2, PRM3, Tnp1 and Tnp2	Testicular cells	Ferraz et al., 2013
4	CRISP2, PEBP1, CCT8, BRP	spermatozoan cells	Arangasamy et al., 2011
5	PRM1, LOC783058, HMGB4, LOC404073, KIF5C, TMSB4X, GSTM3	spermatozoa cells	Card et al., 2013
6	Protamine 1, Protamine 2	spermatozoa cells	Ganguly et al., 2013
7	CCT5, GUK1, CTRB1, SRMS, ISCU, PJA1	spermatozoa cells	Yathish et al., 2018
8	MIR708, VSNL1, SQRDL, CD28	spermatozoa cells	Selvaraju et al., 2017

Hub gene identification and validation:

Following the construction of gene networks from transcriptomic data, hub genes are identified using specialized bioinformatics tools. Tools such as Cytoscape, MCODE (Molecular Complex Detection), and CytoHubba are employed to pinpoint genes that are densely clustered within the networks, indicating their likely role as control points for specific study conditions or sperm functions (Kaur et al., 2022). In buffalo bulls, hub genes were identified for progressive motility, acrosome integrity, functional membrane integrity, and fertility rate, with ribosomal protein gene families being common across several groups, suggesting their role in fertility regulation. The identified hub genes are then subjected to validation to confirm their expression and potential as fertility biomarkers. This typically involves:

- **RT-qPCR (Real-time quantitative polymerase chain reaction):** This molecular technique is used to validate the differential expression of hub genes

- identified from transcriptome analysis in buffalo bulls' semen samples. Genes such as RPL36AL (fold change 14.99) in acrosome integrity, EIF5A (fold change 54.32) in functional membrane integrity, and RPLP0 (fold change 8.55) and RPS28 (fold change 13.42) in fertility rate were significantly up-regulated. Their expression levels showed strong correlations with sperm functional traits and fertility rate, suggesting these genes as potential molecular markers for semen quality and fertility diagnosis in buffalo bulls (Swathi et al., 2023).
- **Quantitative dot blot:** To further validate the findings at the protein level, quantitative dot blot analysis is performed. This method assesses the relative abundance of the proteins corresponding to the hub genes in high and low fertility groups (Tian et al., 2017). E.g., Protein levels of EXT2, RPS18, and RPLP0 were analysed using specific antibodies in high and low fertility buffalo bull groups. It was observed that only EXT2 protein levels were significantly higher in the low fertility group compared to the high fertility group. This discrepancy between protein abundance and RNA-seq/RT-qPCR find-

ings suggests that available transcripts might have already been translated into proteins in sperm, indicating post-transcriptional regulation or protein turnover differences (Swathi et al., 2023).

Conclusion

This review underscores that reliable prediction of buffalo bull fertility is contingent upon the integration of specific semen traits and molecular markers, supported by well-defined cutoff values. Among the semen characteristics, sperm motility is paramount: fertile bulls consistently exhibit total motility above 77% and progressive motility above 73%, while sub-fertile counterparts show rates below these thresholds. Plasma membrane and acrosome integrity are also emphasized, with fertile bulls demonstrating >74% and >92% intactness, respectively, significantly surpassing cutoff values that distinguish them from sub-fertile animals. At the proteomic level, there are 26, 30, and 55 kDa bands in seminal plasma chiefly osteopontin (55 kDa) as reliable fertility-associated markers, with a higher proportion of these proteins linked to superior in vivo conception outcomes. Bulls with conception rates (CR) above 48% are consistently classified as high-fertility, while those below 38% are considered low-fertility, reaffirming the critical role of in vivo CR records in field assessment. In conclusion, optimal fertility assessment in buffalo bulls should prioritize progressive sperm motility (>73%), membrane/acrosome integrity (>74%/>92%), specific biochemical/hormonal markers (LH >0.28 mU/mL, testosterone >0.53 ng/mL), seminal antioxidant status, and dominant protein bands (26, 30, 55 kDa), utilizing these empirically-defined cutoffs for the precise selection of breeding sires.

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