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Comparative Enrichment of Functional and Structural Sperm Characteristics by Swim-up and Density-gradient Centrifugation in Thawed Crossbred Bull Semen

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ABSTRACT

Background: Selection of functionally competent spermatozoa is an important step in bovine reproductive biotechnology. Swim-up (SU) and density-gradient centrifugation (DGC) use different principles for sperm enrichment and may therefore produce populations with different functional characteristics. The present study compared SU and DGC for enrichment of sperm quality characteristics in thawed crossbred bull semen.

Methods: Five crossbred bulls contributed two ejaculates each at a weekly interval, resulting in 10 ejaculates. Each ejaculate was divided into matched Initial, SU and DGC fractions. Sperm motility, viability, hypo-osmotic swelling test (HOST), acrosomal integrity and morphological characteristics were assessed. Computer-assisted sperm analysis (CASA) was used to evaluate motility and kinematic characteristics. Data were analyzed using linear mixed-effects models with processing method as a fixed effect and bull and ejaculate nested within bull as random effects. Pairwise comparisons were adjusted using the Holm method.

Result: Progressive motility increased from 41.10±2.00% in Initial semen to 46.80±2.12% after SU and 54.50±1.66% after DGC (P<0.0001). Viability increased from 48.50±1.95% to 56.50±1.65% and 59.10±1.76%, while HOST-positive spermatozoa increased from 35.10±1.60% to 40.80±1.71% and 46.40±1.53% in Initial, SU and DGC fractions, respectively (P<0.0001). DGC produced higher progressive motility, viability and HOST response than SU. CASA-derived total motility, progressive motility, straightness, linearity and wobble were also higher following DGC. Acrosomal integrity and most morphological characteristics improved after sperm selection but did not differ significantly between SU and DGC. Thus, both methods enriched selected sperm-quality attributes, with DGC providing greater enrichment for several functional parameters under the conditions tested.

Key words: Bovine semen, CASA, Density-gradient centrifugation, Sperm motility, Sperm quality, Sperm selection, Swim-up.

INTRODUCTION

Semen quality is a multifactorial determinant of reproductive performance in cattle and no single parameter adequately represents the functional competence of a sperm population. Motility, viability, plasma-membrane integrity, acrosomal integrity and morphology provide complementary information for evaluating cryopreserved bull semen (Mondal *et al*., 2010; Arias *et al*., 2017). This is particularly important for frozen semen used in artificial insemination and other reproductive technologies because cryopreservation and thawing can adversely affect sperm motility, membrane integrity and cellular structure.

Post-thaw sperm-selection techniques are therefore used to enrich functionally competent spermatozoa from heterogeneous semen populations. Swim-up (SU) is primarily based on the ability of progressively motile spermatozoa to migrate into an overlying culture medium. The method has been widely used for recovering motile and functionally competent spermatozoa, including in bovine semen (Parrish and Foote, 1987). Density-gradient centrifugation (DGC), in contrast, separates spermatozoa according to differences in density and physical characteristics and can concentrate spermatozoa while reducing cellular debris and less desirable sperm populations. The 45%/90% percoll gradient has been used extensively for bovine sperm preparation and has been compared with swim-up in relation to sperm recovery and subsequent *in vitro* embryo production (Parrish *et al*., 1995). Recent studies have further demonstrated that density-gradient selection can influence post-thaw sperm motility,

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membrane and acrosomal characteristics (Arias *et al*., 2017; Duma *et al*., 2025).

Assessment of sperm-selection procedures should therefore consider multiple complementary endpoints rather than motility alone. Eosin-nigrosin staining provides an estimate of sperm viability, while the hypo-osmotic swelling test (HOST) evaluates functional plasma-membrane integrity (Jeyendran *et al*., 1984; Correa and Zavos, 1994). Acrosomal integrity is important for subsequent sperm-oocyte interaction, whereas morphological assessment provides information on structural abnormalities (Watson, 1975; Mondal *et al*., 2010). Computer-assisted sperm analysis (CASA) additionally provides objective measurements of total and progressive motility and sperm kinematics, although the measured characteristics depend on instrument configuration and analytical settings (Kathiravan *et al*., 2011; Yániz *et al*., 2018). Studies published previously have also demonstrated the application of Percoll-based sperm selection and CASA for evaluating bovine sperm quality (Promthep *et al*., 2016; Bhat and Sharma, 2020; Pathak *et al*., 2019).

Despite the established use of both SU and DGC, their comparative effects are not necessarily uniform across sperm-quality attributes and may depend on semen source, cryopreservation status, separation medium and processing conditions. Moreover, when different fractions are generated from the same ejaculate, the observations are inherently related. Consequently, comparison of matched fractions requires an analytical approach that accounts for the repeated structure of the data. A systematic comparison of conventional sperm-quality characteristics together with CASA-derived motility and kinematic parameters can therefore provide a more comprehensive assessment of the enrichment produced by each method.

The present study compared SU and DGC for enrichment of functional, structural and CASA-derived sperm characteristics in thawed crossbred bull semen. Five bulls contributed two ejaculates each, with every ejaculate processed into matched Initial, SU and DGC fractions. Sperm-quality and CASA-derived characteristics were evaluated using a mixed-effects model accounting for matched ejaculates and clustering within bulls. The study focused on laboratory sperm-quality enrichment; fertilization, embryo development and field fertility were not evaluated.

MATERIALS AND METHODS

Study site, animals and research period

The study was conducted at the Animal Physiology and Reproduction Laboratory, ICAR-National Dairy Research Institute, Eastern Regional Station (ICAR-NDRI-ERS), Kalyani, Nadia, West Bengal, India, from December 2024 to March 2026. Five clinically healthy, sexually mature crossbred bulls aged 4-6 years were maintained under standard farm-management conditions. Two ejaculates were collected from each bull at a weekly interval, giving 10 ejaculates in total. Ejaculates were maintained separately and were not pooled. The ejaculate was the experimental unit for within-ejaculate processing comparisons, while bull was retained as the higher-level clustering factor. Frozen semen straws contained approximately 20 million spermatozoa/mL and had initial motility of >70-80%.

Semen evaluation and conventional sperm-quality assessment

Fresh ejaculates were evaluated for mass activity, sperm concentration and progressive motility using standard laboratory procedures. Sperm viability was assessed by eosin-nigrosin staining following Bloom (1950) and Campbell *et al*. (1956). Functional plasma-membrane integrity was assessed by the hypo-osmotic swelling test (HOST) according to Jeyendran *et al*. (1984) and Takahashi *et al*. (1990). Acrosomal integrity was evaluated using Giemsa staining following Watson (1975). Egg-yolk tris-glycerol (EYTG) extender was prepared using a standard Tris-based formulation and stored at 4°C until use.

Thawing and matched allocation

Frozen semen straws were thawed in a water bath maintained at 37°C for 30 s. Immediately after thawing, the straw was wiped dry, opened and the semen was transferred to a sterile tube. Each ejaculate was retained as a matched set comprising an Initial fraction and aliquots subjected to SU and DGC. All three fractions originated from the same ejaculate and were evaluated using the same assessment schedule.

Density-gradient centrifugation

A discontinuous percoll gradient comprising 90% and 45% layers was prepared. A 10× concentrated salt solution was prepared from 2.889 g NaCl, 0.238 g KCl, 0.116 g KH_2PO_4, 0.112 g $CaCl_2$ and 0.163 g HEPES buffer dissolved in 50 mL distilled water. The solution was filtered, autoclaved at 121°C for 20 min and stored at 4°C. Isotonic percoll (100% SIP) was prepared by mixing percoll and 10× buffer at a 9:1 ratio; target density was approximately 1.123 g/mL and osmolality was adjusted to 280-320 mOsm/kg. The 90% layer was prepared by mixing nine parts SIP with one part sperm-washing medium and the 45% layer by mixing one part of the 90% solution with one part washing medium.

The 90% Percoll layer was placed below the 45% layer in a 2.0-mL microcentrifuge tube and thawed semen was gently layered over the gradient. The sample was centrifuged at 4700 rpm for 6 min. Using the 7-cm effective rotor radius used for the centrifuge, this corresponds to approximately 1729 × g. After centrifugation, the supernatant was discarded and the sperm pellet was recovered and transferred to pre-incubated IVF medium. The suspension was centrifuged again at 1700 rpm for 3 min (approximately 226 × g at a 7-cm effective radius). The washed pellet was thoroughly resuspended in IVF medium and used for subsequent sperm assessment. The 45%/90% percoll configuration is consistent with

established bovine sperm-separation methodology (Parrish *et al*., 1995; Promthep *et al*., 2016; Bhat and Sharma, 2020).

Swim-up selection

For SU selection, 2 mL of diluted semen was centrifuged at 500 × g for 5 min. The sperm pellet was resuspended in 1 mL TCM-199 supplemented with antibiotics. Aliquots of 0.25 mL were placed at the bottom of each of four falcon tubes and 1 mL TCM-199 was gently layered above the sperm suspension. The tubes were incubated at 38.5°C for 1 h to permit migration of progressively motile spermatozoa into the upper medium layer. After incubation, the upper 0.25 mL from each tube was carefully aspirated and pooled. The pooled fraction was resuspended in 1 mL TCM-199, supplemented with heparin at 10 µg/mL and maintained in the incubator until subsequent use. The migration-based principle is consistent with established bovine swim-up methodology (Parrish and Foote, 1987; Arias *et al*., 2017).

Computer-assisted sperm analysis

Sperm motility and kinematic characteristics were evaluated using a hamilton thorne IVOS 2 computer-assisted sperm analyser equipped with a Zeiss 10× CM-040GE objective. Samples were loaded into a capillary chamber with 20 µm chamber depth and a 1.3 capillary correction factor. The stage temperature was maintained at 37°C. Following loading, a 30-s equilibration period was used to minimize fluid-wave motion and assessments were completed within 3 min. Video capture was performed at 60 Hz for 30 frames. The minimum total count target was 200 spermatozoa per assessment. Cell-detection settings included head size of 10-54 µm^2, elongation of 5-81%, minimum head brightness of 112, minimum tail brightness of 84 and minimum tail length of 4 µm. Progressive spermatozoa were defined using STR ≥80% and VAP ≥45 µm/s; slow-VAP and slow-VSL cut-offs were 40 and 35 µm/s, respectively, with static VAP/VSL thresholds of 15 and 1 µm/s. Individual sperm tracks were reviewed using playback to exclude non-sperm debris. The same CASA settings and evaluation time window were used for Initial, SU and DGC fractions.

Statistical analysis

Five bulls contributed two ejaculates each, resulting in 10 ejaculates. Each ejaculate generated matched Initial, SU and DGC observations; consequently, the three processing conditions were not treated as independent observations. Conventional sperm-quality and CASA-derived outcomes were analyzed using linear mixed-effects models, with processing method (Initial, SU and DGC) as a fixed effect and bull and ejaculate nested within bull as random intercepts. The ejaculate was considered the experimental unit for comparisons among processing methods. Overall processing effects were evaluated using two-degree-of-freedom Wald tests. Pairwise treatment contrasts were evaluated using two-sided Wald tests, with the three pairwise comparisons for each endpoint adjusted using the Holm method. Statistical significance was defined as adjusted P<0.05. Results are presented as mean±SEM.

RESULTS AND DISCUSSION

Conventional sperm-quality characteristics

All five conventional sperm-quality endpoints showed significant overall processing effects (all P<0.0001; Table 1). Because each fraction originated from the same ejaculate, the mixed-effects model retained the within-ejaculate dependence and clustering of repeated ejaculates within bulls.

Progressive motility increased from 41.10±2.00% in Initial semen to 46.80±2.12% after SU and 54.50±1.66% after DGC. SU increased progressive motility by 5.70 percentage points relative to Initial semen (Holm-adjusted P<0.0001), while DGC increased it by 13.40 percentage points (P<0.0001). DGC also exceeded SU by 7.70 percentage points (P<0.0001). This pattern is consistent with migration-based SU and density-based enrichment by percoll separation (Parrish and Foote, 1987; Parrish *et al*., 1995; Arias *et al*., 2017). The result is best interpreted as enrichment of the recovered population rather than direct enhancement of individual sperm function.

Viability increased from 48.50±1.95% in Initial semen to 56.50±1.65% after SU and 59.10±1.76% after DGC (overall P<0.0001). Both selection procedures increased viability relative to Initial semen (both Holm-adjusted P<0.0001) and DGC exceeded SU by 2.60 percentage points (P=0.0128). The concurrent increase in motility and viability is compatible with preferential recovery of a sperm subpopulation possessing multiple favourable characteristics (Mondal *et al*., 2010; Baruah *et al*., 2013).

Table 1: Progressive sperm-quality parameters (mean±SEM) of spermatozoa recovered from Initial, swim-up (SU) and density-gradient centrifugation (DGC) fractions.

Parameter	Initial	SU	DGC	Overall P
Motility (%)	41.10±2.00^a	46.80±2.12^b	54.50±1.66^c	<0.0001
Viability (%)	48.50±1.95^a	56.50±1.65^b	59.10±1.76^c	<0.0001
HOST (%)	35.10±1.60^a	40.80±1.71^b	46.40±1.53^c	<0.0001
Acrosomal integrity (%)	57.90±2.19^a	68.80±1.41^b	72.30±1.87^b	<0.0001
Abnormality (%)	13.80±1.15^a	7.80±0.63^b	6.80±0.49^b	<0.0001

Values are mean±SEM (n=10 matched ejaculates). Different superscripts within a row denote holm-adjusted pairwise differences at P<0.05. HOST: Hypo-osmotic swelling test. Exact pairwise adjusted P values are given in the results.

HOST-positive spermatozoa increased from 35.10± 1.60% in Initial semen to 40.80±1.71% after SU and 46.40±1.53% after DGC (overall P<0.0001). Both selected fractions differed from Initial semen (P<0.0001) and DGC exceeded SU by 5.60 percentage points (P<0.0001). Because HOST evaluates functional membrane response rather than simple dye exclusion, this finding indicates enrichment of spermatozoa with better functional plasma-membrane integrity (Jeyendran *et al*., 1984; Correa and Zavos, 1994).

Acrosomal integrity increased from 57.90±2.19% in Initial semen to 68.80±1.41% after SU and 72.30±1.87% after DGC (overall P<0.0001). Both selected fractions differed from Initial semen, but DGC and SU did not differ significantly (difference 3.50 percentage points; Holm-adjusted P=0.0837). Thus, both procedures enriched spermatozoa with structurally intact acrosomes, without evidence of a DGC-specific advantage for this endpoint.

Morphological abnormality decreased from 13.80±1.15% in Initial semen to 7.80±0.63% after SU and 6.80±0.49% after DGC (overall P<0.0001). Both selected fractions differed from Initial semen, whereas DGC and SU did not differ significantly (difference -1.00 percentage point; Holm-adjusted P=0.1619). The reduction therefore indicates enrichment relative to the starting population rather than a demonstrated DGC-specific advantage.

Collectively, both procedures enriched several desirable conventional sperm characteristics. DGC produced greater enrichment than SU for progressive motility, viability and HOST response, whereas acrosomal integrity and morphological abnormality did not differ significantly between the two procedures. The comparative effect was therefore parameter-specific rather than evidence of uniform superiority.

CASA-derived motility and kinematic characteristics

CASA provided an objective assessment of sperm movement using the same instrument settings, chamber conditions and evaluation window for all three matched fractions. Total motility increased from 48.40±2.41% in Initial semen to 67.27±1.71% after SU and 77.47±2.07% after DGC (overall P<0.0001; Table 2). Initial-SU and initial-DGC differences were both significant after Holm adjustment (P<0.0001) and DGC exceeded SU by 10.20 percentage points (P=0.0005).

CASA-derived progressive motility increased from 35.47±2.21% in Initial semen to 44.79±1.49% after SU and 62.05±1.02% after DGC (overall P<0.0001). Both selection methods differed from Initial semen (P<0.0001), while DGC exceeded SU by 17.26 percentage points (P<0.0001). Conventional and CASA-derived progressive-motility estimates are not numerically identical because the methods use different measurement criteria and operational thresholds. Importantly, both showed the same ranking: Initial < SU < DGC.

Most absolute velocity and distance measures did not show significant overall processing effects: DAP (P=0.4395), DSL (P=0.1535), DCL (P=0.7527), VAP (P=0.5486),

Table 2: CASA-derived motility, kinematic and morphological characteristics.

Parameter	Initial	SU	DGC	Overall P
TM (%)	48.40±2.41^{a}	67.27±1.71^{b}	77.47±2.07^{c}	<0.0001
PM (%)	35.47±2.21^{a}	44.79±1.49^{b}	62.05±1.02^{c}	<0.0001
DAP (µm)	36.34±1.39^{a}	34.76±1.95^{a}	38.27±2.35^{a}	0.4395
DSL (µm)	31.78±1.60^{a}	29.69±1.85^{a}	34.87±2.21^{a}	0.1535
DCL (µm)	54.15±2.25^{a}	53.31±3.14^{a}	51.17±3.17^{a}	0.7527
VAP (µm/s)	90.05±2.31^{a}	86.45±5.35^{a}	93.33±5.24^{a}	0.5486
VSL (µm/s)	78.17±2.87^{a}	73.68±4.97^{a}	84.74±4.87^{a}	0.1949
VCL (µm/s)	134.40±5.04^{a}	131.70±8.43^{a}	124.38±7.20^{a}	0.5706
STR (%)	85.43±1.41^{a}	85.05±1.18^{a}	90.23±0.84^{b}	0.0006
LIN (%)	59.87±2.52^{a}	58.08±2.35^{a}	69.74±2.39^{b}	0.0009
ALH (µm)	6.69±0.25^{a}	6.45±0.36^{ab}	5.38±0.46^{b}	0.0227
BCF (Hz)	23.83±1.45^{a}	24.56±1.31^{a}	27.59±1.96^{a}	0.2094
WOB (%)	68.51±1.93^{a}	67.09±1.77^{a}	76.27±1.97^{b}	0.0011
Morphologically normal (%)	88.47±0.75^{a}	92.63±0.78^{b}	93.13±0.95^{b}	<0.0001
Bent-tail (%)	3.28±0.55^{a}	1.10±0.18^{b}	1.24±0.35^{b}	<0.0001
Coiled-tail (%)	1.01±0.16^{a}	0.45±0.12^{b}	0.67±0.14^{ab}	0.0183
DMR (%)	6.78±0.66^{a}	5.62±0.66^{a}	4.73±0.55^{a}	0.0673
Proximal droplet (%)	0.14±0.06^{a}	0.14±0.05^{a}	0.05±0.03^{a}	0.2777
Distal droplet (%)	0.70±0.18^{a}	0.33±0.08^{a}	0.37±0.14^{a}	0.1283

Values are mean±SEM (n=10 matched ejaculates). Different superscripts within a row denote holm-adjusted pairwise differences at P<0.05. TM: Total motility; PM: Progressive motility; DAP: Distance average path; DSL: Distance straight line; DCL: Distance curvilinear; VAP: Average path velocity; VSL: Straight-line velocity; VCL: Curvilinear velocity; STR: Straightness; LIN: Linearity; ALH: Amplitude of lateral head displacement; BCF: Beat-cross frequency; WOB: Wobble; DMR: Distal mid-piece reflex.

VSL (P=0.1949) and VCL (P=0.5706). Thus, increased proportions of motile and progressively motile spermatozoa were not accompanied by a generalized increase in swimming velocity.

Trajectory-related variables showed selective responses. STR differed overall (P=0.0006), with DGC exceeding SU by 5.18 percentage points (Holm-adjusted P=0.0017). LIN differed overall (P=0.0009), with DGC exceeding SU by 11.66 percentage points (P=0.0016). WOB differed overall (P=0.0011), with DGC exceeding SU by 9.18 percentage points (P=0.0018). These findings suggest that the DGC-enriched population contained a greater proportion of spermatozoa displaying more directed trajectories.

ALH showed an overall processing effect (P=0.0227), but the DGC–SU contrast was not significant after Holm adjustment (P=0.0701); therefore, no specific DGC advantage is inferred for ALH. BCF did not differ significantly among fractions (P=0.2094). These selective responses reinforce the need to interpret CASA variables individually (Kathiravan *et al*., 2011; Yániz *et al*., 2018).

CASA-derived sperm morphology

The proportion of morphologically normal spermatozoa increased from 88.47±0.75% in Initial semen to 92.63±0.78% after SU and 93.13±0.95% after DGC (overall P<0.0001). Both selected fractions differed from Initial semen, whereas DGC and SU did not differ (Holm-adjusted P=0.6699). Bent-tail spermatozoa decreased from 3.28±0.55% to 1.10±0.18% after SU and 1.24±0.35% after DGC (overall P<0.0001); both selected fractions differed from Initial semen, but SU and DGC did not differ (P=0.7682).

Coiled-tail spermatozoa showed an overall effect (P=0.0183), driven primarily by the Initial-SU contrast (P=0.0150); DGC did not differ significantly from either Initial semen or SU after adjustment. DMR (P=0.0673), proximal cytoplasmic droplets (P=0.2777) and distal cytoplasmic droplets (P=0.1283) were not significantly affected. The results again indicate selective rather than uniform changes across morphological subcategories.

Comparative interpretation and biological relevance

The combined conventional and CASA findings indicate that both SU and DGC enriched selected sperm-quality characteristics relative to Initial semen, while DGC produced greater enrichment for several motility-and membrane-related endpoints. The strongest DGC-versus-SU differences occurred for conventional progressive motility, viability and HOST response and for CASA-derived total motility, progressive motility, STR, LIN and WOB. In contrast, acrosomal integrity, conventional abnormality, CASA-derived normal morphology, bent-tail spermatozoa and most absolute velocity and distance measures did not differ significantly between SU and DGC.

The findings are compatible with the different physical bases of the two methods. SU preferentially recovers spermatozoa able to migrate into the upper medium, whereas DGC concentrates spermatozoa according to density and facilitates removal of less desirable material. Earlier bovine studies established both the quantitative recovery characteristics of SU and the use of Percoll gradients for selecting motile spermatozoa (Parrish and Foote, 1987; Parrish *et al*., 1995). Direct comparisons have subsequently shown that the relative effects of SU and density gradients can extend to membrane and acrosomal integrity and other functional characteristics (Arias *et al*., 2017). The present study adds a matched, hierarchical comparison across conventional and CASA-derived endpoints in thawed crossbred bull semen.

The results should be interpreted in relation to the exact processing protocol. Percoll concentration, centrifugation conditions, semen source, cryopreservation history and selection medium can influence the recovered population. ARCC studies have demonstrated the application of Percoll-based bovine sperm selection and the use of CASA for post-thaw kinematic characterization (Promthep *et al*., 2016; Bhat and Sharma, 2020; Pathak *et al*., 2019). A recent ARCC study directly compared density-gradient and swim-up approaches in the context of bovine sperm quality and *in vitro* embryo development, further supporting the relevance of evaluating these established selection strategies (Varshini *et al*., 2026).

The present study does not demonstrate that DGC improves the intrinsic function of individual spermatozoa or fertility. The observed increases are most appropriately interpreted as changes in the composition of the recovered sperm population. No sperm-recovery/yield measurements were made, so a higher-quality fraction cannot be separated analytically from possible differences in the number of sperm recovered. Likewise, DNA-integrity, oxidative-stress and other molecular endpoints were not assessed and no IVF, embryo-development or field-fertility validation was performed. The findings should therefore be regarded as laboratory enrichment under the tested protocol rather than evidence of improved reproductive performance.

The findings should be interpreted within the scope of the study. The relatively small number of bulls may limit the generalizability of the findings and sperm recovery, molecular sperm-quality parameters and reproductive outcomes were not evaluated. Therefore, the observed improvements represent enrichment of laboratory sperm-quality characteristics and should not be interpreted directly as evidence of improved fertility.

CONCLUSION

Both swim-up and density-gradient centrifugation enriched several functional and structural characteristics of cryopreserved crossbred bull spermatozoa relative to the Initial fraction. Under the conditions tested, DGC produced greater enrichment than SU for progressive motility, viability, functional plasma-membrane integrity and selected CASA-derived motility characteristics, whereas several structural and kinematic endpoints did not differ between the two

procedures. The results support both SU and DGC as laboratory sperm-enrichment approaches, with the relative benefit depending on the specific sperm-quality attribute assessed. The study does not establish an effect on fertilization, embryo development or field fertility. Further work incorporating sperm recovery/yield, molecular damage markers and reproductive validation is warranted.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal Care and Handling Techniques of ICAR-NDRI, ERS, Kalyani, West Bengal [No. 22-P-AP-04 dated 24/06/2024].

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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