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Bioprospecting snakehead fish protein concentrate (SFC) as a non-serum-based albumin source for promoting capacitation in bovine sperm

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ABSTRACT

Capacitation is a prerequisite for successful fertilization with albumin as an inevitable component among the oviductal fluid's constituents. Under *in vitro* environments, albumin's function is replaced by serum-based products, which carry the issue of unsustainability, some allergies, and potential transmission of transmissible spongiform encephalopathies (TSEs) to the unborn fetus. To discover an alternative, sustainable, and innoxious promoter, snakehead fish (*Channa striata*) albumin was examined for its potential to promote capacitation in bovine sperm. Snakehead fish protein concentrate (SFC) with respective albumin concentrations of 3, 6, and 9 mg/mL was added to the bovine capacitation medium. Following the swim-up technique, bovine sperm were incubated in different treatment groups for 90 minutes to stimulate the capacitation process. The results revealed that the application of 3 mg/mL albumin from SFC in Tyrode's-based capacitation medium (T-SFC3) provided sufficient evidence in promoting capacitation, as demonstrated by higher sperm exhibiting hyperactive motility, kinematic parameters, and the percentage of sperm showing B pattern as compared to Tyrode's-based capacitation medium containing 6 mg/mL BSA (T-BSA6) ($p>0.05$) and other levels of T-SFCs ($p<0.05$). In contrast, greater concentrations of SFC application retrieved more viable sperm with intact acrosomes and less in both viable and mortal sperm with reactive acrosomes ($p<0.05$).

Keywords: Albumin content, Bovine sperm, Capacitation promoter, Channa striata, Fish protein concentrate

INTRODUCTION

Capacitation is a prerequisite for successful fertilization, which encompasses significant modification and involves morphological changes to achieve sperm competency for penetrating the oocyte in both domestic and wild animals (Aitken, 2017; Ded *et al.*, 2010; O'Flaherty, 2015). Albumin, an abundant compound in oviductal fluid (OF), is critical in modulating sperm capacitation (Kumaresan *et al.* 2019). By acting as a diffusible carrier protein, albumin promotes capacitation and regulates cytosol alkalization, calcium influx, hyperactivation, microdomain

aggregation, and membrane priming and docking (Flesch *et al.*, 2001; Tsai *et al.*, 2010; van Gestel *et al.*, 2005).

Under *in vitro* conditions, capacitation has been mimicked by incubating sperm in the defined medium containing several compositions imitating the physiological environment of the female reproductive tract in combination with sperm selection protocols such as swim-up, gradient density Percoll, and other techniques. Up to this point, the role of albumin during *in vitro* capacitation is commonly replaced by serum albumin (Chaves *et al.*, 2021). Serum-based albumin source has shown a stabilizing effect on acrosome integrity, which can maintain the acrosome intact. Moreover, its synergistic effect with bicarbonate triggered hyperactivation, tyrosine phosphorylation, and centralization of proteins related to zona binding and zona-induced acrosome reaction (Boerke *et al.*, 2013).

Regardless, it should be emphasized that utilizing any blood-derived products in the culture system poses several potential negative impacts; it raises the issue of possible transmission of transmissible spongiform encephalopathies (TSEs) such as Creutzfeldt-Jakob disease (CJD) (Bungum *et al.*, 2002), bovine spongiform encephalopathy (BSE) (Casalone and Hope, 2018), and sheep's scrapie (Prusiner, 1991), which are possibly transmitted from maternal blood to fetal trophectoderm (Nalls *et al.*, 2017). Not only in domestic animals, these prion-related diseases have also been reported in wildlife species, including Reeves' muntjac deer (Nalls *et al.*, 2017), Rocky Mountain elk (Selariu *et al.*, 2015), cheetah (Bencsik *et al.*, 2009), reindeer, mouflon sheep, Iberian wild goat, and Pyrenean chamois (Pitarch *et al.*, 2018). Besides the above, serum albumin has also been identified as a food allergen and an aeroallergen, causing anaphylaxis and respiratory symptoms (Voltolini *et al.*, 2013). Preliminaries have been attempted to replace serum albumin, such as non-animal macromolecules (Matson and Tardif, 2012) and recombinant albumin (Bungum *et al.*, 2002). However, none of those reported the capacitation status and kinematic patterns of the observed sperm, making the results less convincing.

One potential alternative source of albumin is the Snakehead fish (*Channa striata* (Adamson *et al.*, 2012; Song *et al.*, 2013). This fish has abundant albumin level in the flesh (Asfar *et al.*, 2019) and is now rated as Least Concern (LC) by the International Union for Conservation of Nature and Natural Resources (IUCN) due to its large population in the wild (Chaudhry *et al.*, 2019). The population is also supported by the growth of the snakehead farming industry and intensive culture system using conventional farming techniques and other captive breeding methods (Bich *et al.*, 2020; Kumar *et al.*, 2022). Due to its biochemical constitution, snakehead fish is currently used as a food supplement, traditional medicine, and other pharmacological therapeutics (Sahid *et al.*, 2018; Yuliana *et al.*, 2022). In light of the importance of capacitation for *in vitro* fertilization (IVF) in both domestic and wild animals, as well as the fact that the application of fish-derived non-serum albumin is under-studied, the present study aimed to observe the ability of snakehead fish protein concentrate (SFC) containing fish albumin to promote the capacitation process in bovine sperm and identified its sufficiency to replace the common promoter of bovine serum albumin.

MATERIALS AND METHODS

Ethical Statements

This study does not require ethical approval for animal experimentation, as outlined by institutional or national guidelines. Frozen bovine semen was sourced from a certified commercial supplier; the Snakehead fish used were also purchased from the local market. The researchers did not handle, house, or subject any live animals to procedures throughout the study. The semen samples were treated after collection and were initially obtained in accordance with the regular operating procedures and animal welfare requirements of the commercial provider.

Chemicals and Materials

Unless otherwise stated, all chemicals used in this study were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Fertility-proven frozen semen samples from the breeds of Friesian Holstein and Charolais (~15 straws from different individuals) aged 5 to 11 years were obtained from the Semen Production and Research Centre, Inthanon Royal Project, Chiang Mai, Thailand, in compliance with the

regulations and standardized frozen semen production protocols from the Thailand Ministry of Agriculture and Cooperatives. Snakehead fish (*Channa striata*) was obtained from Mae Hia Fresh Market, Chiang Mai Province, Thailand.

Snakehead Fish Protein Concentrate (SFC)

Freshly purchased snakehead fish (*Channa striata*) were immediately transported to the laboratory in a plastic bag with ice cubes and processed according to our previous study (Setiawan *et al.*, 2024). The fish fillets were prepared by washing and chopping, followed by smoothing with a water-based solvent in a blender at room temperature. The filtrate was freeze-dried using a freeze-dryer DW-10 (Drawell Scientific Instrument, Shanghai, China). The dried powder, henceforth referred to as Snakehead fish protein concentrate (SFC), was analyzed for its total soluble protein and albumin level using Bicinchoninic Acid (BCA) assay following its standard protocol (ThermoScientific, MA, USA) and Bromocresol Purple (BCP) assay.

An equally concentrated sample was also subjected to Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) with a 12% polyacrylamide gel to screen the retrieved protein bands. The percentage of unpurified fish albumin from SFC was quantified with the following equation below and used to determine the albumin concentration in the *in vitro* capacitation medium according to the treatments. Meanwhile, the gel of SDS-PAGE was analyzed using a feature of Raw volume from GelAnalyzer V.19.1 to enumerate the relative quantity of the bands.

1.Fish Albumin Proportion (%) =

$$\frac{Albumin\ Concentration\ (mg/mL)}{SFC\ Yield\ (mg/mL)} \times 100$$

2. Relative Quantity (%) =

$$\frac{Raw\ Volume\ of\ A\ Band}{Total\ Raw\ Volume\ within\ A\ Lane} \times 100$$

3. Relative Quantity (µg/mL) =

$$[Equation\ 2] \times Total\ Protein\ Loaded\ (\mu g/mL)$$

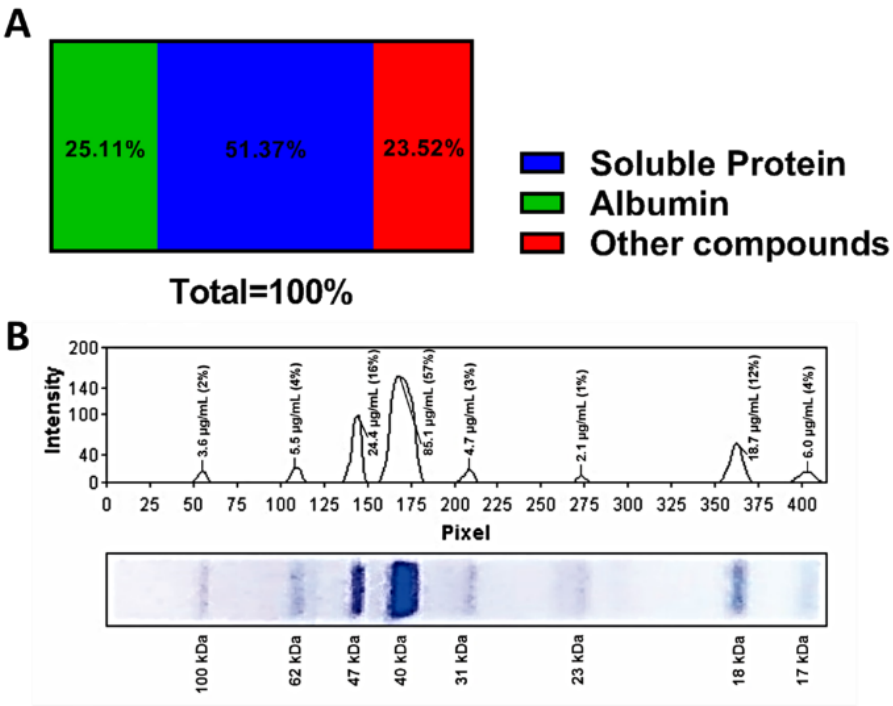

Figure 1. The proportion of protein distribution (A) and densitometric analysis of SDS-PAGE (B) from Snakehead fish protein concentrate (SFC). The extraction process recovered 25.11%, 51.37%, and 23.52% of fish albumin, soluble protein, and other compounds from the yield, respectively. Two major bands were identified at 40 kDa and 47 kDa with the respective proportions of 57% and 16% from the SFC loaded. The fish albumin proportion was calculated within the SFC concentration of 10 mg/mL.

Following this obtained protein distribution (Figure 1A), a twenty-five percent fish albumin proportion was set to quantify the SFC needed for each *in vitro* capacitation medium treatment containing 3 mg/mL, 6 mg/mL, and 9 mg/mL of albumin.

In Vitro Capacitation

Sperm capacitation was done according to Parrish *et al.* (1988) with a slight modification. The capacitation medium used in this study was Tyrode's-based capacitation medium (TALP medium: 100 mM/L NaCl, 3.10 mM/L KCl, 2 mM/L $CaCl_2.2H_2O$, 0.3 mM/L $NaH_2PO_4.H_2O$, 0.4 mM/L $MgCl_2.6H_2O$, 25 mM $NaHCO_3^-$, 10 mM/L Hepes, 1 mM/L Sodium Pyruvate, 21.6 mM/L DL-Lactic Acid 90%, 10 µg/mL Heparin). The respective three straws (0.25 mL dose) of Friesian Holstein and Charolais breeds were thawed in a water bath at 37 °C for 30 s. The frozen-thawed semen was evaluated individually using computer-assisted sperm analysis (CASA) and selected based on its motility to create the exact condition of sperm; the minimum post-thawing motility of 40% was set and used for further steps.

Upon evaluation, all semen was pooled and centrifuged at 200× g for 5 min to remove the extender. The supernatant was discarded, and the pellet was immediately resuspended with the non-capacitation medium (TALP without albumin and heparin) up to ~1 mL. An aliquot 250 µL sperm pellet was further selected using a swim-up procedure by placing at the bottom of 6 mL tubes containing the capacitation medium with the following albumin contents: T-BSA6 (TALP + 6 mg/mL BSA), T-SFC3 (TALP + SFC containing 3 mg/mL fish albumin), T-SFC6 (TALP + SFC containing 6 mg/mL fish albumin), and T-SFC9 (TALP + SFC containing 9 mg/mL fish albumin). The swim-up samples were then incubated for 90 min to capture the entire capacitation process, including hyperactivated motility and acrosome reaction. All capacitation mediums were acclimatized by setting in the incubator under 5% CO_2 at 37 °C before use. At the end of incubation, the upper fraction of each treatment was recovered, centrifuged at 200× g for 5 min, and removed its supernatant, leaving ~250 µL of the sperm pellet. Kinematic parameters, hyperactivation, sperm capacitation status, and acrosome integrity were further assessed.

Computer-assisted Sperm Analysis (CASA)

Sperm kinematic parameters associated with hyperactivated motility, such as the amplitude of the lateral head displacement (ALH), velocity curved line (VCL), and linearity (LIN), were examined on a pre-warmed coverslip-coated slide (37 °C) by using AndroVision software (Minitube, WI, USA) connected to a Zeiss AxioScope (Carl Zeiss, Göttingen, Germany). The other kinematic parameters such as velocity average path (VAP), velocity straight line (VSL), distance average path (DAP), distance curve line (DCL), distance straight line (DSL), wobble (WOB), straightness (STR), beat cross frequency (BCF), and head activity (HAC) were also observed from a total of at least 200 cells observation.

Hyperactivated motility was defined as spermatozoa showing a high amplitude of lateral head displacement and an eight-shaped flagellar beating pattern (Mishra *et al.*, 2019). Ten microliters of the resuspended sperm pellet were taken, placed onto a pre-warmed chamber slide, and subjected to rapid CASA analysis. A single appraiser conventionally determined sperm hyperactivation according to the capacitated sperm swimming pattern as previously recorded (Chung *et al.*, 2017). The number of hyperactivated sperm over the total number of sperm in all fields of reference was considered the percentage of hyperactivation.

Chlortetracycline (CTC) Assay

Sperm suspension (~150 µL) was centrifuged at 2000× g for 2 min, the supernatant was removed, and the pellet was dissolved and incubated in a phosphate-buffered saline (PBS) and daily prepared CTC solution (750 µmol/l CTC in 130 mmol/l NaCl, 5 mmol/l cysteines, 20 mmol/l Tris-HCl, pH 7.8) with a ratio of 45 µl:45 µl for 30 min under dim light. Sperm cells were then fixed by 8 µL of 12.5% paraformaldehyde in 0.5 mol/l Tris-HCl (pH 7.4), and an aliquot of 8 µL solution was smeared onto object glass, overlaid with a coverslip, and immediately observed within 24 h under the Axio Scope A1 fluorescence microscope (Carl Zeiss, Thornwood, USA) with a magnification of 400×. A total of at least 100 sperm or 10 fields were evaluated in each group

of the treatments. Sperm capacitation statuses were classified into three categories based on their acrosomal characteristic: uncapacitated-acrosome intact sperm (F): bright fluorescent over the entire sperm head and positive mid-piece of the tail, capacitated-acrosome intact sperm (B): prominent fluorescent positive equatorial segment, mid-piece of the tail, and fluorescence-free (dark) band in the post-acrosomal region, acrosome reacted sperm (AR): Low fluorescent signal throughout sperm head, with remaining positive signal in the equator segment and mid-piece (Ded *et al.*, 2010).

Imaging Flow Cytometry

The acrosome integrity assessment was done by live imaging flow cytometry (FlowSight, Seattle, WA, USA). The lectin PNA from Arachis hypogaea (peanut) conjugated with Alexa Fluor® 488 and Propidium Iodide (PI) were employed (Thongkham *et al.* 2021). Specifically, a 50 µL sperm suspension was dissolved into 450 µL Phosphate Buffer Saline (PBS). PNA-Alexa 488 (100 µg/mL) and PI (1 mg/mL) were added to the sperm solution, 1.5 µL each (final sperm concentration 2.6×10^6 cells/mL). The mixture was then incubated at 37 °C for 10 min in the dark and followed by centrifugation at 1075× g for 5 min. The supernatant was removed, and the pellet was resuspended in 50 µL PBS before flow cytometric analysis. All sperm solution was loaded into a 96-well plate and visualized with a 60-mW 488-nm laser to do excitation to PNA-Alexa 488 and PI. Acrosome statuses were identified according to several characteristics, live-acrosome-intact sperm (LI): PNA-Alexa 488 negative and PI negative, live-acrosome-reacted sperm (LR): PNA-Alexa 488 positive and PI negative, dead-acrosome-intact sperm (DI): PNA-Alexa 488 negative and PI positive, and dead-acrosome-reacted sperm (DR): PNA-Alexa 488 positive and PI positive. The IDEAS version 6.2 (Amnis, Seattle, USA) data analysis software was used.

Statistical Analysis

All numerical data were presented as mean ± SEM. All values were subjected to the Shapiro-Wilk test to check the normal distribution, followed by Levene's test for homogeneity of variance. Data of sperm kinematic parameters, hyperactivation rate, capacitation status, and acrosome integrity that met the assumption of normality and homogeneity were analyzed using one -way ANOVA, while data that were not normally distributed were analyzed using Kruskal-Wallis's test. Both analyses were proceeded to Tukey's HSD test for multiple comparisons in RStudio version 1.4.1717 (The R Foundation, Vienna, Austria). The difference was considered significant when $p<0.05$. All graphs were constructed in GraphPad Prism version 9.0.0 (GraphPad Software, San Diego, USA).

RESULTS

Albumin Proportion and Densitometric Profile

According to the results of the bicinchoninic protein assay and the bromocresol purple albumin assay, the SFC powder contained 76.48% total soluble protein, of which 25.11% and 51.37% were albumin and other soluble proteins, respectively. Meanwhile, the other compound, which does not belong to soluble protein, was also identified at 23.52% (Figure 1A). The densitometric analysis revealed 40 kDa and 47 kDa as the major bands, accounting for 57% and 16% of the loaded SFC (Figure 1B).

Post-thawing Motility (PTM)

The initial post-thawing motility test was conducted to establish the uniformity and consistency of early sperm motility among the employed breed of cattle, thus avoiding any possible interference following incubation with a capacitation medium. Each of the three straws from two distinct cattle breeds was studied severally. The motility cut-off of 40% was employed as a minimum benchmark for the motility of bovine sperm in subsequent experimental stages. The results demonstrated no statistical difference ($p>0.05$) in the early motility between the two breeds employed, indicating that the initial motility of the two breeds was identical (Figure 2).

Kinematic Parameters and Hyperactivation

The concentration of 3 mg/mL fish albumin from SFC in the capacitation medium (T-SFC3) generated the sperm response with higher values ($p<0.05$) in VCL, LIN, and ALH (18.70 ± 3.81 µm/s, 38.70 ± 4.33%, 0.29 ± 0.04 µm, respec-

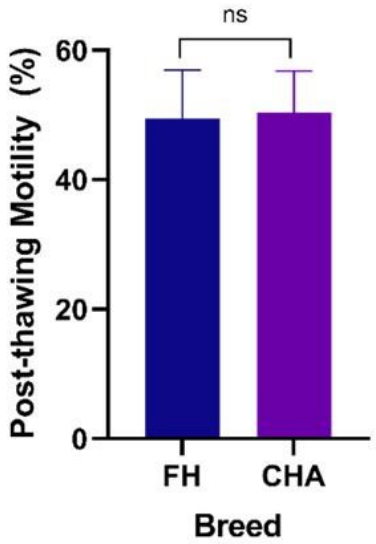

Figure 2. Individual post-thawing motility (PTM) from Friesian Holstein (FH) and Charolais (CHA). No significant differences ($p>0.05$) were observed in the initial motility data between the two breeds of bovine sperm (n = 3 biological replicates, over 200 sperm were analyzed per experimental unit). Data were analyzed using classical Student's t-test and presented as mean ± SEM. ^{ns}Non-significant difference.

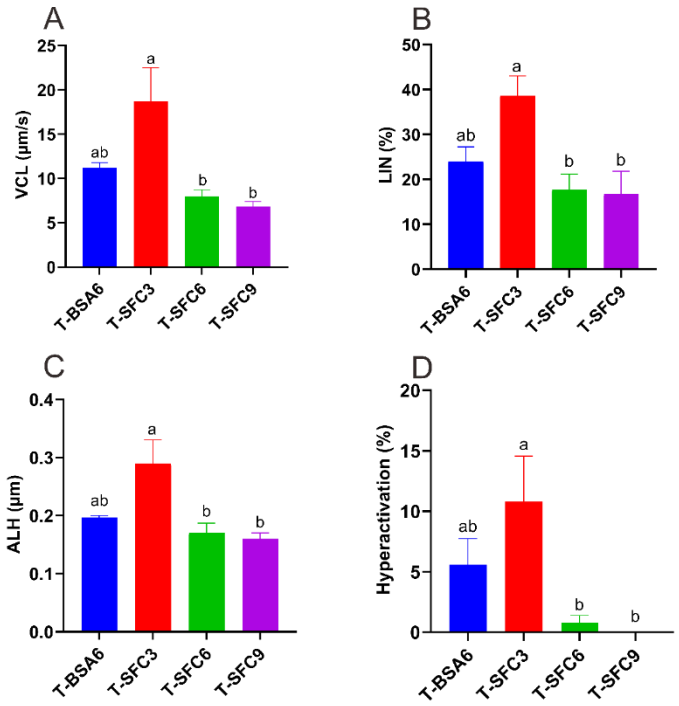

Figure 3. Differences of sperm kinematic parameters related to hyperactivation (A-C) and hyperactivation rate (D) from T-BSA6 (TALP medium + 6 mg/mL BSA), T-SFC3 (TALP + SFC containing 3 mg/mL fish albumin), T-SFC6 (TALP + SFC containing 6 mg/mL fish albumin), and T-SFC9 (TALP + SFC containing 9 mg/mL fish albumin) following the incubation for 90 min at 37 °C under 5% CO_2. The higher value of kinematic parameters related to hyperactivation, i.e. velocity curved line (VCL), linearity (LIN), and amplitude of the lateral head displacement (ALH) (n = 3 biological replicates, over 200 sperm were analyzed per experimental unit), and hyperactivation rate (n = 5 biological replicates, all field of references were analyzed per experimental unit) were observed when sperm were incubated in T-SFC3 as compared to other SFCs ($P < 0.05$) and control treatment ($p>0.05$). Data were analyzed by either one-way ANOVA or Kruskal-Wallis test and presented as mean ± SEM. Bars with different superscripts (^{a,b}) indicate a significant difference ($p<0.05$).

Table 1. Kinematic parameters of sperm following 90 min of incubation in the capacitation medium with different albumin contents.

Parameters	Treatments				P-value
	T-BSA6	T-SFC3	T-SFC6	T-SFC9	
VAP (μm/s)	4.47 ± 0.55^{b}	8.12 ± 1.27^{a}	2.55 ± 0.41^{b}	2.28 ± 0.47^{b}	0.002
VSL (μm/s)	3.08 ± 0.41^{b}	6.26 ± 0.96^{a}	1.44 ± 0.33^{b}	1.19 ± 0.46^{b}	0.001
DAP (μm)	1.76 ± 0.39^{b}	3.78 ± 0.63^{a}	1.20 ± 0.19^{b}	1.05 ± 0.17^{b}	0.004
DCL (μm)	5.33 ± 0.27^{ab}	8.66 ± 1.94^{a}	3.85 ± 0.33^{b}	3.28 ± 0.20^{b}	0.020
DSL (μm)	1.80 ± 0.36^{ab}	2.89 ± 0.48^{a}	0.67 ± 0.16^{b}	0.52 ± 0.16^{b}	0.003
WOB (%)	35.00 ± 2.65^{ab}	50.30 ± 5.49^{a}	31.3 ± 3.28^{b}	33.00 ± 4.73^{ab}	0.043
STR (%)	68.30 ± 3.76^{ab}	77.30 ± 0.33^{a}	54.70 ± 6.39^{ab}	49.00 ± 9.45^{b}	0.038
BCF (Hz)	1.28 ± 0.12^{ab}	3.20 ± 0.96^{a}	0.91 ± 0.04^{b}	0.29 ± 0.08^{b}	0.015
HAC (rad)	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.00	0.02 ± 0.00	0.278

Note: Data of kinematic parameters were analyzed by either one-way ANOVA or Kruskal-Wallis test and presented as mean ± SEM. ^{a,b}Different superscript in the same row indicates significant differences ($P < 0.05$, n = 3 biological replicates, over 200 sperm were analyzed per experimental unit). Abbreviations: T-BSA6 (TALP + 6 mg/mL BSA), T-SFC3 (TALP + SFC containing 3 mg/mL fish albumin), T-SFC6 (TALP + SFC containing 6 mg/mL fish albumin), T-SFC9 (TALP + SFC containing 9 mg/mL fish albumin), VAP (Velocity average path; μm/s), VSL (Velocity straight line; μm/s), DAP (Distance average path; μm), DCL (Distance curve linear; μm), DSL (Distance straight line; μm), WOB (Wobble; %), STR (Straightness; %), BCF (Beat cross-frequency; Hz), and HAC (Head activity; rad).

tively) among all SFC treatments (Figure 3A-C). These values were consistent with the percentage of sperm that experienced hyperactivation (Figure 3D), which was higher by ~2-fold ($p>0.05$) as compared to the control treatment when a capacitation medium with BSA (T-BSA6) was used (10.80 ± 3.77% vs 5.60 ± 2.14%). However, the increment of fish albumin concentrations (T-SFC6 and T-SFC9) decreased ($p<0.05$) the kinematic parameters related to hyperactivation as well as the hyperactivation rate.

Likewise, variations were also seen in additional kinematic parameters such as VAP, VSL, DAP, DCL, DSL, WOB, STR, and BCF ($p<0.05$) after 90 min of incubation, except for HAC ($p>0.05$). As expected, sperm incubated in T-SFC3 also showed the highest values among all treatments. In direct contradiction, greater concentrations of SFC's albumin adversely influenced the sperm swimming pattern, as indicated by decreased kinematic parameters (Table 1).

Capacitation Status

In order to corroborate the observation of hyperactivation, sperm from the treatments with which the hyperactivation was observed were proceeded to assess the capacitation status. According to the chlortetracycline (CTC) assay, the percentage of the capacitation status was clustered into three patterns (Figure 4A-C). The data revealed that the application of BSA and SFC's albumin in a capacitation medium did not differentiate ($p>0.05$) the number of bovine sperm exhibiting F pattern upon 90 min of incubation, with the individual rate of 78.00 ± 7.09%, 62.30 ± 0.33%, and 67.00 ± 2.08% for T-BSA6, T-SFC3, and T-SF with the individual rate of 78.00 ± 7.09%, 62.30 ± 0.33%, and 67.00 ± 2.08% for T-BSA6, T-SFC3, and T-SFC6 each (Figure 5).

In contrast, a higher number of capacitated sperm (B pattern) was obtained upon incubation in T-SFC3 among all treatments, although the value did not differ significantly ($p>0.05$) as compared to T-BSA6 (36.70 ± 0.33% vs 21.00 ± 7.02%). Similar to the result of hyperactivation rate, lower sperm with B pattern and higher sperm with AR pattern were observed upon incubation in a capacitation medium with a higher concentration of SFC's albumin, among other treatments ($p<0.05$).

Acrosome Integrity

The acrosome integrity of live and dead sperm after incubation was further examined (Figure 6A-E). After the incubation of sperm in a capacitation medium with different albumin content, the percentage of live sperm with intact acrosome (LI), live sperm with reacted acrosome (LR), and dead sperm with reacted acrosome (DR) analyzed from the imaging flow cytometry

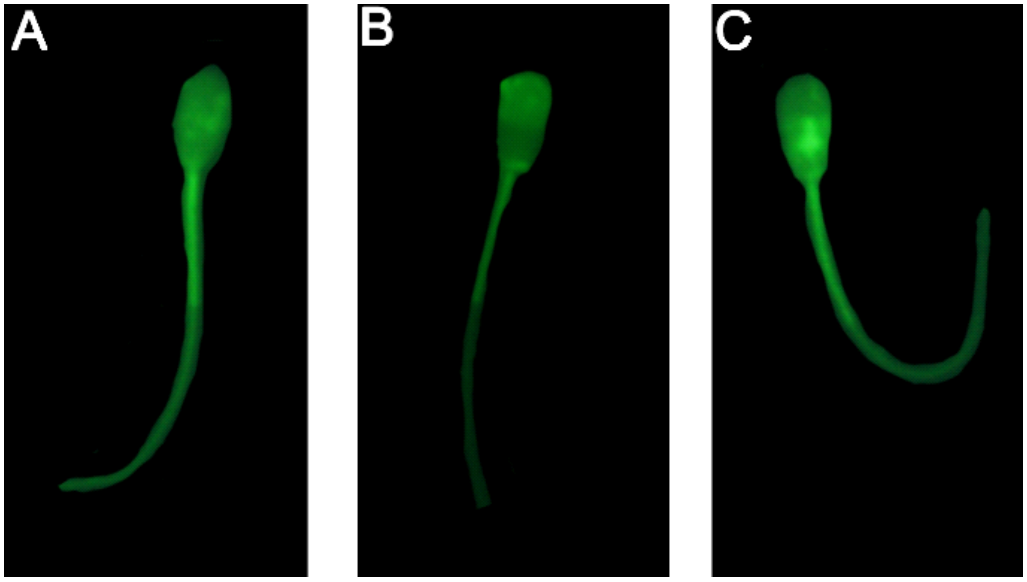

Figure 4. Representation of sperm showing different capacitation statuses. (A) Uncapacitated-acrosome intact sperm (F pattern): Bright fluorescent over the entire sperm head and positive mid-piece of the tail, (B) Capacitated-acrosome intact sperm (B pattern): Prominent fluorescent positive equatorial segment, mid-piece of the tail, and fluorescence-free (dark) band in the post-acrosomal region, (C) Acrosome reacted sperm (AR pattern): Low fluorescent signal throughout sperm head, with remaining positive signal in the equator segment and mid-piece.

did not differ ($p>0.05$) between T-BSA6 and T-SFC3 (Table 2). However, better results were achieved when sperm were treated with T-SFC6 and T-SFC9 ($p<0.05$). The higher concentration of albumin in SFC resulted in a higher percentage of LI and a lower percentage of both LR and DR. Additionally, no difference was observed from dead sperm with acrosome intact (DI) among the treatments ($p>0.05$).

DISCUSSION

In its natural condition, oviductal fluid plays a crucial role in facilitating sperm chemotaxis, rheotaxis, and thermotaxis, as well as in achieving sperm capacitation, as it contains complex composition; one of which is albumin (Kumaresan *et al.*, 2019). Albumin has been evidenced to promote capacitation through various identified mechanisms; the previous studies reported that sperm could not acquire capacitation without albumin (Chaves *et al.*, 2021), indicating the crucial role of albumin in modulating subsequent capacitation processes. Even though there are several alternatives such as recombinant oxysterol-binding protein-related protein (ORP-1 and ORP-2) (Suchanek *et al.* 2007) and methyl-β-cyclodextrin (MBCD) that act as sterol depletion, albumin from bovine serum is still prominent for achieving sperm capacitation (Boerke *et al.*, 2013).

By using a conventional staining method, our lab has previously confirmed that SFC's albumin possesses a similar ability to maintain bovine sperm in viable conditions as compared to BSA (Setiawan *et al.*, 2023). The differentiation between live and dead sperm was essential to identify the adverse effects that may arise due to the toxicity of snakehead fish protein concentrate (SFC). Moreover, the frozen semen used in this study can also result in cryo-capacitation, characterized by the spontaneous acrosome reaction, which is likely affecting sperm survival ability (García-Álvarez *et al.*, 2014). Thus, the previous result represented an equal percentage of recovered viable sperm among all treatments that may or may not experience capacitation.

In this preliminary study, applying albumin from snakehead fish protein concentrate (SFC) in Tyrode's medium showed sufficient evidence to promote the capacitation and hyperactivation of bovine sperm, indicating the potent ability to be

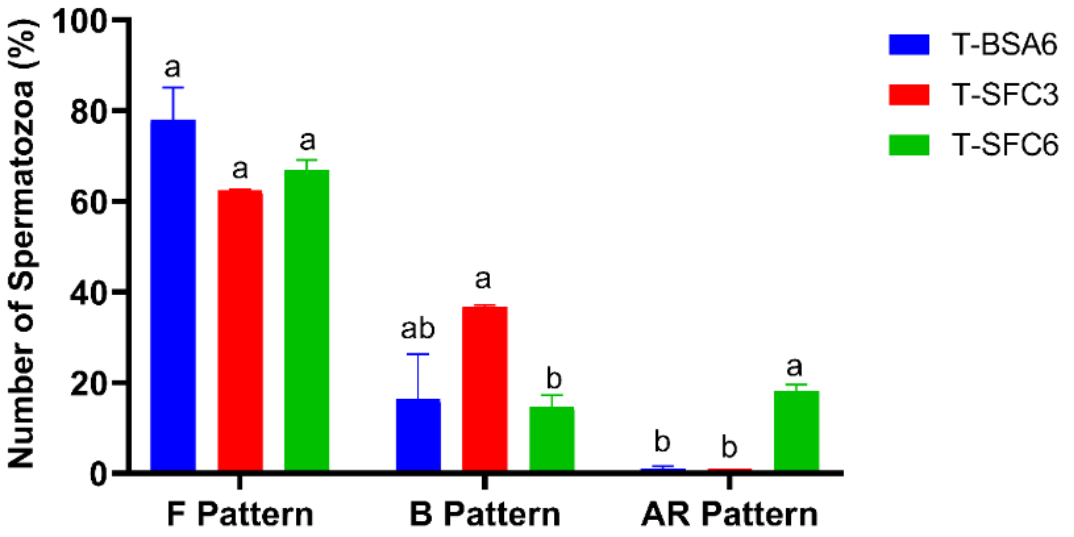

Figure 5. The percentage of spermatozoa with different capacitation statuses from T-BSA6 (TALP medium + 6 mg/mL BSA), T-SFC3 (TALP + SFC containing 3 mg/mL fish albumin), and T-SFC6 (TALP + SFC containing 6 mg/mL fish albumin) following the incubation for 90 min at 37 °C under 5% CO_2. A higher percentage of sperm showing B pattern was observed when sperm were incubated in T-SFC3 among other treatments (n = 3 biological replicates, over 10 fields or 100 sperm were analyzed per experimental unit). Sperm patterns: F (uncapacitated-acrosome intact sperm), B (capacitated-acrosome intact sperm), AR (acrosome-reacted sperm). Data were analyzed by one-way ANOVA and presented as mean ± SEM. Bars with different superscripts (^{a,b}) indicate a significant difference ($p<0.05$).

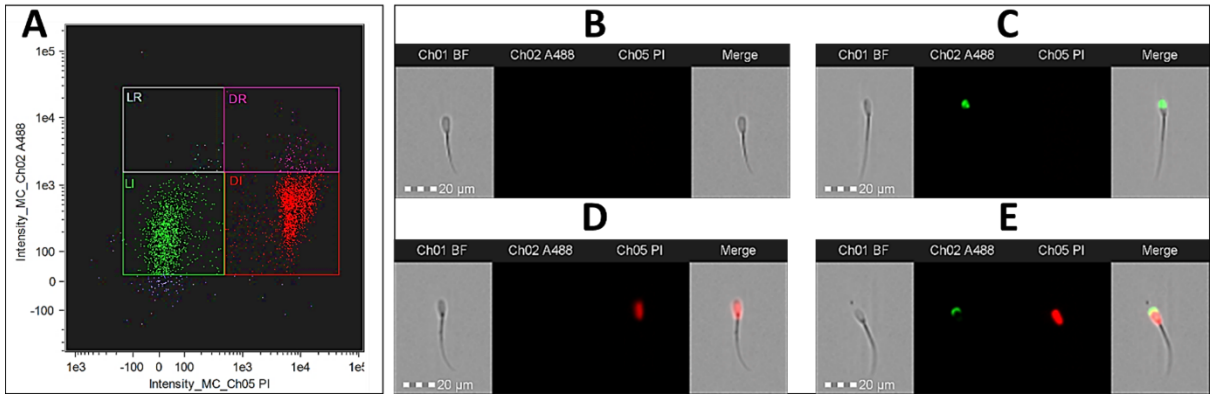

Figure 6. Representative density plots for imaging flow cytometry analysis of sperm acrosomes. (A) Regions used to analyze the acrosome integrity. Areas in each dot plot represent: Live acrosome-intact sperm (LI), live-acrosome-reacted sperm (LR), dead-acrosome-intact sperm (DI), and dead-acrosome-reacted sperm (DR). (B–E) Patterns of sperm observed with A488 PNA/PI: (B) LI, (C) LR, (D) DI, (E) DR.

used for serum albumin replacement. More sperm were capacitated from the chlortetracycline (CTC) assay supported by the high appraisal of hyperactivated motility, which rose ~2-fold when the albumin source was replaced by 3 mg/mL fish albumin from SFC (T-SFC3). This result was also affirmed with a high amplitude of both flagellar wave and head displacement, characterized by higher VCL and ALH among all treatments, and a low linear trajectory (LIN) than the previous report (Ryu *et al.*, 2019; Setiawan *et al.*, 2022).

The acquisition of hyperactive motility in sperm is modulated by the synergistic effect of albumin and bicarbonate, which trigger cAMP generation and Protein Kinase-A (PKA) activation, thereby inducing the phospholipid scrambling (Flesch *et al.*, 2001). Following this activa-

Table 2. Acrosome integrity of sperm following 90 min of incubation in the capacitation medium with different albumin contents.

Acrosome integrity (%)	Treatments				P-value
	T-BSA6	T-SFC3	T-SFC6	T-SFC9	
LI	9.38 ± 0.96[b]	19.10 ± 2.73[b]	54.20 ± 9.06[a]	48.30 ± 7.95[a]	0.000
DI	44.30 ± 1.53	39.70 ± 1.46	30.60 ± 5.57	40.80 ± 6.06	0.151
LR	1.68 ± 0.16[ab]	2.88 ± 0.59[a]	1.38 ± 0.27[b]	0.84 ± 0.11[b]	0.002
DR	42.40 ± 1.21[a]	37.00 ± 2.16[a]	12.70 ± 5.01[b]	8.83 ± 3.77[b]	0.000

Note: Data of acrosome integrity were analyzed by either one-way ANOVA or Kruskal-Wallis test and presented as mean ± SEM. [a,b]Different superscript in the same column indicates significant differences ($P < 0.05$, n = 3 biological replicates, over 10,000 were analyzed per experimental unit). Abbreviations: T-BSA6 (TALP + 6 mg/mL BSA), T-SFC3 (TALP + SFC containing 3 mg/mL fish albumin), T-SFC6 (TALP + SFC containing 6 mg/mL fish albumin), T-SFC9 (TALP + SFC containing 9 mg/mL fish albumin), LI (live-acrosome-intact sperm), DI (dead-acrosome-intact sperm), LR (live-acrosome-reacted sperm), DR (dead-acrosome-reacted sperm).

tion, albumin facilitates reverse cholesterol transport (RCT) by removing cholesterol, desmosterol, and oxysterol from the sperm surface to the extracellular environment (Bernecic *et al.*, 2019), which appears to be essential for fertilization (Boerke *et al.*, 2013). In the later phases of capacitation, PKA also regulates a rise in tyrosine phosphorylated proteins of sperm in the flagellum catalyzed by protein tyrosine kinase (PTK) activation (Parrish, 2014), leading to hyperactivation (Sati *et al.* 2014).

Besides, initiating hyperactivation of sperm motility demands intracellular alkalinization, which can be achieved by activating a voltage-gated proton channel (Hv1) (Lishko *et al.*, 2010). As previously reported, direct activation of this channel by albumin (Zhao *et al.*, 2021) led to the elevation of intracellular calcium influx via CatSper (Jin *et al.*, 2007), which alters sperm active motility patterns (Carlson *et al.*, 2007; Marquez and Suarez, 2007). In addition, the exhibition of hyperactive flagellar movement in sperm is also correlated to the chelation of zinc by albumin from the sperm plasma membrane, particularly in the principal piece, where Hv1 is located (Lishko *et al.*, 2010); evidenced by the identification of four different seminal zinc patterns, which represent the capacitation status of sperm (Kerns *et al.*, 2018).

Chlortetracycline assay distinguishes the capacitation status by penetrating the sperm membrane and, upon entering intracellular compartments (Gillan *et al.*, 1997), it becomes negatively charged and forms a CTC-Ca^{2+} complex that preferentially binds to hydrophobic areas, resulting in fluorescence-rich staining patterns (Pérez *et al.*, 1996; Rathi *et al.*, 2001). This mechanism confirms that T-SFC3 can modulate the intracellular calcium influx and distribution to more bovine sperm than T-BSA6 and other T-SFCs, leading to the highest sperm showing B pattern and hyperactive motility. However, the mechanism by which SFC's albumin elevates intracellular calcium in bovine sperm cannot yet be elucidated in this study and requires further intensive identification.

This study utilized crude protein from SFC that contains not only fish albumin but also impurities that cannot be neglected. The preliminary research for the albumin extraction revealed the other compound accounting for ~24%, which refers to the number of impurities from SFC (Setiawan *et al.*, 2024). Some minerals, including calcium and other proteins, are thought to be present in SFC and interfere with the CTC sperm staining due to their high affinity to the dye (Pérez *et al.*, 1996). Noteworthy, the elevation of albumin concentration up to 9 mg/mL from SFC content in the capacitation medium results in a broad area of fluorescence that covers and reduces the visibility of sperm; thus, the purification step and/or the utilization of different assays are highly required for better observation and more reliable findings.

The result of the acrosome integrity showed that T-BSA6 and T-SFC3, which induced high capacitation and hyperactivation, possessed significantly higher dead sperm with reacted acrosome (DR) than the higher concentration of T-SFCs. This discovery provides evidence that the acrosome reaction can only occur when the sperm have undergone capacitation (Bernecic *et*

al., 2019), as shown by changes in intracellular pH and calcium influx (Kerns *et al.*, 2018) that are accumulated in the acrosome store. However, as T-SFC3 generated a higher LI over two-fold than T-BSA6, an alternative theoretical mechanism is also suggested.

According to the previous report, an increase in sperm pH, particularly in the acrosome store, was not always followed by the acrosome reaction; this appears to be dose-dependent on the calcium flux (Chávez *et al.*, 2018). Thus, T-SFC3 may moderately increase the calcium influx due to its poorer ability than T-BSA6 to permeate the store-operated calcium channel (SOC). The study amplifies the notion that the acrosome reaction occurs only after a sustained high calcium influx, with SOC as the responsible channel (Felix *et al.*, 2004). Alternatively, T-SFC3 may induce a rapid transient calcium influx through a voltage-gated calcium channel (VGCC) that does not trigger the acrosome reaction; the lower occurrence of the acrosome reaction in live sperm was, therefore, observed in T-SFC3 than in T-BSA6. This mechanism also seems to extrapolate on the result of the high number of LI from the greater application of albumin in T-SFC6 and T-SFC9, as the higher involvement of impurities in SFCs decreases the permeability of those calcium channels.

Besides, as no stimulant was added, acrosome reaction is most likely to occur spontaneously (Parrish, 2014) or as a consequence of cell degeneration during sperm processing, including centrifugation and flow cytometry sorting (Flesch *et al.*, 2001); both resulted in sperm death. Therefore, the addition of high albumin concentration may protect the sperm from environmental damage as albumin adheres to the exterior surface of the cell membrane by non-specific adsorption and provides cell protection to prevent physical cell damage induced by hydrodynamic stress (Shahin *et al.*, 2020). Moreover, higher albumin concentrations are beneficial in neutralizing reactive oxygen species (ROS) (Farrugia, 2010), particularly hydrogen peroxide, which can trigger acrosome reaction, lipid peroxidation, and sperm death (Aitken and Nixon, 2013; Aitken, 2017; Setiawan *et al.*, 2022). Finally, as the high capacitation and hyperactivation rate was followed by microdomain aggregation and soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins in the apical ridge (Tsai *et al.*, 2010; van Gestel *et al.*, 2005) responsible for the high number of sperm bound per oocyte and high fertilization rate (Boerke *et al.*, 2013), further applications in the IVF steps are worth trying to confirm the fertilization ability of this new albumin source.

CONCLUSION

The result of the present study overall provided sufficient evidence that the application of snakehead fish concentrate (SFC) containing 3 mg/mL fish albumin in Tyrode's-based capacitation medium (T-SFC3) can promote the capacitation in bovine sperm with better results than control, proven by the higher hyperactivation rate, kinematic parameters, percentage of sperm with B pattern, and live-acrosome-intact sperm as compared to bovine serum albumin (BSA). However, this initial exploratory study has limitations in isolating and purifying fish albumin from SFC and lacks evidence in the *in vitro* fertilization (IVF) stage. Therefore, this study still demands subsequent explorations for more reliable findings.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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AUTHOR CONTRIBUTIONS

HS: Conceptualization, Methodology, Software, Formal analysis, Investigation, Data cura-

tion, Writing—original draft preparation, Visualization, Project administration. **TL:** Software, Investigation. **MT:** Methodology, Software. **PC:** Methodology, Software, Validation, Resources, Writing—Review and editing, Supervision. **KS:** Methodology, Software, Validation, Resources, Writing—Review and editing, Supervision. **MI:** Methodology, Software, Validation, Resources, Writing—Review and editing, Supervision. **AS:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing—Review and editing, Supervision, Funding acquisition. All authors have read and agreed to the manuscript.

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