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Effect of Skim Nanoparticles on the Motility and Kinematics of Simmental Cattle Frozen Semen

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ABSTRACT. Nanotechnology has positively impacted the development of extenders used in the cryopreservation of spermatozoa in livestock. The inclusion of nano skim in the extender is expected to preserve semen quality after thawing. This study aimed to determine the optimal concentration of nano skim. Various concentrations of nano skim (0%, 1.66%, 3.33%, 5%, 6.66%, 8.33%, and 10%) with the addition of 10% nano egg yolk in each extender were compared. Fresh semen samples were collected from three three-year-old Simmental bulls using an artificial vagina. The study parameters included motility (total motility, progressive motility) and kinematics (velocity curve linear (VCL), velocity straight linear (VSL), velocity average pathway (VAP), amplitude lateral head (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN), and wobble (WOB). A completely randomized design was employed, and data were analyzed using analysis of variance (ANOVA). Fresh semen dilution results indicated that only the control group (0%) and the nano skim group with concentrations of 6.66% and 8.33% were eligible for further processing into frozen semen. The results showed no significant difference between the three treatment groups on sperm motility and kinematics of Simmental cattle after thawing ($p>0.05$). The control group exhibited the highest percentage of total motility, VCL, VAP, and ALH values, while the 6.66% nano skim group outperformed the 8.33% nano skim group. The highest percentage of progressive motility and VSL value was observed in the 6.66% nano-skim group compared to the 8.33% nano-skim and control groups. The highest values of BCF, WOB, LIN, and STR was observed in the nano-skim group at 8.33% compared to the nano-skim group at 6.66% and control. The study concluded that 6.66% nano skim was the optimal concentration to maintain the quality of frozen semen of Simmental cattle.

Keywords: extender, nanoparticles, semen, Simmental, skim

INTRODUCTION

Artificial insemination (AI) is commonly used to increase livestock production (Abdel-Khalek *et al.*, 2018). Simmental cattle are one of the exotic cattle widely used for AI activities due to their fast growth, good performance, docility, easy to control, large body size, good muscle tone, and low fat contents (Kurnia *et al.*, 2020; Shoimah *et al.*, 2021). Bulls play a role in improving the quality of offspring produced genetically through spermatozoa. However, cryopreservation causes loss of membrane integrity, DNA fragmentation, or impaired cell function, reducing sperm motility and fertilization potential. Bovine spermatozoa are susceptible to peroxidative damage and oxidative stress due to the high polyunsaturated fatty acids content (Ismail *et al.*, 2020). Therefore, extenders with cryoprotectants and additives contained are needed to stabilize sperm cells and prevent damage during cryopreservation and freeze-thawing (Arif *et al.*, 2022).

Many studies have confirmed the benefits of milk and egg yolk as extracellular cryoprotectants in semen preservation across various species, including bovine (Novita *et al.*, 2021), buffalo (Pramanik *et al.*, 2021), sheep (Galarza *et al.*, 2020), goat (Wahjuningsih *et al.*, 2019), chicken (Saleh *et al.*, 2022), fish (Malik *et al.*, 2018), and others. Milk and egg yolk are economical, and effective cryoprotectants still favored by inseminators (Bustani & Baiee, 2021; Pytlik *et al.*, 2022). A concentration of 10% skim and 5% egg yolk in the extender provides a protective effect on bull semen cryopreservation (Sukirman *et al.*, 2020).

Nanotechnology has introduced new ways to improve extender media to maintain sperm viability during storage and cryopreservation (Tsakmakidis *et al.*, 2021). A lecithin nano-extender has been developed for bovine spermatozoa, showing positive effects compared to conventional extenders (Mousavi *et al.*, 2019). Sun *et al.* (2021) reported a similar case using nano lecithin in chicken semen. Meanwhile, the development of nano egg yolk plasma in dog semen has also shown encouraging results (Da Silva *et al.*, 2022). However, the development of nano skim extenders for bovine semen cryopreservation has not been explored. Therefore, this study aims to determine the optimal concentration of nano skim on the motility and kinematics of frozen Simmental cattle semen.

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MATERIALS AND METHODS

Material

The samples were collected from Simmental cattle semen owned by the Lembang Artificial Insemination Center, West Bandung Regency, West Java Province. The bulls were 3 years old, with a body weight of 760±20.

Method

Semen was collected from each bull using an artificial vagina and the semen quality was assessed. Only fresh semen with motility criteria ≥70% and concentration ≥1.000x10^6 ml was used. The experimental method was used with a completely randomized design (CRD). Nano skim concentration treatments (NS), namely P1=1.66%, P2=3.33%, P3=5%, P4=6.66%, P5=8.33%, P6=10%, and P7=control (10% skim and 5% fresh egg yolk). Each treatment was prepared in an egg yolk nano plasma extender with 0.05% fresh egg yolk, 1% glucose, 8% glycerol, and 0.66% stock glutathione. Each treatment was repeated three times. Dilutions were conducted according to the extender treatment, followed by microscopic analysis using the CASA Androvison® machine (Minitüb GmbH, Tiefenbach, Germany). Treatment groups with motility ≥55% were stored and equilibrated in a cool top (4-5^{o}C) for 4 hours, then packaged in straws that had been identified, frozen in liquid nitrogen vapor for 10 minutes in an automatic freezing machine to a temperature of - 140^{o}C, and then dipped in liquid nitrogen at - 196^{o}C. Samples were stored in liquid nitrogen for 24 hours, thawed, and analyzed. Parameters measured included the percentage of motility, progressive motility, VCL, VSL, VAP, ALH, BCF, LIN, STR, and WOB. Data were analyzed by ANOVA using the IBM SPSS Statistics program 29. followed by the Duncan test for differences between treatments.

Preparation Nanoparticles

The nanoparticles were derived from skim milk (Mugro®) and powdered egg yolk (Ovobel®), prepared by the wet milling method, as described in the literature, with slight modifications (Florena *et al.,* 2021). The particles were measured using a Particle Size Analysis (PSA) instrument, mixed with water, and placed in Beadsmill machine tube with a fifty-micron (30:70) Zirconia ball. The milling process was carried out for approximately 2 hours, filtered, and measured again with PSA. Skim milk and egg yolk nanoparticle measurements averaged 92.3 nm and 91.3 nm, respectively. The samples were dried to obtain nanoparticles in powder form and stored at room temperature until used.

RESULTS AND DISCUSSION

Fresh Semen Quality

Evaluation of fresh semen samples was conducted macroscopically and microscopically (Moradpour, 2019), as shown in Table 1.

The results are normal according to the research conducted by Kurnia *et al.* (2018) with a volume of approximately 5.0±1.04 to 7.33±2.08 ml, the semen is creamy, pH 6.4, and medium to thick consistency. Examination of individual motility of 66.66% - 73.33%, sperm concentration 1336.33±196-1373.66±218x10^6 per ml, and mass movement was 2.33±0.57. The average percentage of individual motility in fresh semen exceeded 70% meeting the requirements of SNI 4869.1:2017 for frozen bovine semen. Meanwhile, CASA machine evaluation provided better results compared to manual analysis using a light microscope as shown in Table 2.

Table 1. Fresh semen quality of Simmental cattle

Macroscopic	
Volume (ml)	5.7 ± 1.74
Color	Milk-cream-yellow
Consistency	Currently
pH	6.63 ± 0.15
Microscopic	
Individual motility (%)	72.08 ± 2.57
Spermatozoa concentration (x10^6/ml)	1306.83 ± 151.68
Mass movement	+2

Table 2. Characteristics of fresh semen motility of Simmental cattle

TM (%)	78.29 ± 7.25
PM (%)	76.81 ± 7.92
VCL (µm/s)	123.92 ± 29.41
VSL (µm/s)	58.81 ± 11.72
VAP (µm/s)	66.22 ± 12.94
ALH (µm)	1.33 ± 0.35
BCF (Hz)	16.04 ± 3.82
WOB	0.54 ± 0.04
LIN	0.48 ± 0.05
STR	0.89 ± 0.04

Description:
TM (total motility); PM (progressive motility); VCL (velocity curve linear); VSL (velocity straight linear); VAP (velocity average pathway); ALH (amplitude lateral head); BCF (beat cross frequency); WOB (wobble); LIN (linearity); STR (straightness).

Sperm Motility

Motility is the progressive movement of spermatozoa and is one of the determinant of semen quality and fertilization success (Prastika *et al.*, 2018). The CASA machine is used because the results are more objective and detailed for quality assessment and fertility estimation (Pipan *et al.*, 2020). The percentage of motility after dilution is presented in Table 3.

The percentage of motility decreased in the P1, P2, P3, and P6 groups compared to the control ($p<0.05$). However, no significant difference in the percentage of TM and MP between P4, P5, and control groups ($p>0.05$, see data in Table 3). The highest percentage of motility among the NS treatments was observed in the P4 group. Only the P4, P5, and control groups showed a percentage of motility above 55%, meeting the requirements to continue the semen freezing process.

These results indicate that supplementation of 6.66% and 8.33% nano skim in egg yolk extender was able to maintain the motility and kinematics of Simmental bovine spermatozoa after dilution. The presence of casein and lactose in skim milk in sufficient quantities is thought to protect spermatozoa during storage. Casein can bind to BSP (protein binder of sperm) thus preventing the loss of lipids from the sperm cell membrane. Meanwhile, lactose is likely to be used for energy needs during the process of sperm metabolism (Khaeruddin *et al.*, 2020).

Table 3. The effect of nano skim concentration on sperm motility and kinematics of Simmental cattle after dilution

Variable	Nano Skim Concentration Treatment						
(Unit)	P1	P2	P3	P4	P5	P6	P7
TM (%)	33.06±7.45^{a}	36.98±2.79^{a}	40.00±9.68^{a}	73.44±10.25^{c}	66.70±46.5^{bc}	46.5±3.30^{ab}	78.29±2.24^{c}
PM (%)	20.01±4.76^{a}	27.00±4.76^{ab}	35.39±9.72^{abc}	67.89±15.38^{de}	59.48±13.34^{cde}	41.14±7.34^{bcd}	77.05±3.27^{e}
VCL (µm/s)	32.36±6.10^{a}	42.04±4.00^{a}	53.13±10.46^{ab}	93.14±63.31^{ab}	64.05±19.64^{ab}	48.57±4.92^{a}	123.93±18.20^{b}
VSL (µm/s)	6.89±0.62^{a}	14.65±5.74^{ab}	21.87±6.82^{ab}	36.53±19.80^{c}	27.34±8.38^{bc}	20.34±0.95^{ab}	58.82±7.51^{c}
VAP (µm/s)	10.78±0.84^{a}	18.70±5.46^{ab}	26.49±7.28^{ab}	46.56±29.04^{bc}	32.81±8.35^{abc}	25.21±1.23^{ab}	66.22±8.83^{c}
ALH (µm)	0.46±0.09^{a}	0.50±0.01^{a}	0.62±0.09^{ab}	1.16±0.66^{ab}	0.81±0.20^{ab}	0.71±0.09^{ab}	1.33±0.22^{b}
BCF (Hz)	6.58±0.23^{a}	10.81±3.68^{ab}	8.90±2.07^{a}	10.17±1.31^{ab}	11.14±3.01^{ab}	6.94±0.76^{a}	16.04±2.13^{b}
WOB	0.34±0.04^{a}	0.44±0.09^{ab}	0.49±0.06^{ab}	0.51±0.05^{b}	0.52±0.04^{b}	0.52±0.07^{b}	0.54±0.02^{b}
LIN	0.22±0.05^{a}	0.34±0.105^{ab}	0.41±0.01^{b}	0.42±0.06^{b}	0.43±0.01^{b}	0.42±0.06^{b}	0.48±0.02^{b}
STR	0.64±0.07^{a}	0.77±0.09^{ab}	0.82±0.04^{b}	0.81±0.06^{b}	0.82±0.06^{b}	0.81±0.01^{b}	0.89±0.01^{b}

Description: TM (total motility); PM (progressive motility); VCL (velocity curve linear); VSL (velocity straight linear); VAP (velocity average pathway); ALH (amplitude lateral head); BCF (beat cross frequency); WOB (wobble); LIN (linearity); STR (straightness). Different superscripts in the row indicate differences ($P<0.05$).

Previous research by Khaeruddin *et al.* (2020) found that using 6% and 9% skim milk in Ringer's lactate, was effective in maintaining spermatozoa motility of native chickens up to 48 hours of storage compared to controls. Other results reported that the use of 10% skim milk in Aceh cattle semen which was stored at 4˚C for 24 hours was able to maintain sperm motility (Novita *et al.*, 2021). Different results were shown by Raji *et al.* (2022) on the use of skim milk, which caused a drastic decrease in motility, damage to the acrosome, and death of epididymal sperm cells in goats due to goat bulbourethral secretions.

This report also showed that the low concentration of nano skim (1.66-5%) reduced its ability to maintain sperm viability. Small amounts of casein are thought to be insufficient to prevent large amounts of lipids from being lost from the sperm membrane, causing many sperm to die from cold shock. Conversely, the high concentration of nano skim (10%) is thought to cause hypertonicity in the extender. This condition results in release of water from the sperm cells, decreasing motility and viability, leading to death (Khaeruddin *et al.*, 2020). Feugang *et al.* (2019) argued that high concentrations of nanoparticles were identified as the cause of toxicity resulting in decreased sperm motility and viability. This finding is consistent with the negative effects caused by the use of high concentrations of nano-lecithin (1.5% and 2%) in chickens, as they tend to be toxic, cause pro-oxidant effects, exacerbate reactive oxygen species (ROS) production, and excessive free radical scavenging (Sun *et al.*, 2021). Other reports also mention a decrease in the quality of goat semen because the high concentration of 4% nano lecithin causes excessively modifies the coating or membrane (Nadri *et al.*, 2019).

The results of the motility parameters after freezing and thawing are shown in Table 4. There was no significant difference in the motility parameters between the three treatment groups ($p>0.05$). A higher percentage of TM was observed in the control group. However, the P4 group was still superior to its P5 group. A higher percentage of MP was observed in the P4 group than in the P5 and control groups.

Table 4. Effect of nano skim concentration on sperm motility and kinematics of Simmental cattle after thawing

Variable	Nano Skim Concentration Treatment		
(Unit)	P4	P5	P7
TM (%)	53.98±4.97	47.01±3.61	57.75±4.65
PM (%)	49.96±5.26	44.01±4.15	46.20±8.01
VCL (µm/s)	57.77±8.83	53.50±11.91	66.24±16.99
VSL (µm/s)	25.79±3.63	26.75±10.94	26.71±7.57
VAP (µm/s)	30.59±3.89	31.08±10.50	33.51±7.83
ALH (µm)	0.75±0.08	0.68±0.08	0.81±0.20
BCF (Hz)	10.07±1.04	10.68±2.86	10.37±2.19
WOB	0.53±0.03	0.57±0.06	0.51±0.01
LIN	0.45±0.03	0.48±0.09	0.40±0.03
STR	0.84±0.02	0.85±0.06	0.79±0.06

Description: TM (total motility); PM (progressive motility); VCL (velocity curve linear); VSL (velocity straight linear); VAP (velocity average pathway); ALH (amplitude lateral head); BCF (beat cross frequency); WOB (wobble); LIN (linearity); STR (straightness).

These findings indicate that the concentration of 6.66% nano skim is the best concentration for cryopreservation of Simmental bovine semen, with a mean particle size diameter of 92.3 nm. Khalil *et al.* (2019) noted that the unique properties of nanomaterials allow the use of less material but are more efficient compared to conventional materials. Reducing particle size to <100 nm can affect the physicochemical properties of a substance (Khan *et al.,* 2019). The solubility and biological activity are higher than conventional particles, making them more optimal for protecting the sperm cell membrane (Ariantie *et al.,* 2021). Nano skim and nano egg yolk plasma are thought to be able to work together to produce better sperm interaction or coating so that damage due to cold shock during the freezing and thawing process can be minimized (Nadri *et al.,* 2019). Progressive motility, which was higher at 6.66% nano skim concentration compared to the conventional one, might be due to the smaller particle size providing less resistance to movement, making it easier for sperm to move (Ariantie *et al.*, 2021).

These results are different from other studies using 1% nano lecithin supplementation in Lake extender using the sonication method, which increased motility, plasma membrane, and acrosome integrity, mitochondrial activity, percentage of fertility, and hatchability of chicken sperm after thawing compared to conventional (Sun *et al.,* 2021). Another similar study on dog semen with nano supplementation of egg yolk plasma containing 8% LDL in tris extender using the ultrasonication probe method significantly improved total and progressive sperm motility, sperm membrane integrity and fluidity, and intracellular reactive oxygen species levels (Da Silva *et al.*, 2022). The varying results may be due to differences in nanoparticle preparation methods, extender, cryoprotectants, and species used (Ugur *et al.,* 2019).

Sperm Kinematics

Kinematic evaluation characterizes the physiological state and potential fertilization capacity of sperm (Arruda *et al.,* 2021). The results of sperm kinetic parameters after dilution are shown in Table 3. In this study, no significant differences were found in sperm kinematics after sample dilution between the control and treatment groups, except for P1 (VSL, VAP, WOB, LIN, and STR) ($p<0.05$). The highest kinematic value was observed in the control. However, the VCL, VSL, VAP, and ALH were higher in the P4 group than in the control group. Similarly, a higher BCF value was observed in P5 than in the other treatment group. Meanwhile, higher WOB values were observed at P5 and P6.

These results indicate that the 6.66% and 8.33% nano skim as well as the control demonstrated the ability to maintain all sperm kinematics parameters (VSL, VCL, VAP, ALH, BCF, STR, WOB, LIN). Spermatozoa diluted in the extender are more efficient in maintaining the structure of the sperm flagella or their cellular metabolism, which is the main characteristic of sperm kinematics (Fernandez-Novo *et al.,* 2021). According to Arif *et al.* (2022), estimates of fertility can be evaluated using the parameters VCL, VSL, and VAP. Arruda *et al.* (2021) added that VAP, VCL, and BCF have been shown to correlate with fertility while the efficiency of sperm migration in cervical mucus is related to LIN. Sun *et al.* (2021) stated that VAP, VSL, STR, and LIN were positively correlated with sperm development, while VCL, ALH, and BCF were effective indicators of sperm strength.

The results of sperm motility and movement parameters after frozen thawing are shown in Table 4. There was no significant difference between the three treatment groups in terms of kinematics parameters (p>0.05). Higher VCL, VAP, and ALH values were observed in the control group. However, the P4 group was still superior to the P5 group. A higher MP percentage and VSL values were observed in the P4 group compared to the P5 and control groups. The values for BCF, WOB, LIN, and STR were higher in the P5 group than in the P4 and control groups.

This finding is similar to the research report by Da Silva *et al.* (2022) with supplementation of 8% nano plasma LDL 8% egg yolk, VSL, STR, LIN, ALH, WOB, and BCF values were not significantly different in all treatment groups. Another similar report by Sun *et al.* (2021) found that nano lecithin in extenders had no significant effect on ALH or WOB. Different results were reported by Sun *et al.* (2021) who found that low concentrations of nano lecithin (0.5% and 1.0%) increased the values of VAP, VSL, VCL, LIN, STR, and BCF. The higher BCF value is thought to be because lecithin can increase ATP production so that it correlates with heart rate. In line with this, Nadri *et al.* (2019) reported the positive effect of all lecithin nano treatments compared to their lecithin counterparts on goat semen cryopreservation. Two percent nano lecithin supplementation increased VCL, VSL, LIN, and WOB values.

CONCLUSION

Nano skim at 6.66% is the best concentration to maintain the quality of frozen semen of Simmental cattle. Further evaluation is needed on other semen quality parameters to determine their effectiveness.

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