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Specific-Protein Sperm Membrane Supplementation on Freezing Medium Maintain Post-Thawed Bull Sperm Quality

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Abstract : Based on previous study, plasma membrane protein of spermatozoa weighed around 55 kDa was found in fertile *Friesian Holstein* bull. Observation on the semen of 8 bulls indicated that higher specific protein sperm membrane weighed 55 kDasuggested as significant marker of fertility. Detrimental effects caused by cryopreservation have been widely studied. Therefore, it needs to be added into the semen diluter in order to improve the semen quality The objective of this research was to describe the mechanism of male cattle fertility improvement through the addition of specific protein into spermatozoa stored in freezing medium to increase the numbers of offspring which resulting the rapid improvement of dairy cattle population in Indonesia. To achieve this goal, sequences of gradual researches was conducted. The first phase consist of biochemistry characterization of specific protein sperm membrane in isolated bull spermatozoa and seminal plasma which involved specific protein sperm membrane expression in bull semen using specific protein sperm membrane 55 kDa monochlonal antibody and determining the weight of specific protein sperm membrane molecule using SDS-PAGE method which was then confirmed by Western blot. Receptor binding test based on immunohistochemistry and specific protein sperm membrane activity test to determine the optimum condition of isolated bull semen were also conducted.
The second research consist of laboratory and field test involving sequences method to measure the quality of frozen semen with specific protein sperm membrane addition from isolated bull semen including determining standard additional protein dose using ELISA method, determining optimum dose of specific protein sperm membrane should be added into freezing medium and examining the effect of protein addition on the qualities of spermatozoa after thawing, including motility, viability, plasma membrane integrity, dizziness, in vitro fertility, and in vivo fertility.
Keywords : bull semen, specific protein sperm membrane, freezing medium, semen quality, fertilization.

Introduction

Recent dairy cattle husbandry businesses face many problems resulting relatively low cattle productivity. One of these problems is reproduction disorders which causes cow infertility. The disorder can be identified from low calving rate[9].Repeat breeding may be the main factor of infertility. Repeat breeding refers to a condition when a cow has not been pregnant after being bred twice or three times. In normal and fertile cattle with normal fertility rate as much as 50-55%, about 9-12% of the cows undergo repeat breeding. One of the main cause of repeat breeding is low quality of frozen semen causing incapability of spermatozoa to fertilize the ovum. The quality of frozen semen plays a significant role in cow conception.

So far, semen examination to indicate bull fertility was only done by macroscopic and microscopic examinations. Fertility examination can also be measured from its pedigree –through a selection which is based on ancestral reputation of each young bull. However, this method was not very accurate because bloodline of individuals with good characteristics does not necessarily inherit the good characteristics through breeding[5]. Another bull fertility test can be done through progeny test by examining heritability of the bulls. However, the test takes 4-6 years to conduct therefore the test is not very efficient[1]. Semen examination needs to be completed by molecular examination considering certain substancein seminal plasma may indicate semen fertility. Before fertilizing the ovum, spermatozoa undergo capacitation inside female cattle reproductive duct.

According to[3] specific protein sperm membrane plays a significant role in successful fertilization and prevention topolyspermia. Hence, specific protein sperm membrane content of seminal plasma is associated with quality and fertility of a bull[4,·]A bull whose semen contains specific protein sperm membrane weighed 55 kDa has higher fertility rate (about 20%-25% times) compared to a bull whose semen does not contain certain protein[12,7].A research by[10]suggested that adding specific protein collected from milk to the frozen semen dilutersuccessfully increase the rate of fertilized ovum in vitro.This condition may also be applied on artificial insemination. Therefore, an attempt intended to improve frozen semen fertility by adding isolated specific protein sperm membrane into freezing medium needs to be conducted.

Experimental

This research consists of two stages of examination. The first stage of examination requires bull semen with separated spermatozoa and seminal plasma to obtain spermatozoa and seminal plasma proteins. Meanwhile, the second stage of examination requires bull semen with diluter and isolated specific protein sperm membrane.

Materials and reagents used in first phase examination are pH 7 Phosphate Buffer Saline (PBS), tween 20 (polyoxyethylenesorbitantmonolaurate Art. 822184, Merck-Schuchardt, Muenchen), phenyl methane sulphonyl (PMSF, Biorad), absolute ethanol (SIGNALCHEM), Western Blue Stabilized Substrate for alkaline phosphatase cat #S 3841 (Promega Co., USA), Anti IgG Rabbit AP (Anti Rabbit IgG C (Fc), AP Conjugate, Catalog #S 3731, Promega Co. USA), Anti IgG Rabbit SA-HRP (Catalog #S 3731, Promega Co. USA), nitrocellulose (Hybond-C pure, nitrocellulose membrane, Amersham Life Science- England), tissue, Adenosine triphosphate (ATP) BM 551.2 g/mol, histon (sigma H 5505), TCA (Trichloroacetic Acid) 8%, BO caffeine (Bricket and Olliphant Caffeine), TrisCl, KCl, NaCl, KH_2PO_4, Tween 20, Magnesium Chloride ($MgCl_2$), NaH_2PO_4, absolute ethanol (C_2H_5OH), sterile deionized water, bis-acrylamide, Sodium Dodecyl Sulphate (SDS), Ammonium per sulphate (APS), *N,N,N',N'*tetramethyletylenediamina (TEMED), and bromophenol blue. The equipment used in this examination are Blotter (Bio-Dot Apparatus, BIORAD-USA), microplate, vacuum pump, vortex, scissors, epfendorf pipet, Pasteur pipet, millipore, tip plastic, cellophane bags, centrifuge, reaction glass, refrigerated centrifuge, freezer, autoclave, measurement glasses: 10 mL, 100 mL, 250 mL, and 1000 mL, pipet volume 10 mL, beaker glass 100 mL, 250 mL and 1000 mL, dripping pipet, glass stirrer, watch glass, magnetic stirrer, digital pH-meter, analytic scales (sartorius basic P-160), centrifugation glass and centrifugation tools (Denley type BR 401), incubator (memmert), vortex (Guo-Huq), sonication (Branson 200), UV spectro-photometer, mini 2D electrophoresis protein II (Biorad), autoclave, stirrer, funnel, suction flask, sprayer, eppendorf, and refrigerator.

The materials and reagents used in second phase examination consist of: TCM-199, Bovine Serum Albumin (PBS), physiologic saline, glycerol, skim milk, egg yolk, eosin-negrosin, liquid nitrogen, gentamycin, and tissue paper. Meanwhile, the equipment used in this phase are: cool-top, water bath, artificial cow vagina, laminar flow, filling-sealing, goblet, canister, container, petri dish, Pasteur pipet, reaction flask, dissecting microscope, inverted microscope, optical microscope, object glass, cover glass, and insemination gun.

1. Procedures

The second phase of the research was intended to determine the optimum dosage of specific protein sperm membrane should be added into freezing medium to improve spermatozoa quality after being thawed. This phase consist of several steps. They are:

2. Determining Specific protein sperm membrane using ELISA method

Micro plates were coated by Antigen (standard Specific protein sperm membrane, SIGNAL CHEM) as much as 50 L per well and being incubated at 4 C temperature for 24 hours. The micro plates were washed by PBS 0.05%- Tween 20 4 times. The process was followed by adding blocking buffer as much as 50 L per well into the micro plates and incubated at room temperature for 2 hours. After being washed with PBS 0.05% - Tween 20 4 times, the micro plates were reacted with MAb-Specific protein sperm membrane (ABGENT) which had been solved into blocking buffer BSA 1% with solution series 1/250, 1/500, 1/1000, 1/2000, 1/4000, and 1/8000 as much as 50 L per well and incubated at 4C temperature for 24 hour. Micro plates were rewashed with PBS 0.05% - Tween 20 as many as 4 times and were reacted with secondary antibody (anti Rabbit IgG Biotin Labelled) which had been solved into TBS Tween 20 with solution 1/2500. Micro plates were incubated at room temperature for 1 hour and being washed with PBS 0.05%-Tween 20 as many as 4 times. Finally, SA-HRP substrate was added into the micro plates as much as 50 L per well. Then the micro plates were incubated in dark condition and at room temperature for 30 minutes added by HCl as reaction stopper. The measurement was based on titer read by ELISA reader in 450 nm wave-length[2].

3. Frozen Semen Diluter

Semen diluter used in this research was egg yolk-skim milk solution. Diluter used in this study was skim milk with egg yolk combination. Antibiotic of penicillin (dose: 1000 IU/mL) and streptomycin (dose: 1 mg/mL) were added into diluter and stored in the refrigerator. Those diluter which had been added with antibiotic were divided into two part, diluter A and diluter B. Diluter B was composed by diluter A added with glycerol 16% and fructose 25.

4. Mixing Semen and Diluter

Fresh semen was added into 20 ml of diluter A and then stored in cool-top with 3-5°C temperature as long as 35 minutes. Several treatments in this research are:controllgroup (P0): without specific protein sperm membrane addition, Treatment 1 (P1): 5g/ml specific protein sperm membrane addition, Treatment 2 (P2): 10 g/ml specific protein sperm membrane addition, Treatment 3 (P3): 20 g/ml specific protein sperm membrane addition. After water jacket being taken from isolate, the isolate were left for 50 minutes and added by the rest of diluter A and being left for 15 minutes.

Diluter B was addedgradually into each treatment (P0, P1, P2, and P3) and then put inside the cool top for an hour to achieve equilibration. These processes were continued by pre-freezing process by putting the straws on a rack about 4-5 cm above liquid nitrogen as long as 10 minutes. Finally, the straws were fully dipped into liquid nitrogen at -196 C.

Diluter volume estimation was done based on artificial insemination dosage referring to the concentration of spermatozoa per straw (0.25 ml). The sperm dosage per straw was 25-30 millions.

5. Examining Frozen Spermatozoa Viability Percentage

10 ml of semen suspension was dripped onto the tip of object glass added by eosin-negrosin dripping and blended until become homogenous. The tip of another object glass was scratched on the colored semen suspension in inclined position to obtain a thin layer of colored suspension and the object glass was aerated until dry. The object glass was examined under light microscope with 400X magnification. Spermatozoa with colorless head indicates living spermatozoa while pink-headed spermatozoa indicatesthe dead ones[13].

6. Examining Frozen Spermatozoa Motility Percentage

Frozen spermatozoa motility examinations consist of qualitative examination and quantitative examination. 10□L semen suspension of each treatment group was dripped on concave object glass. The object glass was covered by cover glass and observed under light microscope with 400 times magnification to examine spermatozoa motility. Quantitative examination of spermatozoa motility was determined by level of Spermatozoa motility was measured by comparing the numbers of motile and immotile spermatozoa on a view field randomly. Spermatozoa motility percentage was estimated based on average motility percentage of all examined view fields.

7. Examining Frozen Spermatozoa Plasma Membrane Intactness

Frozen spermatozoa plasma membrane and acrosome cover intactness examination was done based on Hypo-osmotic Swelling Test (HOST) method. 0.1 ml spermatozoa suspension taken from each treatment P0, P1, P2, and P3 was added with 9.9 ml hypo-osmotic solution (made from 7.35 g sodium citric and 13.52 g fructose diluted with 1 L aquadest) and incubated inside incubator CO_2 at 37□ C temperatures for an hour. Smear preparation was made by mixing a drop of the solution with a drop of eosin and observed under light microscope with 400 times magnification. Spermatozoa with complete plasma membrane integrity were indicated by swollen curved tails emitting bright color while spermatozoa with damaged plasma membrane were indicated by flat head and straight tails.

8. Measuring Fertilization Rate In Vitro

The measurement was conducted through laboratory test on frozen bull semen which had been supplemented by various dosages of specific protein sperm membrane on in vitro fertilization process. **a. Oocyte Collection**

Cow ovaries collected from slaughter houses were stored in NaCl 0.89% and added by gentamycin sulphate 50 □g/ml at 30-35□ C. The ovaries were washed in physiological saline added by gentamycin sulphate several times until the solutions became clear. Oocyte aspiration was conducted using G-18 needle connected to syringe 5 ml containing 1 ml PBS added by 0.3% BSA and gentamycin 50 □g/ml. the oocyte were washed three times in PBS medium and three times in TCM 199 consecutively. Only grade A and B oocyte put into in vitro maturation.

b. Oocyte Maturation

Oocyte in vitro maturation process was done in TCM 199 medium added by 0.01 □g/ml FSH, 0.01 □g/ml LH 3% BSA, and 50 □g/ml gentamycine sulphate. Oocyte were stored in petri dish containing 50 □L dripping medium and covered by mineral oil, each drop contained 10 oocytes. Oocyte maturation process was conducted on CO_2 5% incubator at 38.5□ C for 20-22 hours.

c. In Vitro Fertilization

Frozen bull semen of each treatment (P0, P1, P2, and P3) were used during in vitro fertilization preparation. Frozen semen were rinsed in 6 ml BricketOlliphant (BO) medium which had been added by 20 □g/ml heparin 3% BSA and 50 □g/ml gentamycin. Spermatozoa were diluted in fertilization medium and incubated in CO_2 5% incubator for an hour to optimize spermatozoa motility. Incubated spermatozoa were injected into maturated oocyte with dosage $1x10^6$spermatozoa/drop. Oocyte which had blended with spermatozoa were incubated in CO_2 5% incubator at 38.5□ C temperature for 24 hours. Fertilization rate was determined based on the numbers of zygote, two-cell embryo, and four-cell embryo developed after 24, 48, and 72 days of incubations. Successful fertilization was indicated by polarized cell bodies which was clearer, male and female pro-nuclei merge, and formed zygote.

9. Measuring Cow Conception Rate

The measurement was done on field test through artificial insemination using supplemented frozen semen by various doses of specific protein sperm membrane. Forty cows were estrous-synchronized using PGF2α and became estrous 48-72 hours after injection. The cows were inseminated using supplemented frozen semen with various dosages of specific protein sperm membrane. 10 cows were inseminated using frozen semen without specific protein sperm membrane supplementation (P0); (10 cows were inseminated using frozen semen supplemented with specific protein sperm membrane 5 µg / 50 millions spermatozoa) P1); 10 cows were inseminated using frozen semen supplemented with specific protein sperm membrane 10 µg / 50 millions spermatozoa) (P2); and 10 cows were inseminated using frozen semen supplemented with specific protein sperm membrane 20 µg / 50 millions spermatozoa) (P3). Conception diagnose was conducted on 75th day after insemination using rectal palpitation.

10. Data Analysis

Obtained data were analyzed descriptively. Spermatozoa viability and motility percentages were determined by Univariate Test. Tukey Test was done to detect any errors which may happen on previous test. Fertilization rate of in vitro fertilization and conception rate were measured using Kruskal-Wallis test continued by Mann Witney test to confirm any deviations[14].

Results and Discussion

Result of Post-Thawing *Frisian Holstein* Spermatozoa Motility Data Analysis of Each Treatment Group

Table 5.1: Post-Thawing Spermatozoa Motility Average Rate and Standard Deviations of the Treatment Groups

Treatment Groups	Average Motility Percentage (%) ± Standard Deviation
P0	$36,25^{a} \pm 4,4320$
P1	$41,875^{b} \pm 2,5877$
P2	$50^{c} \pm 2,6726$
P3	$51,25^{cd} \pm 3,5355$

Different superscripts indicated significant results of the four treatment groups.

Note:
P0 = Fresh semen + OPN 0 µg / 50 millions spermatozoa + PBS
P1 = Fresh semen + OPN 5 µg / 50 millions spermatozoa + PBS
P2 = Fresh semen + OPN 10 µg / 50 millions spermatozoa + PBS
P3 = Fresh semen + OPN 20 µg / 50 millions spermatozoa + PBS

One way ANOVA test result on the four treatment groups indicated significant differences ($p < 0.05$). Post-thawing spermatozoa motility average rate ordered from the highest to the lowest are: P3, P2, P1, and P0. This result was compared to BNT 5% test which indicated significant difference ($p < 0.05$) between P3 treatment group and three other treatment groups. There was insignificant difference ($p > 0.05$) between P2 and P3 treatment groups.

Result of Post-Thawing *Frisian Holstein* Spermatozoa Viability Data Analysis of Each Treatment Group

The following are the results of post-thawing spermatozoa viability data analysis using Analysis of Variance (ANOVA) test continued by BNT 5% test:

Table 5.2: Post-Thawing Spermatozoa Viability Average Rate and Standard Deviations of the Treatment Groups

Treatment Groups	Viability Average (%) ± Standard Deviation
P0	$43{,}375^{a}$ ±2,82
P1	$50{,}375^{b}$ ± 5,95
P2	56^{c} ± 3,07
P3	$64{,}875^{d}$ ± 5,16

Different superscripts indicated significant results of the four treatment groups.

Note:
P0 = Fresh semen + OPN 0 □g / 50 millions spermatozoa + PBS
P1 = Fresh semen + OPN 5 □g / 50 millions spermatozoa + PBS
P2 = Fresh semen + OPN 10 □g / 50 millions spermatozoa + PBS
P3 = Fresh semen + OPN 20 □g / 50 millions spermatozoa + PBS

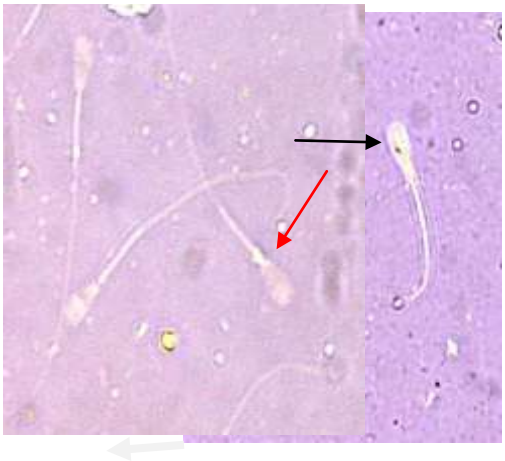

Figure 1: Observation of living and dead spermatozoa under light microscope using eosinnegrosin coloration and 400 times magnification: a. Colorless living spermatozoa (black arrow); b. dead purple-ish spermatozoa (red arrow)

Result of Frisian Holstein Post-Thawing Spermatozoa Plasma Membrane Intactness

The following bellow is post-thawing spermatozoa plasma membrane intactness examination analyzed using ANOVA (Analysis of Variance) test continued by BNT 5% test:

Table 5.3: Average Value and Standard Deviation of intact spermatozoa plasma membrane on each treatment group

Different superscripts indicated significant difference among treatment groups ($p < 0.05$).

Result of In Vitro Fertilization Examination of Each Treatment Group after Freezing

Examining the fertility of frozen Frisian Holstein semen which had been supplemented by various dosage of specific protein sperm membrane was conducted by using in vitro. Embryo resulted from in vitro fertilization was described in Figure 5 below:

Treatment Group	Average Intact Spermatozoa Plasma Membrane (%) ± Standard Deviation
P0	$41,13^{a}$ ± 1,49
P1	$42,63^{a}$ ± 1,85
P2	$45,63^{b}$ ± 2,26
P3	$50,63^{c}$ ± 1,69

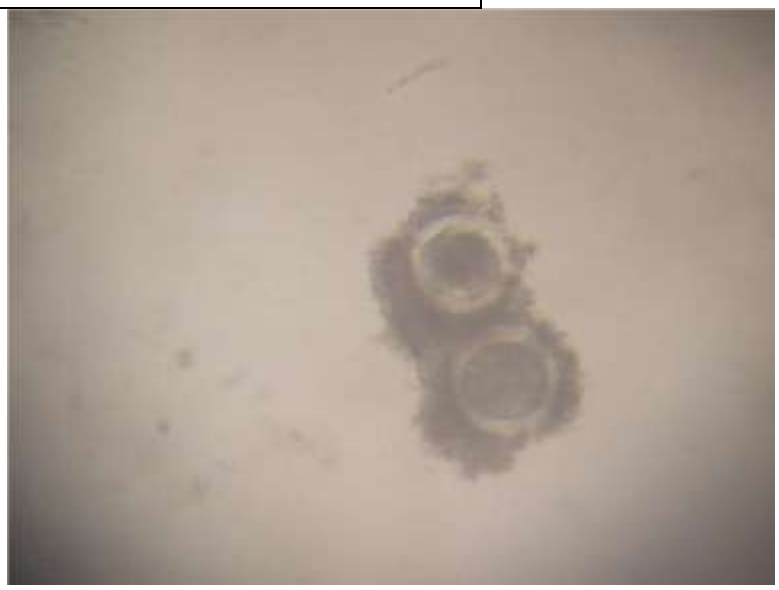

Figure 2: Zygote formation after In Vitro Fertilization

The numbers of embryo and in vitro fertilization percentages using frozen Frisian Holstein spermatozoa was presented in Table 5.4 below:

Table 5.4: The numbers of zygote resulted by In vitro fertilization using frozen Frisian Holstein spermatozoa supplemented by various concentrations of specific protein sperm membrane

Treatment Group	Oocyte	Examination on zygote formed after 24-hour in vitro fertilization (%)
P0	20	10(50)
P1	20	18(90)
P2	20	18 (90)
P3	20	20 (100)

Note:
P0: Controlled group (without specific protein sperm membrane supplementation)
P1: Supplemented by specific protein sperm membrane 5 □g / 50 millions spermatozoa
P2: Supplemented by specific protein sperm membrane 10 □g / 50 millions spermatozoa
P3: Supplemented by specific protein sperm membrane 20 □g / 50 millions spermatozoa

The result of statistical test measured using Kruskal Wallis test indicated different result between controlled group and treatment groups which had been supplemented by various dosages of specific protein sperm membrane in terms of numbers of zygote. To examine the difference among treatment groups, MannWhitney test was conducted. Based on the test, there was no significant difference between treatment group 1 and 2 (although the values were different). Meanwhile, there was significant different between controlled group and treatment groups ($p < 0.05$).

Cow Conception Examination after being Inseminated by Various Treatment Frozen Semen

Frozen Frisian Holstein semen was inseminated into cows which determined on several criteria, namely not in pregnant condition, had ever calved, and had no reproductive disorders. The results were presented on Table 5.5 below:

Table 5.5: Frisian Holstein Conception Percentage after being Artificially Inseminated by Various Treatment Frozen Semen (done on 75th day after insemination)

Treatment Groups	Number of Cows	Pregnant/(%)	Not Pregnant/%
P0	10	5 (50)	5
P1	10	9 (90)	1
P2	10	9 (90)	1
P3	10	10(100)	0

Note:
P0: Controlled group (without specific protein sperm membrane supplementation)
P1: Supplemented by specific protein sperm membrane 5 g / 50 millions spermatozoa
P2: Supplemented by specific protein sperm membrane 10 g / 50 millions spermatozoa
P3: Supplemented by specific protein sperm membrane 20 g / 50 millions spermatozoa

The result of statistical test measured using Kruskal Wallis test indicated different result between controlled group and treatment groups which had been supplemented by various dosages of specific protein sperm membrane in terms of numbers of conception. To examine the difference among treatment groups, Mann-Whitney test was conducted. Based on the test, there was no significant difference between treatment group 1 and 2 (although the values were different). Meanwhile, there was significant different between controlled group and treatment groups ($p< 0.05$).

Discussion

Progressive spermatozoa motility was one of important indicators determining good quality spermatozoa because progressive motile spermatozoa would be able to reach infundibulum enhancing ovum penetration. Individual progressive motility referred to individual spermatozoa ability in moving forward. Progressive motility was needed by spermatozoa to reach the location of fertilization inside female reproductive duct, mainly during penetrating ovum pellucid membrane.

The findings of this research indicated the highest Frisian Holstein spermatozoa motility percentage was obtained after being supplemented by specific protein sperm membrane in 20 g/ml dosage as much as 51.25 ± 3.5355 (%) (P3). This finding suggested that 20 g/ml was the most optimum dosage of specific protein sperm membrane in improving spermatozoa auto-phosphorilation which was associated with motility improvement. According to[16], tyrosine kinase phosphorilation in spermatozoa principal piece was associated with spermatozoa motility hyper-activation needed in penetrating ovum pellucid zone. This finding confirmed the previous research conducted by[17] indicating spermatozoa tyrosine phosphorilation is very important in motility hyper-activation and pellucid zone reaction.

Specific protein sperm membrane supplementation on freezing medium stimulated specific protein sperm membrane receptors spermatozoa membrane improvement. Eventually, specific protein sperm membrane receptors induced transduction signal by increasing adenylatecyclase. Adenylatecyclase activated cAMP which stimulated Protein Kinase A (PK-A). Increased PK-A induced tyrosine kinase and tyrosine phosphorilation. At last, increased tyrosine phosphorilation induced spermatozoa capacitation and motility hyper-activation[15]. Spermatozoa capacitation improved spermatozoa plasma membrane and ovum pellucid zone bond which stimulated acrosome reaction on spermatozoa[18].

Previous study[6] proved that specific protein sperm membrane supplementation can improve mitochondria activity indicated from MitoTracker coloration. Mitochondria activity was expected to provide more energy which improved spermatozoa motility. This statement was in conformity with the finding of this research which indicated specific protein sperm membrane supplementation may improve post-thawing spermatozoa motility.

Spermatozoa viability percentage was one of semen quality indicators needed in improving fertilization. Eosin-negrosin coloration was applied to examine whether the spermatozoa were dead or alive. The principle of spermatozoa coloration laid on difference between dead and living spermatozoa affinity to absorb eosinnegrosin

coloration. Eosin-negrosin coloration was not able to permeate the membrane of spermatozoa head because of intact spermatozoa membrane permeability resulting spermatozoa head appeared clear/colorless. Meanwhile, dead spermatozoa head would appear reddish because of damaged membrane which causing the coloration easily permeate spermatozoa cytoplasm[19].

The finding of this research indicated Specific protein sperm membrane supplementation may improve viability rate of post-thawing spermatozoa. The percentages of spermatozoa viability order (arranged from the lowest to the highest) were: P0, P1, P2, and P3. This finding was in accordance with previous research conducted by[6] indicated that spermatozoa with specific protein sperm membrane supplementation had higher viability compared to spermatozoa without specific protein sperm membrane supplementation. Specific protein sperm membrane was one of several protein which highly contributed in cell resilience[21]. It was found that pig semen with high freezeability contained more specific protein sperm membrane.

Several factors of spermatozoa death caused by permeability damage were described in the previous study[20]. First, Ca^{2+} ion which initiated endonuclease enzyme activation which destroyed DNA inside spermatozoa nucleus and transglutaminase which formed covalent bond with membrane proteins resulting isopeptide causing cell death. Second, spermatozoa death may also be caused by changes on membrane structure –particularly phospholipid structure of membrane- as a result of phosphatidylserine translocation from inner layer of membrane onto outer layer. Third, spermatozoa death may also be caused by freezing temperature.

Specific protein sperm membrane supplemented on freezing medium may function as membrane stabilizer by forming bonds between hydrogen and amino acid composing membrane protein preventing membrane protein denaturation. The accumulation of membrane protein denaturation could kill cells. Denaturation inhibited physiological functions of membrane protein as enzyme, receptor, and in cell communication. These functions could not operate normally due to membrane protein denaturation[22].

Both plasma membrane and mitochondria membrane were vulnerable to cryopreservation. Cryopreservation biggest effects on spermatozoa are viability and motility decrease, changing permeability, and alteration on membrane lipid component. Lipid peroxidation during freezing process could cause spermatozoa membrane damage. Spermatozoa physical damage could be identified from acrosome plasma and membrane damages. Plasma membrane was cell's wall membrane functioned to circulate substances needed in metabolism process and cellular activities. It consisted of proteins, carbohydrate, and lipids which functioned as receptors for several substances. Intact spermatozoa membrane functioned to protect the whole cell and maintaining cell survivability. Intact spermatozoa membrane provided protection for cellular organelles from mechanical and chemical damages. It also functioned as filter in intracellular and extracellular circulation by maintaining substances which are still needed in metabolism process[8]. Spermatozoa membrane intactness is also examined on spermatozoa quality evaluation done by frozen semen industries[23].

Hypo-Osmotic Swelling Test (HOST) was a test conducted to identify spermatozoa with intact plasma membrane. Intact spermatozoa plasma membrane was characterized by bent (curving) tail as a result of good membrane permeability which was able to absorb water from hypotonic environment. Meanwhile, spermatozoa with damaged plasma membrane were indicated by straight tail.

Data analysis on spermatozoa plasma membrane intactness (as presented on Table 5.3 above) indicated significant difference ($p < 0.05$) between control group (P0) and treatment groups (P1, P2, P3). The percentages of spermatozoa plasma membrane intactness arranged from the lowest order were: P0, P1, P2, and P3. This result was in conformity with spermatozoa viability percentage. It proved that the higher specific protein sperm membrane concentration supplemented into frozen semen diluter the higher Frisian Holstein viability percentage.

In Vitro Fertilization

A test to examine the fertility of Frisian Holstein frozen semen with specific protein sperm membrane supplementation was conducted through in vitro fertilization. Fertilization referred to interaction between spermatozoa and ovum happened on several phases on the surface of both cells initiated by attaching extracellular matrixes followed by plasma membrane attachment. This was followed by spermatozoa and ovum (pellucid zone) involving ligands and receptors on both spermatozoa and ovum. Before spermatozoa fertilized ovum, it underwent several biochemical and physiological alterations (called *capacitation*). On in vitro fertilization, spermatozoa used to fertilize ovum underwent capacitation process in vitro manner involving complex medium containing organic

and non-organic substance needed in the process. One of capacitation parameters was tyrosine phosphorilation improvement inducing spermatozoa hyper-activation and acrosome reaction[18].

Fertilization process was initiated by spermatozoa penetration into cumulus oophorus mass surrounding the ovum. The interaction involved receptors both on spermatozoa and ovum. Ovum possessed extra-cellular layers called pellucid zone (PZ)[8]. Pellucid zone consisted of three parts: PZ 1. PZ 2, and PZ 3 which are reacted with spermatozoa plasma membrane during initial and secondary interactions[24]. PZ 3 was the first receptor functioned to attach spermatozoa while PZ 2 was the second receptor functioned to keep spermatozoa attached on the ovum during fertilization process[8].

Based on laboratory application of Frisian Holstein frozen semen samples with specific protein sperm membrane supplementation 5 µg/50 millions spermatozoa (P1), 10 µg/50 millionsspermatozoa (P2), and 10 µg/50 millions spermatozoa incubated in CO_2 5% for 22 hours which are used on in vitro fertilization test, the highest percentage of zygote formed was resulted in P3 as much as 100% (See Table 5.4). The results of Kruskal-Wallis statistical examination indicated different numbers of zygote resulted in controlled group (P0) and treatment groups (See Appendix 3). To describe further differences among each treatment group, MannWhitney test was conducted. Based on the test, it was indicated that there was no significant difference among controlled group (P0), P1, and P2 ($p > 0.05$). Meanwhile, there was a significant difference ($p < 0.05$) between controlled group and P3.

Based on in vitro fertilization, the highest percentage of zygote formed was found on P3. It may be caused by chemical and physical characteristics of the medium. Chemical characteristics of medium included solubility, purity, stability, and ion contents while physical characteristics of medium consisted of pH, osmotic concentration, surface tension, and buffer.

Field test of frozen Frisian Holstein spermatozoa supplemented with specific protein sperm membrane fertility was measured through artificial insemination. Parameters used to measure cattle reproductive efficiency were first calving age should be less than 24 month, the period between first calving and conception (day open) should be less than 100 days, conception percentage on first insemination (first service conception rate) should be around 70%, calving intervals should be less than 380 days, the numbers of straw given in insemination to impregnate the cow (service per conception) should be less than two, the number of pregnant cow in a herd (conception rate) should be around 95%, and the numbers of calf birth on a herd (calving rate) should be around 90%[8].

Artificial insemination done on forty cows using supplemented frozen semen in various concentrations. Descriptively, all cows which were inseminated by frozen semen supplemented with specific protein sperm membrane 20 µg / 50 millions spermatozoa (P3) were pregnant (100%). However, the percentages of pregnant cows on P2 and P1 were decreased into 90% while the conception percentage of cows inseminated by frozen semen without specific protein sperm membrane supplementation was decreased up to 50% only.

The successful of artificial insemination was always associated with application of other reproductive technologies including sexual stimulation. According to Inskeep et al. (1998), the applications of Progesterone Release Intra-vaginal Device (PRID) combined with estradiol benzoate, PRID combined with prostaglandin F2 alpha (PGF2α), or PGF2α injected on one injection pattern were able to increase conception rate as much as 60%, 50%, and 51% respectively.

Successful artificial inseminations mostly related to technical factors such as estrous detection, insemination dosage, semen deposition on servix, and the skill of inseminator; and non-technical factors, such as anatomical condition of female reproductive organs and changing chemical substances inside the body during follicular phase caused by hormonal effects. High estrogen concentration induced contraction on myometrium and stimulating chemical substance secreted by uterus which influenced spermatozoa viability and motility to reach fertilization area[13]. Another function of specific protein sperm membrane was to activate transduction signal enabling receptors on pellucid zone 3 (PZ-3) to recognize spermatozoa (CD44 which bound integrin. Specific protein sperm membrane contents on spermatozoa could improve spermatozoa fusion with ovum (fertilization)[11].

Based on this study we can conclude that the highest percentage of spermatozoa viability after specific protein sperm membrane supplementation was as much as 64.28%.The highest percentage of individual spermatozoa motility after specific protein sperm membrane supplementation on freezing medium was as much as 51.25%.The highest percentage of intact spermatozoa plasma membrane rate after specific protein sperm

membrane supplementation on freezing medium was as much as 50.63%.At last, the conception rate (up to 100%) could be reached after artificial inseminationusing supplemented frozen semen with specific protein sperm membrane.

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