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Comparison Between Oviduct Fluid Protein and Oviductal Epithelia Cell As Supplements In Capacitation Media To Improve Sheep's Spermatozoa Quality

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Abstract : Embryo transfer technique is a breakthrough technology that has been applied in reproduction to increase livestock population through in vitro fertilization (IVF) to produce embryos in large numbers. Constraints are often encountered in producing embryos through IVF techniques. Successful fertilization is still low, especially with regard to the quality of spermatozoa after capacitation process. Previous studies suggests that supplementation of Oviductal Fluid Protein (OFP) in oocyte maturation media provided better oocyte quality outcome than supplementation of Oviductal Ephitelia Cell (OEC). However, further study is needed on the comparison of both supplementations on the improvement of spermatozoa quality during in vitro capacitation, which can be seen by identifying viability, motility and spermatozoa membrane permeability after capacitation. The results of this study showed that, compared to supplementation with Oviductal Ephitelia Cell, Oviductal Fluid Protein supplementation in capacitation media showed significantly different results in increasing the percentage of viability, motility and spermatozoa membrane permeability after capacitation. Improving the quality of sperm for in vitro fertilization program may support successful frozen embryos production in goats and sheep, as well as improve the genetic quality of goats and sheep in Indonesia.
Keywords : Oviduct Fluid Protein, Oviductal Ephitelial Cell, spermatozoa quality.

Introduction

One of Indonesian farm commodity that meet the criteria in assisting the development of the national economy is commodity of goats and sheep. However, in fact the development of goats and sheep breeding until then is relatively undeveloped. The growth of production and productivity showsalmost no significant progress. It is feared to cause a decrease in national sheep and goat population, due to the development of goat and sheep population not currently in line with the increase in consumption demand and the development of goat and sheep population.

Develop goats and sheep in Indonesia is wide open, because approximately 30 percent of food needs in agriculture, livestock and agrobusiness sectors are met by livestock, so therefore existence of cattle is very strategic in life and human life.[12] Artificial insemination (AI) technology and the technique of frozen semen and liquid semen is a technology that can be used to address the shortage of breeding stock. The cost efficiency of superior male maintenance can immediately increase the population of superior breeding stock at a relatively affordable cost. In goats and sheep the application of TE technology is not impossible, especially the making of

frozen embryo in straw has now been experiencing rapid development. Even media maturation has been studied to determine the potential of improving the quality of oocytes in vitro.[1]

Related to the function of spermatozoa, during capacitation there is increasing physiological and fluidity of the membrane which causes increased motility,[5] and changes of the physical and chemical characteristics, as well as lipid changes are also taking place, which is known to help the entry of the calcium ions into the cell. Increased calcium ions into the membrane may stimulate membrane bond with intracellular cAMP.

Mammal's oviductal fluid provides biological environment for fertilization and early stage of embryos cleavage. These changes pose a particular microenvironment in the oviduct for gametes maturation, sperm capacitation, gamete and embryo transport, fertilization and early embryo cleavage[13]. The function of this complex depends on the activity of the epithelium, both secretory and ciliated and non-ciliated, which is lining the oviduct mucosa. Detection and analysis of proteins synthesized and secreted from the oviduct epithelium show temporal and spatial distribution of macro molecules as well as differences in the distribution of proteins in various species.

One attempt made on IVF technique is trying to modify the medium used to improve the viability of spematozoa so as to fertilize the egg. Several previous studies showed that the addition of several compounds in sperm capacitation in vitro media can reduce the amount of dead sperm due to longer incubation period and to support sperm quality prior to fertilizing mature oocytes.

The addition of protein to the frozen semen dilution associated with improved sperm fertility has been studied,[6,7,8,14] but studies on supplementation of either oviduct fluid or oviduct epithelium in sperm capacitation media remains rare. This study aims to determine the effect of supplementation in spermatozoa capacitation media on the percentage of viability, motility and membrane permeability in goat spermatozoa in vitro.

Procedures

Goat semen collection

Semen collection methods in goats can be done using an artificial vagina, since this method is easy to do and can accommodate sperm with good quality and in the highest concentration compared to other methods. The following is the procedure for carrying out goat semen collection by artificial vagina:

Artificial vagina is properly prepared, so that the temperature of the artificial vagina reaches 40-45^{o}C and stored in an incubator in temperature of 45-50^{o}C. Inner sheath is smoothed with a little vaseline, pressure is adjusted with by pumping the air into it and then semen collecting tube is installed. Cattle teaser was prepared in advance by keeping it within squeeze cage. The male whose semen will be collected is cleaned first, especially on the part where the penis is extended. If hair around the prepuce, it should be shaved before the collection. The males is brought closer to the teaser. False mounting is done 3-5 times. Semen is collected and immediately examined macroscopically, and then taken to the laboratory as soon as possible for further examination.

Oviduct fluid protein collection from oviduct fluid and oviductal epithelial cells from shredded oviduct tissue

Oviduct fluid and shredded oviduct tissue collection was done using goat oviduct from a slaughterhouse and transported to the laboratory at room temperature with physiological NaCl media. Only oviduct derived from animals slaughtered not more than 3 hours (Lapointe et al., 1998) that was used. The oviduct was then washed two times using physiological saline, placed in a petri dish and then dissected. Oviduct fluid collection was done as follows: oviduct fluid was collected from goat's oviduct with follicle diameter between 3-5 mm, by means of flushing using stock TCM 199 solution of 0.5 cc to 10 cm along the oviduct. The result of the flushing were then centrifuged at 7000 rpm for 10 min at 4°C to remove cell debris. Supernatant resulting from centrifuge was then precipitated with ethanol. The obtained supernatant was then mixed with ethanol at a ratio of 1: 2, the mixture was then kept at 20°C overnight, and the next day it was centrifuged again at 3000 rpm. Pellet was the part that would be used (Van-Oss, 1989). Shredded oviduct tissue collection to obtain oviduct epithelial cells was done in the following manner: 10 cm oviduct was dissected and then shredded until smooth

and then the shred was added with TCM 199 stocks solution as much as 0.5 cc. After that, the results of the shredding were centrifuged at 7000 rpm for 10 min at 4°C to remove cell debris.[3] The supernatant resulting from centrifugation was then precipitated with ethanol. Obtained supernatant was mixed with ethanol at a ratio of 1: 2. The mixture was then stored at 20°C overnight, and the next day it was centrifuged again at 3000 rpm. Pellet was the parts that would be used.

Sperm capacitation

Spermatozoa capacitation was done by using a modified Brackett Oliphant medium (m B-O) which was added with oviduct Fluid Protein (P1), Oviductal Ephitelia Cell (P2) and a control group without any additions (P0). A total of 1 ml medium was added to the test tube containing 250 µl semen and then incubated at a temperature of 38°C for 30 minutes. Then it was centrifuged and taken as many as 500 mL of the upper part and examined under a microscope to observe the characteristics of spermatozoa. Observed change was the percentage of viability, percentage of motility, the percentage of spermatozoa cell membrane integrity of with Hypo Osmotic Swelling Test.

Data analysis

Analysis of Variance (ANOVA) test was conducted to determine the effect of Oviduct Fluid Protein and Oviductal Ephitelia Cell addition in the capacitation media of goat/sheep spermatozoa on the percentage of viability, percentage of motility, and percentage of spermatozoa cell membranes integrity using Hypo Osmotic Swelling Test. If there was significant differences among the treatment groups, the test was followed with Duncan test ($p < 0.05$).

Results

Goat semen collection

Table 1. Macroscopic and microscopic characteristics of fresh goat semen collected using artificial vagina

Characteristics	Results
Macroscopic	
Volume	1.1 ml
Color-odor	Milky white (creamy)-normal
pH	Normal
Viscosity	Thick
Microscopic	
Concentration/ml	1.9x109
Motility (%)	++/80%
Viability (%)	83%
Abnormalitt (%)	12%

After obtaining the results of macroscopic and microscopic goat semen quality examination, the step was followed by counting the number of fresh semen samples to be taken, for further preparation process of sperm capacitation.

Semen capacitation preparation

Fresh semen was taken as much as 200 mL and then added to BO (Brackett and Oliphant) media of 2.5 ml which had been added with caffeine benzoate of 2.5 mM. Then, the supernatant was removed with a sterile pipette, and BO-caffeine media was added again as much as 0.5 cc, and swimmed-up to select the sperm based on motility in CO2 incubator for 60 minutes. However, prior to the swim-up, the media was added with treatment as follows: K-: Fresh semen + BO caffeine media, P1: Fresh semen + BO caffeine media + 10% OEC supplementation , P2: Fresh semen + BO caffeine media + 10%OFP supplementation

Post incubation, samples were taken for examination of study parameters. Samples taken were at the highest part of the supernatant at the top of the tube (surface), then motility, viability and integrity of sperm cell

membrane after capacitation were examined. Tables 2, 3, and 4 present the results of the three parameters of this study.

Table 2. Results of post-capacitation sperm motility examination

Parameters	Mean (%) ± SD
K-	45^{a}± 14.33
P1	53^{b}± 6.82
P2	54^{b}± 6.29

Notes: different superscript in the same column indicate significantdifference ($p<0.05$)
K- : Fresh semen + BO caffein media
P1 : Fresh semen + BO caffein media + 10% OEC supplementation
P2 : Fresh semen + BO caffein media + 10% OFP supplementation

Table 2 shows the individual motility and treatment group K- had significantly different results from treatment groups P1 and P2. It shows significant differences between groups with the oviductal supplement and those without, with the highest percentage of motility in group P2, although it does not differ significantly from that of P1 ($p>0.05$).

Table 3. The results of sperm viability examination post capacitation

Parameter	Mean (%) ± SD
K-	53^{a}± 4.13
P1	55^{a}± 8.41
P2	61^{b}± 6.22

Notes: different superscripts in the same column indicate significant differences ($p <0.05$)

The results of data analysis showed that treatment group K- was not significantly different from group P1 ($p>0.05$), but significantly different from treatment group P2 ($p<0.05$). Each treatment group showed that groups P1 and P2 were significantly different ($p>0.05$), and the order of viability percentage of from the lowest to highest are respectively K-, P1 and P2.

Table 4. Results of HOS-Test examination of sperm post capacitation to determine sperm membrane integrity

Parameters	Mean (%) ± SD
K-	28^{a}± 14.33
P1	31^{b}± 6.82
P2	32^{b}± 6.82

Notes: different superscripts in the same column indicate significant differences ($p <0.05$)

Data analysis on spermatozoa MPU percentage shown by Table 4 shows significant difference between control group P0 and treatment groups P1and P2 ($p <0.05$), but P1 was not significantly different from group P2 ($p>0.05$). Sequentially, MPU percentage value of all treatment groups ranging from the lowest to the highest are P0, P1 and P2.

Discussion

A decrease in spermatozoa motility is allegedly due to the lack of energy during incubation the process for 1 hour. Energy is required by spermatozoa to actively move and the energy is derived from the decomposition of ATP to ADP and AMP in the mitochondria inside the sperm tail.[9] If the ATP and ADP expires, fibril contraction of spermatozoa tail will cease, so that the spermatozoa do not move. To maintain the continuity of sperm motility, the ATP and ADP should be set up again in the form of reversible reaction.

In vitro spermatozoa vitality is influenced by substances contained in the diluent and quality of spermatozoa. Spermatozoa with good quality have the ability to survive longer, because it has greater ability to absorb nutrients. The results of data analysis in Table 3 indicate that the treatment group K- was not

significantly different from group P1 ($p>0.05$), but significantly different from treatment group P2 ($p<0.05$). Each treatment group showed that groups P1 and P2 were significantly different ($p>0.05$), and the order of viability percentage from the lowest to highest, respectively, are K-, P1, and P2.

Viability percentage can be interpreted as the percentage of living spermatozoa, which is an indicator of semen quality to improve fertilization. To find spermatozoa alive or dead we used eosin-nigrosin staining. The principle of the staining is the difference of affinity in color absorbing between viable and dead permatozoa. Viable spermatozoa does absorb the color or the head will not be stained or appears clear, while the dead spermatozoa will absorb the color or the head will be reddish.

These results indicate that CO addition in capacitation media BO-caffeine may increase the rate of spermatozoa viability after capacitation. However, EO addition in the media did not show remarkable results with regard to viability percentage compared to K-.

Data analysis on spermatozoa MPU percentage tested with HOS-test (Table 4) showed significant differences between control group P0 and treatment groups P1 and P2 ($p <0.05$). However, P1 was not significantly different from group P2 ($p>0.05$). Sequentially, MPU percentage values of all treatment groups ranging from the lowest to the highest are P0, P1, and P2.

Hypo-Osmotic Swelling Test (HOST) is a test that can be performed to determine spermatozoa intact plasma membrane. Spermatozoa with intact plasma membrane are marked with circular spermatozoa tail because the membrane is still functioning in absorbing water in hypotonic environment. Damaged spermatozoa membrane is characterized by a straight tail.

Spermatozoa surface has high polarity and has five main membrane areas that are closely related to part of each cells related to the functions of different parts of the cell.[9] Head acrosome cell membrane has a function for capacitation, acrosome reaction, and ovum penetration in fertilization process. Rear acrosomal membrane (postacrosomal region) serves to hold the first ontact and become one with ovum oolema in the fertilization process, while the membranes in the central part of the tail (mid piece) has a function to obtain substrates important for spermatozoa energy and dissipates the wave motion, and the main membrane serves for spermatozoa movement. The composition of spermatozoa membrane composed of lipids, proteins, carbohydrates or other molecules joined together by non-covalent bonds that are very sensitive to external factors, such as temperature, ionic strength and polarity of the solvent. Lipids are components of spermatozoa membrane structure that plays an important role in maintaining the stability and viability of spermatozoa overall, including capacitation and ability to fertilize the egg, as well as the instability of spermatozoa in cooling and freezing process.[11]

The liquid in the oviduct of mammals provide a biological environment for fertilization and early stage of embryos cleavage. This change creates a specific microenvironment in the oviduct for gametes maturation, sperm capacitation, gamete and embryo transport, fertilization and embryo early cleavage.[13] This complex functions depend on epithelium activities, both secretory and ciliated and non-ciliated that line the oviduct mucosa. Detection and analysis of proteins synthesized and secreted from the oviduct epithelial found temporal and spatial distribution of macro molecules as well as differences in proteins distribution in various species. Moreover, this analysis has allowed the identification of the major secretory products of oviduct specific glycoprotein, whose secretion depends on estrogen, and has a high molecular weight. In addition, glycoprotein of oviduct fluid has been demonstrated in pigs and cows to participate in functional modification of the zona pellucida that occurs before fertilization, helping sperm penetration of into the ovum, and provide control over polyspermia on sheep IVF.[3]

The spermatozoa was capacitated in vitro earlier so that they may undergo physiological preparation and alteration process, so as to heightens it fertility. The process of sperm capacitation used BO media by centrifugation.

The success of in vitro fertilization (IVF) is not only influenced by the oocyte, but also by sperm used to fertilize. Spermatozoa capacitation method also determines FIV success. It has been recognized that the production of spermatozoa by a male (spermatogenesis) is a long process, which takes place in the testes, and also undergo capacitation process in the female reproductive tract, so that the sperm is able to penetrate the zona pellucida of the oocyte and eventually form a zygote/embryo. Spermatozoa motility, acrosome reaction and

competition between spermatozoa in the female reproductive tract are all factors that are important for successful fertilization.[5] Capacitation is a complex biochemical and physiological reactions process, including the removal of a component derived from seminiferous tubule, epididymis, vas deferens and seminal plasma absorbed through the spermatozoa membrane. During the process from the testes, through the epididymis, sperm cells are modified to become fertile and stored in the epididymal tail until release during ejaculation to avoid urine contamination.

It should be noted that spermatozoa maturation and modifications process occurs on the surface. Spermatozoa that leave the testes and enter the epididymal caput have not been able to penetrate the oocyte. Capacitation process is needed by spermatozoa to be able to penetrate the oocyte. Before fertilization, the sperm must migrate through female reproductive tract. In this journey spermatozoa surface is protected by a protective glycoprotein secreted by epididymal spermatozoa that serves to protect the surface when exposed to seminal plasma during ejaculation. Capacitation process should run gradually to remove part of the acrosome.

In vitro fertilization is a technique of oocyte penetration by spermatozoa that occurs outside the body in a cell culture that includes oocyte maturation and sperm capacitation in vitro. Medium used for capacitation still need to be modified to obtain better FIV results. One attempt made on IVF technique is to modify the medium used to improve spematozoa viability so as to fertilize the egg. Several previous studies showed that the addition of several compounds in the sperm capacitation media in vitro can reduce the amount of dead sperm due to too long incubation period and to support sperm quality before it fertilizes mature oocytes.

During capacitation process, membrane physiology and fluidity increase, which causes increased motility.[5] Physical and chemical characteristics change, and so does lipid, which known to help calcium ions influx into the cell. Increased calcium ions into the membrane may stimulate membrane bonding with intracellular cAMP. Calcium ion and cAMP are known as regulators of tail movement. The results of this study indicate that an increase is taking place in sperm motility, viability, plasma membrane intactness and the percentage of MPU after the administration of oviduct fluid and oviduct epithelium in capacitation media of goat sperm. With the increasing quality of spermatozoa, in vitro fertilization rate will also increase.

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