Insulin Like Growth Factor – I Complex Of Goat Seminal Plasma Increasing The Percentage of Embryo Cleavage on The In Vitro Fertilization Process

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ABSTRACT
Biomolecular researches prove that with addition some seminal plasma’s protein can improve fertilization process, maintain cell survival and has positive act for capacitation process also spermatozoa acrosomal reaction. Protein contains in seminal plasma are Insulin Like Growth Factor-I (IGF –I) Complex. IGF – I Complex is a glycoprotein that consist from molecule combination with molecule weight 85 kDa, binding protein with molecule weight 53 kDa and IGF build complex. Addition protein IGF – I Complex could improve spermatozoa’s motility percentages, beside it also regulate spermatozoa function before and after ejaculation specially increase motility and spermatozoa capacitation. The aim of this research is to increase adult spermatozoa (capacitated) to increase percentages of fertilization in vitro. The benefit of this research is to improve ram’s embryo quality in fertilization media in vitro. Semen with good quality (motility and viability ≥ 70%), 0,5 ml added BO media 1 ml and centrifugated 1800 rpm for 10 minutes. Spermatozoa 3 x 10⁷ for be given treatments. Centrifugated spermatozoa divided into 3 group; tube I consists of spermatozoa and BO media; tube II consists of spermatozoa, BO media and IGF-I Complex 12 ng ; Tube III consists of spermatozoa and IGF-I Complex 12 ng. Each tube incubated for 15 minutes and observe the motility, viability with eosin negrosin staining, plasma membran integrity and also observation of capacitation and acrosomal reaction with ChlorTetracyclin (CTC). Observation done with florescent microscope 1000 x. The result are : spermatozoa’s head all fluoresced are the spermatozoa hasn’t capacitated. Spermatozoa’s head upper half fluoresced is the spermatozoa that capacitated for next fertilization in vitro. The result of this research shows that highest motility, viability, plasma membrane integrity, capacitation percentages and lowest acrosomal reaction from three treatments is in P2. The highest embryo split percentages after fertilization are in P2 group in IGF-I Complex media. In Conclusion, ram’s seminal plasma Insulin Like Growth Factor – I Complex can increase the number of division in fertilization in vitro process.

INTRODUCTION

Processing (manipulation) spermatozoa is one of factors that influence the success rate for in vitro fertilization process. Spermatozoa’s manipulation in vitro include centrifugation method. In their vitro fertilization process required the presence of ova and spermatozoa that have undergone maturation or capacitation. The process of invitro capacitation of spermatozoacan be achieved by adding an appropriate medium [8]. One such medium is aprotinin found in the seminal plasma of goats. Seminal plasma consists of various components that regulate specific biochemical function of spermatozoa. The composition ofsemen plasmas composed of organic compounds consisting of fructose, citric acid, sorbitol, inositol, glycerylphosphorylcholin (GPC), ergotominandprostaglandins. The inorganic compounds include bicarbonate, potassium, calcium and carbonate [9]. Biomolecular research proves that with the additional of several seminal plasma proteins can improve the fertilization process, cell survival and has a positive role to the process of spermatozoa capacitation and acrosomal reaction [4].

Protein scountained in the seminal plasma is Insulin Like Growth Factor-I (IGF-I) Complex. IGF-I Complex is a glycoprotein consisting of a combination of molecules with a molecular weight of 85 kDa, binding protein
with a molecular weight of 53 kDa and IGF are forming complex. InsulinLike Growth Factor-I in addition to work on the process of spermatogenesis is also functioning in the process of steroidogenesis [12] with the increase in luteinizing hormone receptor expression / Human Chorionic Gonadotropin. The addition ofIGF-I Complex protein can increase the percentage of motile spermatozoa, while also regulating the function of spermatozoa before and after ejaculation significantly improve sperm motility and capacitation [13,19]. Growth research on Insulin Like Factor-I Complex goat seminal plasma were added to the diluent in goat semen freezing process also shows the percentage of sperm motility and viability can be maintained [20]. The mechanism of IGF-I Complex in maintaining sperm motility is through energy metabolism as indicated by an increased in glucose uptake, increased production of lactic acid, increased activity of pyruvate dehydrogenase and has antioxidant effects. The benefit of this research is to increase spermatozoa undergone capacitation so the fertilization in vitro can be improved. Fertilization is the process of oocytes activation by spermatozoa. Without to oocyte activation are vitellus ink, release discharge and migrate into the perivitelline space. At the same time, the head of spermatozoa in the vitellus, swell with gel-like consistency, characteristic shape disappear until finally form structure of the nucleus, named male pronucleus [5]. The time required for penetration of oocytes in vitro fertilization process is less than 4 hours, followed by the head of spermatozoa condense happens in next 1-2 hours and the male pronucleus develops after 3-5 hours later.

MATERIALS AND METHODS

Materials:

Protein IGF-I Complex obtained from semen of goat 3 to5 years old with high libido using an artificial vagina followed by purification.

In outline this study consisted of two phases:

Phase I is aqualitative exploratory laboratory research. At this stage, the identification, isolation of proteins Insulin Like Growth FactorI Complex (IGF-I Complex) goat seminal plasma. Identification is to determine the constituents of seminal plasma proteins in goats. The stages of the first phase of this study consisted of goat semen collection, seminal plasmmaparation, seminal plasma protein purification, Native Polyacrylamide Gel Electrophoresis (Native -PAGE) of seminal plasma, electro elution for isolation of protein IGF-I Complex.

Phase II is the application of protein Insulin Like Growth Factor - I Complex which was eluted in the first stage in the maturation medium (capacitation), followed by fertilization in vitro.

Stages of Research:

Semen collection and Seminal Plasma Protein Purification:

Semen collection of the etawa hybrid was using an artificial vagina. Semen is then coupled with Phosphate Buffer Saline (PBS) and then centrifuged at a temperature of 5 °C at a rate of 1800 rpm for 10 minutes, then the supernatant (seminal plasma) taken with a micropipette inserted into eppendorf tubes. Purification is done by adding PBS and Phenylmethanesulfonylfluoride (PMSF) and then in homogenize for 5-10 minutes 4 °C and then are-homogenize and centrifuged at 6000 rpm for 10 minutes. The supernatant was taken and added with absolute ethanol in the ratio of 1: 1 and subsequently precipitated overnight (until no smell of ethanol), disposed ethanol then added pellets with Tris HCl for 1-2 times the volume of pellets [1].

Protein Isolation of Insulin Like Growth Factor-I Complex:

Isolation started with goat seminal plasma protein identification by a native polyacrylamide gel electrophoresis (Native-PAGE). Gel staining is done by soaking the gel in Coomassie Blue staining solution R-250 for 30-60 minutes [1]. To determine the molecular weight bands formed done by calculate RF (Retardation Factor) valueo feach bands. Isolasi Insulin Like Growth Factor Protein Complex-I was conducted by electroelution. Electroelution performed by inserting a piece band in Native-PAGE gel into a cellophane bag along approximately 10 cm to be maintained in order to position the gel not curved. The top and bottom cellophaned with wine, then put a horizontal electrophoresis apparatus (Bio-Rad). Electrophoresis instrument is filled with a buffer of 500 ml. Running electroelution performed on condition 130 volts, 30 mA for 1 hour. Fluid electroelution results collected in eppendorf tubes, stored at-70° C, ready to be used for subsequent research [1].

Applications of Protein IGF-I Complex on Spermatozoa Suspension:

Good quality of goat’s semen (motility and viability ≥70 %), 0.5 ml of medium plus BO (Bracket and Oliphant) of 1 ml and centrifugated at 1800 rpm for 10 minutes 5° C temperature. Spermatozoa for treatments is 3x10⁶. Spermatozoa centrifugation divided into 3 groups: the first tube filled with spermatozoa 3 x 10⁶ in BO medium (0.5 ml), the second tube is filled with spermatozoa 3 x 10⁶ in BO medium (0.5 ml) and IGF-I Complex.
12 ng. Tube III is filled with spermatozoa 3 x 10^6 in the medium IGF-I Complex 12 ng. Further incubation for 15 minutes at 20°C and perform motility, viability, plasma membrane intact and capacitation examination.

**Oocyte Collection and Oocyte Maturation:**

Goat ovaries collected from Slaughter House (RPH) in Surabaya, East Java was then taken to the laboratory, put a glass beaker containing saline and antibiotics in the water bath at 37°C. Oocytes collected by aspiration, the size of the ovarian follicular fluid of 3-5 mm in diameter, using sterile syringes and needles size 18 Gauge oocyte containing washing Solution (OWS). Follicular fluid is then inserted into a test tube carefully so that the cumulus cells are not damaged and kept in a water bath at 37°C for 10 minutes until the oocyte settles. Then the liquid is evaluated by placing the bottom of the petridish under a steriomicroscope with a magnification of 100 X. Only oocytes with complete cumulus are used, then washed twice with a solution of OWS (Oocyte Washing Solution) and one time with maturation medium. Tissue culture media used maturation consisting of TCM 199 supplemented with 1% Fetal Calf Serum (FCS), 100 μl pyruvat, 25 μg gentamicin and added 10% Bovine Serum Albumin (BSA). Fife until ten oocytes matured in the media in the form of drops (50 ml/drops). The drops then covered with paraffin oil and equilibrated in 5% CO2 incubator at a temperature of 38°C with a humidity of 95% for at least 24 hours and spermatozoa added into group P0, P1 and P2.

- Mature oocytes is an oocytes with expanded cumulus cells.
- The percentage of cleavage = number of embryos that cleave
- The number of oocytes used fertilization.

**Results:**

Macroscopic and microscopic examination of fresh goat semen used for seminal plasma protein purification, isolation and identification of Insulin Like Growth Factor-I (IGF-I) Complex.

As a material for the isolation of protein IGF-I Complex, semen collected from goats 3-5 years old, healthy and high libido. Macroscopic examination (color, smell, consistency, pH and volume) and microscopic examination (concentration, mass motility, progressive individual motility and percentage of live spermatozoa) must be done as a basis for determining the feasibility of fresh semen to isolate the protein. Semen with ≥70% motility, ≥70% viability, and mass motility+++ eligible for isolated protein (Table 1)

**Table 1: Macroscopic and microscopic examination of fresh goat semen.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Character</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color</td>
<td>Cream</td>
</tr>
<tr>
<td>Smell</td>
<td>Typical</td>
</tr>
<tr>
<td>Consistency</td>
<td>Thick</td>
</tr>
<tr>
<td>PH</td>
<td>7,00</td>
</tr>
<tr>
<td>Volume (cc)</td>
<td>1,45</td>
</tr>
<tr>
<td>Concentration (milliom)</td>
<td>3750x10^6</td>
</tr>
<tr>
<td>Mass Motility</td>
<td>+++</td>
</tr>
<tr>
<td>Individual Motility (%)</td>
<td>Progressive (94±6.16)</td>
</tr>
<tr>
<td>Viability (%)</td>
<td>92.35±4.30</td>
</tr>
<tr>
<td>Abnormality (%)</td>
<td>3.50±1.70</td>
</tr>
<tr>
<td>Plasma Membrane Intact (%)</td>
<td>90.60±3.15</td>
</tr>
</tbody>
</table>

Goat seminal plasma in Native-PAGE electrophoresis produce 7 band. Based on the sequence of the next molecule weight of the band's seventh in a row was the first band (BM 150,288 kDa), the second band (BM 103.486 kDa), the third band (BM 76.155 kDa), the fourth band (BM 53.249 kDa), fifth band (BM 35.378 kDa), the sixth band (BM 14,099 kDa) and seventh band (BM 11.492 kDa) (Figure 1).

**Results calculated levels of IGF-I Complex goat seminal plasma:**

Determination of IGF-I Complex goat seminal plasma by using a standard curve Complex IGF-I standard. Measurements were made with a spectrophotometer and the resulting computed the mean levels of IGF-I Complex of 1.179 g/ml.

**Macroscopic and microscopic examination semen fresh goat for treatment:**

The results of macroscopic examination of fresh semen collected from the male goat that will be used for treatment are as follows: semen volume range 1.20 cc to 1.90 ml with an average of 1.54 cc. On examination of color, smell, pH and consistency there is no deviation. Semen color is normal that is cream. Semen smell typical for goat and do not stinkorrancid. Semen pH range from 6.4 to 6.6 with an average of 6.48.

The results of microscopic examination of fresh semen collected from the male goat that will be used for treatment are as follows: the concentration of spermatozoa range 2350 to 2550 million/ml with an average of 2410 million/ml. The mass movement of spermatozoa very well that showed great movement and a lot of

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*Suhermi Susilowati et al, 2015, Advances in Environmental Biology, 9(3) February 2015, Pages: 451-456*
spermatozoa is progressive with an average of 92%. The percentages of live spermatozoa with an average of 93%, if the results are satisfactory (percentage individual motility and live of spermatozoa ≥ 70%) then performed in vitro fertilization.

**Fig. 1:** Native-PAGE12% Profile of goat seminal plasma (M: marker, 2, 3: goat seminal plasma samples).

The percentage of motility, live, intact plasma membrane, capacitation and acrosome reaction of spermatozoa after treatment:

Addition of Insulin Like Growth Factor Complex-I goat seminal plasma in goat semen centrifugation results maintain motility, viability and intact spermatozoa plasma membrane and increase the percentage of spermatozoa undergo capacitation and acrosome reaction decrease (Table 1). Statistical analysis using the One-Way ANOVA followed by Duncan test significant differences (p <0.05) between control and treatment.

**Table 2:** Results of the percentage of motility, live, intact plasma membrane, capacitation and acrosome reaction of spermatozoa after treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>(P0) Tanpa IGF-I Complex (BO)</th>
<th>(P1)BO + IGF-I Complex</th>
<th>(P2)IGF – I Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motilitas (%)</td>
<td>31.85 ± 2.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>46.45 ± 2.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>78.80 ± 3.45&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hidup (%)</td>
<td>35.80 ± 2.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50.65 ± 2.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>80.25 ± 2.50&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Membran plasma utuh (%)</td>
<td>33.65 ± 1.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48.90 ± 3.50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>80.90 ± 1.60&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kapasitasi (%)</td>
<td>33.35 ± 1.90&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.50 ± 2.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50.35 ± 2.45&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Reaksi/Akrosom (%)</td>
<td>20.55 ± 1.65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.45 ± 2.55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.15 ± 2.35&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Notation different letters in the same row differ significantly (p <0.05)

Specification:
- P0: control treatment (spermatozoa + BO medium)
- P1: spermatozoa+BO medium+IGF-I Complex
- P2: spermatozoa+IGF-I Complex

**Table 3:** Percentage of cleavage embryos after treatment

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Embryo Cleavage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0 (BO medium)</td>
<td>46.90 ± 4.50&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>P1 (BO + IGF-I Complex medium)</td>
<td>48.75 ± 5.50&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P2(IGF-I Complex ) medium</td>
<td>60.85 ± 2.10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Notation different letters in the same column differ significantly (p <0.05)

**Discussion:**

From Table 2 the percentages of motility, viability, plasma membrane intact, the highest capacitation and the lowest acrosome reaction of the three treatments medium is treatment II (P2) (the medium Insulin Like Growth Factor I Complex). Based on statistical analysis by ANOVA showed significant differences (p <0.05). It is because of Insulin Like Growth Factor-I Complex have receptors on the plasma membrane resulting in a bond between spermatozoa with its ligand. The bond will activate membrane molecules which then activates cytosolic molecules spermatozoa with the help of transport proteins that exist in the membrane which in turn will have an effect on sperm motility. Insulin Like Growth Factor -I Complex have a role as an antioxidant with a chain breaker mechanism so that the chain reaction caused by reactive oxygen species (ROS) in spermatozoa resulted...
from centrifugation can be muted. Due to the role of IGF-I plasma membrane Complex is running normally, the production of ATP (AdenosineTri Phosphate) maintained results in increased life spermatozoa. Insulin Like Growth Factor I Complex that had a role as antioxidant ultimately lowers the concentration of malondialdehyde (MDA), which is the end result of a chain reaction of poly unsaturated fatty acids.

From table 3 the percentages of embryo splitting after fertilization the highest in the group P2 in the medium IGF-I Complex. Statistical analysis by ANOVA test on embryo cleavage showed significant differences (p <0.05). According to Hafez (2000), the successful implementation of in vitro fertilization in addition influenced by the environment and maturation of oocytes also influenced by the level of sperm motility were used in the process of fertilization. Besides motility, two processes that are fundamental to the occurrence of fertilization is capacitation and acrosome reaction. There are so many factors that affect sperm motility both endogenous and exogenous. Endogenous factors include the circumstances of individual spermatozoa were closely related to the age of spermatozoa, sperm maturation level includes morphological, physiological and biochemical properties, as well as factors relating to the procurement of such energy transport through the membrane of spermatozoa. Protein Insulin Like Growth Factor - I Complex goat seminal plasma is able to increase the percentage of intact plasma membrane thereby increasing the percentage of live sperm and sperm motility. IGF-I Complex also able to increase the percentage of spermatozoa undergo capacitation. Capacitation is the head of the sperm membrane changes especially the acrosome. Due to the process of capacitation of spermatozoa increases the body's metabolism so that the tail of spermatozoa will be more active and causes the forward motion strength increases [7]. From the above results indicate that the addition of IGF-I Complex protein in sperm washing medium is very effective for improving fertilization in goats, the results also showed a positive correlation between the percentage of motility and the percentage of cleavage embryos. Insulin Like Growth Factor I Complex protect in vitro conditions such as fatty membrane-lipid peroxidation as an antioxidant that is related to the sensitivity of the early embryo (embryo newly splitting) against damaging free radicals, which may impede or even prevent cleavage embryos.

Protein IGF-I Complex were added in capacitation medium can increase the percentage of mature oocytes and improve embryo cleavage, this is due in accordance with the opinion of Donald et al (1998) which states that the IGF-I Complex acts as an antioxidant in which the production of ROS (Reactive Oxygen species) in the maturation medium can be prevented, the reaction between the hydroxyl radical (OH) with components of polysaturated fatty acids does not occur, the chain reaction does not occur, the chain reaction does not occur, lipid peroxidation did not happen. Finally bond fatty acid chains do not break and aldehyde compounds including malondialdehyde (MDA) is not formed. Suryohudoyo (2000) antioxidants are compounds that can reduce the negative impact oxidants including enzymes and metal binding proteins. IGF-I Complex acts as a chain-breaking antioxidant (chain-breaking antioxidant) which have antioxidant serves to reduce the negative effects of reactive oxygen species by preventing the chain reaction or also as an antioxidant to prevent (preventive antioxidants) basically prevents the formation of new free radicals. Antioxidant convert free radicals into molecules have less chance to react or to prevent the formation of new free radicals from other molecules.

Conclusion:

a. Insulin Like Growth Factor Complex-I goat seminal plasmacan be used as an indicator of sperm washing medium can increase the percentage of motility, viability, plasma membrane intact and capacitation.

b. Insulin Like Growth Factor-I Complex goat seminal plasma can increase the percentage of mature oocytes and cleavage embryos invitro fertilization process.

REFERENCES