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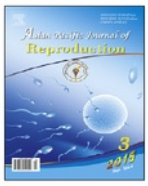

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The effects of insulin-like growth factor I (IGF-I) complex from seminal plasma on capacitation, membrane integrity and DNA fragmentation in goat spermatozoa

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ABSTRACT

To evaluate the effects of the insulin-like growth factor I (IGF-I) complex from seminal plasma on capacitation, membrane integrity and DNA fragmentation.

Methods: A total of 0.5 mL of fresh semen was added to 1 mL of Bracket–Oliphant (BO) medium, and the sample was then centrifuged at a speed of 1 800 rpm for 10 min. The samples were analyzed before and after centrifugation for sperm viability, motility, membrane integrity and capacitation. The centrifuged samples were divided into three groups, each consisting of 3×10^6 spermatozoa. BO medium was added to group 1, BO + 12 ng IGF-I complex medium was added to group 2, and 12 ng IGF-I complex was added to group 3. Then, the samples were incubated for 15 min.

Results: The result showed that sperm motility, viability and membrane integrity were significantly higher ($P < 0.05$) after centrifugation. Furthermore, the sperm capacitation was significantly lower ($P < 0.05$) after centrifugation. The percentages of sperm capacitation, membrane integrity and DNA fragmentation were significantly different ($P < 0.05$) in all media, including BO, BO + IGF-I complex and the IGF-I complex alone.

Conclusions: Sperm quality include motility, viability and membrane integrity were lower after centrifugation. Whereas DNA fragmentation after incubation in the IGF-I complex medium also was lower compared to that of specimens in the BO and BO + IGF-I complex media.

1. Introduction

Sperm manipulation for improving fertilization is undertaken not only *in vivo* but also *in vitro* and underlies the manipulation of spermatozoa for clinical *in vitro* fertilization (IVF). One method for sperm manipulation is the centrifugation of spermatozoa. The process of capacitation of spermatozoa involving biochemical and physiological processes involves complex reactions. During capacitation, modification and characterization of the membrane occur, in addition to enzyme activation and spermatozoa motility [1]. One negative result of centrifugation of semen is the increased formation of reactive oxygen species (ROS) by the spermatozoa. The increase in ROS production after separation by centrifugation is thought to be a complex process and can be derived from chemical processes in organelles inside the cell or even from processes outside the cell [2]. Reactive oxygen species are an important mediator of the function of spermatozoa and are involved in hyperactivity induction, capacitation and acrosome reaction, in addition to spermatozoa and oocyte fusion [3]. However, when excessive ROS are produced, they cannot be neutralized by the antioxidant defense systems of spermatozoa or seminal plasma. This excess will lead to fatty acid damage, especially among polyunsaturated fatty acids, which are essential components of the sperm membrane phospholipid layer, the inactivation of glycolytic enzymes, DNA chain termination, and decrease in sperm motility and sperm death.

Semen consists of spermatozoa suspended in a fluid medium called seminal plasma. Seminal plasma is a complex fluid that mediates the chemical function of the ejaculate. One component of seminal plasma is insulin-like growth factor (IGF-I). This growth factor has been suggested to have a direct or indirect role in spermatogenesis/steroidogenesis in the testes, and its derangement may be involved in male infertility [4,5]. The protein contained in seminal plasma includes the insulin-like growth factor I (IGF-I) complex.

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Insulin-like growth factor-I forms a complex that binds with another molecule that has a molecular weight of 150 kDa and consists of three protein molecules, including one molecule of insulin-like growth factor-I (sub unit α) with a molecular weight of 7.6 kDa, one molecule of insulin-like growth factor binding protein (sub unit β) with a molecular weight of 53 kDa and one molecule of an acid-labile subunit with a molecular weight of 85 kDa (sub unit β) [6]. The acid-labile subunit that binds with IGF-I increases the molecular weight by adding a complex function to the bond between IGF and IGF-3. In seminal plasma, IGF-I has been identified in the testes and is secreted by Leydig and Sertoli cells [7]. IGF-I has been reported to be a significant factor for germ cell development, maturation and the motility of spermatozoa [8,9].

Selvaraju *et al.* [10] reported that variations in IGF-I levels in the seminal plasma can influence germ cell stimuli, including the development, maturation and motility of the spermatozoa. Hence, this study was conducted to determine the possibilities of improving sperm quality with the insulin-like growth factor I (IGF-I) complex after incubation. The objective of the present study was to evaluate the effects of the insulin-like growth factor I (IGF-I) complex from seminal plasma on capacitation, membrane integrity and DNA fragmentation in goat spermatozoa.

2. Materials and methods

2.1. Experimental animals

A total of three male goats were used for semen collection in this study. The average body weight was 45 kg, and the average age was 3–4 years. Semen was collected from the goats two weeks after start of adaptation to the location with the aid of an artificial vagina. Immediately, after collection, the semen was kept in a water bath (37 °C), and semen parameters were assessed, including volume, pH, consistency, color and concentration of the semen. A total of 0.5 mL of fresh semen was added to 1 mL of Bracket–Oliphant (BO) medium, and the sample was then centrifuged at a speed of 1 800 rpm for 10 min. The samples were analyzed before and after centrifugation for sperm viability, motility, membrane integrity and capacitation. The centrifuged samples were divided into the three groups, each consisting of 3×10^6 spermatozoa. BO medium was added to group 1, BO + 12 ng IGF-I complex medium was added to group 2, and 12 ng IGF-I complex was added to group 3. Then, the samples were incubated for 15 min. The samples were then analyzed for sperm viability, motility, membrane integrity, capacitation and DNA fragmentation. For identification, constituents from goat seminal plasma proteins were assessed via native polyacrylamide gel electrophoresis (Native-PAGE) with a concentration of 12% using an electrophoresis mini protein gel (Bio-Rad), and the IGF-I complex protein was isolated from seminal plasma by electro-elution.

2.2. Sperm viability

Eosin-nigrosin staining was used to evaluate sperm viability as described by Malik *et al.* [11]. After thawing, one drop of semen was placed on a tempered glass slide, and this sample was mixed with one drop of eosin-nigrosin solution (0.2 g of eosin and 2 g of nigrosin were dissolved in a buffered saline solution, mixed for 2 h at room temperature and filtered to obtain the staining media). The mixture was smeared on the glass slide and allowed to air dry. One hundred spermatozoa were evaluated in at least five different fields in each smear under a light microscope. Eosin penetrates non-viable cells, which appear red, and nigrosin offers a dark background for facilitating the detection of viable, non-stained cells.

2.3. Assessment of motility

The motility of the spermatozoa was analyzed by mixing the semen gently and placing a 10 µL drop of diluted semen on a warm slide covered with a glass cover slip (18 × 18 mm) from five selected representative fields. Samples were selected randomly from 10 fields, for a total of 200 cells. Individual sperm were recorded as being viable or dead.

2.4. Assessment of sperm membrane integrity

Membrane integrity was determined using the hypo-osmotic swelling test (HOST) described by Malik *et al.* [12]. A total of 100 µL of semen was mixed with 1 mL of hypotonic solution (osmotic pressure 100 mOsm/kg) containing 13.51 g of fructose and 7.35 g of sodium citrate in 1 000 mL of distilled water. The mixture was incubated at 37 °C for 60 min. Following incubation, 15 µL of the sample was placed on a slide, covered with a cover slip and observed under a differential interference microscope (Olympus CK2, ULWCD 0.30) at a magnification of 400×. The spermatozoa were categorized according to the presence or absence of a swollen tail. At least 200 spermatozoa were observed, and the results were recorded as percentages. The membrane integrity after HOST was classified into two groups: normal spermatozoa that displayed coiled tails and abnormal spermatozoa without coiled tails.

2.5. Assessment of sperm capacitation

Sperm capacitation was assessed using chlortetracycline (CTC). Coloration with CTC showed that when the spermatozoa underwent visible capacitation, 2/3 of the equator appear to be yellow sperm heads that are lighter due to the increased distribution of Ca^+, the spermatozoa that underwent acrosome reactions were colorless and had only the yellow tape on the equator of the spermatozoa head. Observations of the capacitation and acrosome reaction of the spermatozoa were performed using a fluorescence microscope at a magnification of 400×.

2.6. Assessment of sperm DNA fragmentation

DNA fragmentation was assessed by a terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) assay adopted by Natalia-Rougier *et al.* [13]. Briefly, all elements were fixed in 2% formaldehyde in 1× PBS solution (pH 7.4; Gibco) for at least one hour. Each sample was placed into one well of a multiwell plate (4-mm diameter). After 2–3 h, each well was washed with 1× PBS (three times, 5 min each), and the cells were permeabilized with cold methanol. Before incubation with the TUNEL solution, each well was washed again with 1× PBS. For each sample, one extra well was incubated with DNA (1 U/mL; Sigma) for 30 min at 37 °C as a positive control, and in another well, the TUNEL

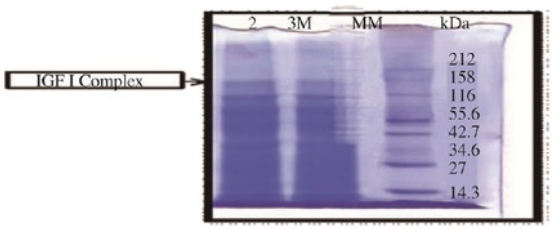

Figure 1. Get analysis with Native-PAGE by concentration 12% on seminal plasma of goat (M = marker, 2.3 = samples of seminal plasma).

Table 1
Percentages of the sperm motility, viability, membrane integrity and capacitation before and after centrifugation with 1 800 rpm for 10 min.

Parameters	Before centrifugation	After centrifugation
Sperm motility (%)	91.25 ± 5.25[a]	75.60 ± 4.35[b]
Sperm viability (%)	93.40 ± 2.25[a]	84.50 ± 2.25[b]
Membrane integrity (%)	90.50 ± 5.50[a]	74.85 ± 6.75[b]
Capacitation (%)	10.45 ± 5.80[a]	21.10 ± 4.50[b]

[a, b]Values in the same row with different superscripts indicate significant difference at $P < 0.05$ (n = 12).

"enzyme" solution was omitted as a negative control. The total sample was incubated in TUNEL solution for one hour at 37 °C. All samples were finally washed with 1× PBS (three times, 5 min each), and mounted in Vectashield H-1000 medium (Vector Laboratories). A total of 400 spermatozoa were counted by fluorescence microscopy for each fraction.

2.7. Statistical analysis

All data were expressed as the mean values ± S.E.M. The statistical significances of the effects of membrane integrity and DNA fragmentation after centrifugation were determined by ANOVA (S-PLUS Statistical Program, Insightful Corporation Seattle, WA, USA). *P*-values <0.05 were considered to be significantly different.

3. Results

Based on the evaluation of fresh ejaculation, the mean semen volume was (1.10 ± 1.26) mL, and the sample was cloudy to creamy white in color, had a pH of (7.00 ± 0.07), and was of thin to thick consistency. The mean mass activity was 2.28 ± 0.41 with a percentage of motile and viable sperm of (90.00 ± 8.40)%. The percent of sperm capacitated was (10.85 ± 87.00)%, and the sperm concentration was 397 × 10^6 spermatozoa. The results of analysis using a native polyacrylamide gel electrophoresis with a concentration of 12% indicated the presence of a protein and IGF-I complex of 150 (kDa) molecular weight (mw) (Figure 1).

The percentages of spermatozoa motility, viability, membrane integrity and capacitation of the semen before and after centrifugation were shown in Table 1. Sperm motility, viability and membrane integrity were significantly higher ($P < 0.05$) after centrifugation. Furthermore, the sperm capacitation was significantly lower ($P < 0.05$) after centrifugation. The percentages of sperm capacitation, membrane integrity and DNA fragmentation after centrifugation were shown in Table 2. The percentages of sperm capacitation, membrane integrity and DNA fragmentation were significantly different ($P < 0.05$) in all media, including BO, BO + IGF-I complex and the IGF-I complex alone.

4. Discussion

The results of this study indicate that centrifugation decreased sperm motility, viability and membrane integrity. This was probably due to the induction of *reactive oxygen species* (ROS) formation by the spermatozoa. These findings confirmed several studies reported by Sharma *et al.* [14] and Alvarez *et al.* [15] who stated that the effects of centrifugation have been attributed to the generation of ROS, which can irreversibly damage the spermatozoa. The toxicity effects of ROS eventually result in protein inactivation and inactivation, unsaturated lipid peroxidation and DNA damage, which destabilize the spermatozoa plasma membrane [16,17].

Moreover, the capacitation, membrane integrity and DNA fragmentation results after incubation for 15 min were significantly different in groups 1, 2 and 3. The percentage of sperm capacitation after incubation in group 3 (IGF-I complex) was higher compared with those of group 2 (BO + IGF-I complex) and group 1 (BO medium). This result strengthened the findings reported by Maxwell and Johnson [18] and La-Falci *et al.* [19] in which the seminal plasma function in goat is dependent on sperm capacitation. Maxwell and Johnson [18], Yanagimachi [20], and Miller *et al.* [21] reported that spermatozoa acquire many proteins during the epididymis transit and during ejaculation, possibly affecting their fecundity capacity.

Acrosome and membrane integrity are the main indicators of spermatozoa capability and membrane functionality [22]. In this study, the membrane integrity after incubation for 15 min with 12 ng of IGF-I complex (group 3) medium was higher compared to that of specimens incubated with BO + IGF-I complex (group 2) and BO medium (group 1). Schoneck *et al.* [23] revealed that one of the functions of seminal plasma in goats is protection against plasma membrane lipid peroxidation.

The evaluation of sperm DNA fragmentation has become an important method for the evaluation of semen quality. In this study, DNA fragmentation after incubation in group 3

Table 2
Percentages of sperm capacitation, membrane integrity and DNA fragmentation after incubation for 15 min.

Parameters	BO medium (3 × 10^6 sperm)	BO medium + IGF-I complex 12 ng (3 × 10^6 sperm)	IGF-I complex medium 12 ng (3 × 10^6 sperm)
Capacitation (%)	57.25 ± 1.35[a]	60.20 ± 1.90[b]	76.10 ± 1.55[c]
Membrane integrity (%)	50.15 ± 4.50[a]	57.12 ± 3.75[b]	65.75 ± 2.15[c]
DNA fragmentation (%)	49.80 ± 2.80[a]	40.25 ± 7.75[b]	40.90 ± 4.15[c]

[a,b,c]Values in the same row with different superscripts indicate significant difference at $P < 0.05$ (n = 12).

(40.90 ± 4.15%) and group 2 (40.25 ± 7.75%) was lower compared with that in group 1 (49.80 ± 2.80%). Decreasing levels of DNA fragmentation after incubation in groups 2 and 3 might be due to the effect of the IGF-I complex in each medium. The IGF-I complex in the seminal plasma contains many proteins in male bovine [24], swine [25], horse [26], and buffalo [27]. Conversely, several researchers have reported a negative correlation between high DNA fragmentation levels and assisted reproductive technology outcomes [28–31].

Based on the results of these experiments, it may be concluded that sperm motility, viability and membrane integrity were lower after centrifugation for 1800 rpm for 10 min. Furthermore, pure IGF-I complex medium increased capacitation and membrane integrity after incubation, whereas DNA fragmentation after incubation in the IGF-I complex medium was lower compared to that of specimens in the BO and BO + IGF-I complex media.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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