Transformation of ram sperm nuclei in oocytes cytoplasm during in vitro fertilization

Y. Dzulfiqor 1, M. A. Setiadi 1,2 and N. W. K. Karja 1,2,*

1 Post Graduate Program in Reproductive Biology, 
Faculty of Veterinary Medicine, IPB University, Dramaga Campus, Bogor 16680 - Indonesia 
2 Reproduction and Obstetrics Division, Department of Veterinary Clinic, 
Reproduction and Pathology, Faculty of Veterinary Medicine, IPB University, 
Jl. Agatis Raya, Kampus IPB Dramaga, Bogor 16680 - Indonesia 
*Corresponding E-mail: karjanwk13@gmail.com

Received November 11, 2018; Accepted April 29, 2019

ABSTRACT

The aim of present study was to understand the transformation of ram sperm nuclei within oocyte cytoplasm during in vitro fertilization. The oocytes were collected from slaughterhouse ovaries. Before fertilization, the oocytes were maturated in vitro for 24 hours in the incubator with 5% CO2 at 38.5°C. Then the oocytes (n= 635) was fertilized by incubating the oocytes with sperm (5x10^6 spermatozoa/ ml) for 3, 6, 9, 12, and 15 hours. At the end of incubating period, the oocytes were fixed and stained with aceto-orcein 2% before evaluated under phase contrast microscope. Sperm nuclear transformation was evaluated according to sperm nuclear status of sperm, such as condensation, decondensation, and formation of prepronuclei and pronuclei. Sperm condensation and decondensation were seen at 3 hours after incubation. Prepronuclei and pronuclei were found at 6 hours of incubation. Pronuclei formation was significantly increased in the 9 hours after incubation (P<0.05). The incidence of polyspermy was significantly increased at 12-15 hours after incubation (P<0.05). In conclusion penetration of sperm into oocytes has been occurred at 3 hour of fertilization period. The formation of pronuclei was found at 6 hours after incubation and the incidence of polyspermy was increased when the fertilization period
INTRODUCTION

Fertilization is defined as a process of haploid gametes fusion to produce a new diploid individual which have inherited genetic trait from its parent (Bianchi and Wright, 2016; Gilbert, 2010). Fertilization process occur within several stages which begin with penetration of sperm into the oocyte through zona pelucida (ZP) (Hirohashi and Yanagimachi, 2018; La Spina et al., 2017), acrosomal reaction (Jin et al., 2011; Hino et al., 2016), transformation of sperm which is followed by oocyte meiosis resume, fusion of genetic material from sperm and oocyte (Elder and Dale, 2011), and oocyte metabolic activation to induce embryonic development (Gilbert, 2010).

The process of nuclear transformation of sperm after fertilization occur in several steps. The sperm which has passed perivitelline space will fuse with oocyte membrane through the microvilli and equatorial postacrosomal part of spermatozoon (Sharma and Rao, 2018; Miyado et al., 2018). This event known as sperm condensation (Hafez and Hafez, 2013) that resulted in sharpened of sperm head (Dozortsev et al., 1994). The transformation of sperm be continued by decondensation process that indicated by sperm head swelling and increasing in size (Tesarik and Kopecny, 1989). The head of decondensed sperm is surrounded by vesicle from cytoplasm of oocyte (Lassalle and Testart, 1991). After decondensation, sperm will transform into prepronuclei. Prepronuclei is a reform stage of the nuclear envelope. When the nuclear envelope seen clearly, the transformation known as pronuclei (Rajabi et al., 2017). Pronuclei is the last stage of sperm transformation with conspicuous of nuclear envelope, completion of nucleolus precursor development, advance change on chromatin distribution, and nuclear envelope modification (Lassalle and Testart, 1991). On the other hand, as a result of sperm stimulation leads to meiosis resumption of oocyte and female pronuclei formation (Elder and Dale, 2011). Cytoskeleton assists the migration of two pronuclei to the equatorial part of the oocyte and ended with syngami (Wan-Hafizah et al., 2015).

The sperm nuclear transformation during in vitro fertilization closely related to incubation time of sperm with oocytes and varies among species depend also on the laboratory. In cattle the incubation time for in vitro fertilization takes 16 hours (Xu and Greve, 1988), 14-18 hours in porcine (Laurincik et al., 1994) and there still no report in the sheep oocytes. It is possible for each species to have different needs in incubation time, caused by species specific. However, the prolongation of incubation time increases the incident of polysperma (Sattar et al., 2011). The understanding of transformaton process of sheep sperm is necessary to increase the fertilization rate and decrease polysperma that which cause the increasing of embryo development rate. Therefore, the objective of this result was to analyze the transformation process after fertilization and time is required for incubation of sperm and oocyte in the in vitro embryo production.

MATERIALS AND METHODS

Oocytes Collection and In Vitro Maturation

The maturation process was carried out in incubator at 38.5°C with 5% CO₂.

**In Vitro Fertilization and Evaluation of Transformation Sperm Nuclei**

The fertilization process was carried out according to Pamungkas et al. (2012) with minor modifications. The frozen semen was thawed in warm water at 35°C for 30 seconds. Semen then placed in fertilization medium (Suzuki et al., 2000) then setrifuged in 630g for 5 minutes. The supernatant then removed and 200 µl of remaining pellet diluted with fertilization medium until the final concentration reach 5 x 10⁶ sperm/ml (Kang et al., 2015). The matured oocytes washed 3 times with fertilization medium without sperm and then placed into 100 µl drop fertilization media which covered with mineral oil (Sigma-Aldrich, USA) (Hasbi et al., 2017). Oocytes and sperm then incubated for 3, 6, 9, 12, 15 at 38.5°C with 5% CO₂.

At the end of each fertilization period, the oocytes were fixed in methanol and acetic acid solution (3:1) for 48 hours and then were stained with 2% aceto-orcein (Yasmin et al., 2015). Sperm nuclear transformation was evaluated according to sperm nuclear status of sperm, such as condensation, decondensation, and formation of prepronuclei and pronuclei. Condensation of sperm characterized by sharpening of the sperm head (Dozortsev et al., 1994). Decondensation indicated by increasing size and swelling of the sperm's head (Tesarik and Kopcey, 1989). Prepronuclei is a reform stage of the nuclear envelope. Pronuclei is the last stage of sperm transformation with conspicuous of nuclear envelope (Lassalle and Testart, 1991; Rajabi et al., 2017). Evaluation was conducted using phase contrast microscope (Olympus XI, Japan).

**Data Analysis**

The sperm nuclear transformation were described qualitatively by pictures. Quantitative data obtained from the percentage of sperm transformation. The data is showed as percentage and standard error means (SEM). The percentage of sperm transformation were analyzed with ANOVA and the differences between treatments were analyzed using Duncan test.

**RESULTS AND DISCUSSION**

**The Journey of Ram Sperm Transformation**

The transformation process of sperm in oocyte cytoplasm after in vitro fertilization in this research was observed every 3 hours for 15 hours. The indicators of transformation stages are observed by the transformation of sperm head, existence of second polar body and nuclear envelope as shown in Figure 1.

The first stage of sperm transformation was sperm condensation which was observed after 3 hours fertilization period. In that stage, inner acrosomal membrane fused with oocyte cytoplasm, resulted in disappearance of sperm nuclear envelope and acrosomal membranes and elongation of sperm head (Figure 1a). The first step of sperm transformation which can be seen is condensation (Hafez and Hafez, 2013) or decondensation (Lassalle and Testart, 1991) of sperm head. Sperm condensation characterized by leakage of sperm membrane (Tesarik and Kopcey, 1989). The sperm’s head that have been undergone condensation were seen elongated as a result of nuclear envelope loss and acrosomal membrane fusion with oocyte cytoplasm (Figure 1a).

The transformation process then continued to sperm nuclear decondensation. Sperm nuclear decondensation occurred after 3 hours after fertilization period which was characterized by swelling of the sperm head (Tesarik and Kopcey, 1989) (Figure 1b). In this research, sperm condensation and decondensation was seen at 3 hours after fertilization period. Sperm transformation process of condensation into decondensation happened rapidly, hence the decondensation often seen at the first observation (Crozet, 1988; Lassalle and Testart, 1991; Tesarik and Kopcey, 1989). At the same time, the nuclear status of oocyte resumed second meiotic division toward anaphase II and telophase II (Figure 1c). Sperm head transformation occurred the oocyte became activated due to the increase of intracellular Ca²⁺ (Ickowicz et al., 2012; Miao and Williams, 2012).

Prepronuclei was the next sperm transformation form. The formation of male and female prepronuclei almost occurred at the same time at 6 hours after fertilization period. Prepronuclei characterized by a set of chromatin and nuclear envelope when still incomplete (Figure 1d). Wu et al. (2017) argue that prepronuclei formation which characterized by flattened vesicle that was adjacent with nucleoplasm hence nuclear envelope was faintly visible.

The last stage of sperm transformation was
the formation of complete pronuclei. The formation of pronuclei was found at 6 hours after the interaction sperm-oocytes. A second polar body was extruded along with pronuclei formation (Figure 1e). Six hours after incubation, pronuclei had been formed which was characterized by increase in size, complete formation of nuclear envelope which became clearly visible (Figure 1e).

Transformation of sperm until pronuclei formation in porcine takes 14-18 hours (Laurincik, 1994), while in cattle is 16 hours (Xu and Greve, 1988). Period of sperm transformation process varies among species, therefore the information regarding optimum time of fertilization is necessary. The rapid transformation of sperm to be decondensed was influenced by sperm nucleus-decondensing factor (SNDF) (Hirao and Yanagimachi, 1979). The existence of SNDF in cytoplasm begin with gerimal vesicle breakdown and continue to increase until oocyte maturation (Mahi and Yanagimachi, 1976). Reported by Nasr-Esfahani et al., (2010) and Alvarez et al., (2013) proved that fertilization of immature oocytes lead to sperm transformation failure. Penetration of sperm into mature oocyte cytoplasm leads to oocyte activation (Aarabi et al., 2014; White et al., 2010), hence at the same time oocyte resumes its second meiosis (Figure 1a and 1b). The process of second meiosis leads to an advanced status of oocyte which characterized by second anaphase-telophase formation. Sitiayu et al., (2005) confirmed that the formation of clear second telophase was an indication of chromatin distribution. Sperm activates phospholipase C which leads to inositol 1, 4, 5-triphosphate (IP3) activation and Ca^{2+} release (Amdani et al., 2013; Kashir et al., 2012; Zhang et al., 2011). The increase of Ca^{2+} leads to decrease in MPF so, the

Figure 1. Sperm nuclear transformation in oocyte cytoplasm. (a) condesed sperm after 3 hours, (b) sperm decondesed and anaphase II of nuclear oocyte, PBI appear, (c) appearance of TII which spread oocyte nucleus, (d) 2 prepronuclei with PBI formed after 6 hours, (e) 2 pronucleus have formed after 6 hours & PBII appear, (f) >2PN indicated polyspermy. KS= condensed sperm; DS= decondensed sperm; ANA= anaphase II; TII= telophase II; = polar body (PB); PPN= prepronuclei; PN= pronucleus.
second meiosis is resumed and completed, then female pronuclei and second polar body is released (Elder and Dale, 2011; Sanders and Swann, 2016).

**Ram Sperm Transformation after Incubation**

Penetration of sperm with oocytes was penetrated 3 hours after sperm-oocytes incubation. Sperm have been transformed to pronucleus 6 hours and the percentage of pronucleus was increased as the fertilization period prolonged (Table 1).

The percentage of sperm transformation for each fertilization period was showed in Table 1. Three hours after fertilization period no sperm were turned into pronucleus, 28.7% became condensed while 50.9% were decondensed. Six hours after fertilization period, the formation of pronuclei was significantly increased to 30.3% (P<0.05), while condensation and decondensation of sperm were significantly decreased (P<0.05) to 7% and 23.5% respectively. Those result are consistent with Elder and Dale (2011) which reported that at 6 hours after fertilization period, some male and female pronuclei located on the oocyte edge, and both pronuclei have completely migrated to the center of oocyte at 9 hours after incubation. These microtubules play a role in directing the male pronucleus migration to the center of oocyte and contact with female pronucleus (Almonacid et al., 2018; Chaigne et al., 2016). Furthermore, actin and cytoskeleton inside cytoplasm also involves in pronucleus migration (Chaigne et al., 2016) and greater developmental competence to cleavage rate (Wan-Hafizah et al., 2015). It shows that microtubules play a role to assist migration process at 6 hours after incubation period, hence at 9 hours of fertilization pronuclei have located at the center of oocyte.

Prepronuclei formation began at 6 hours and increased until 9 hours of incubation. Prepronuclei (14.3%) was significantly increased (P<0.05) followed by pronuclei formation (47.1%) at 9 hours of incubation. In prepronuclei stage, nuclear envelope was not clearly seen. Nuclear envelope was formed by fusion of vesicles which needs GTP (Lete et al., 2017; Ungricht and Kutay, 2017). The binding process of vesicles within chromatin was mediated by lamin B receptor (LBR). In nucleus, lamin B associated with LBR and leads to pronucleus (P<0.05) with prolonged incubation time. Six hours after fertilization period, some male and female pronuclei located on the oocyte edge, and both pronuclei have completely migrated to the center of oocyte at 9 hours after incubation.

### Table 1. Ram Sperm Transformation after Interaction of Sperm-oocytes for 15 Hours

<table>
<thead>
<tr>
<th>IVF period (hours)</th>
<th>Number of Oocytes (n)</th>
<th>Sperm Transformation % ± SE (n)</th>
<th>Fertilization Rate % ± SE (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>KS</td>
<td>DS</td>
</tr>
<tr>
<td>3</td>
<td>129</td>
<td>28.7 ± 7.8&lt;sup&gt;a&lt;/sup&gt; (38)</td>
<td>50.9 ± 5.8&lt;sup&gt;a&lt;/sup&gt; (65)</td>
</tr>
<tr>
<td>6</td>
<td>123</td>
<td>7.0 ± 4.1&lt;sup&gt;b&lt;/sup&gt; (8)</td>
<td>23.5 ± 8.1&lt;sup&gt;b&lt;/sup&gt; (28)</td>
</tr>
<tr>
<td>9</td>
<td>129</td>
<td>0.8 ± 0.8&lt;sup&gt;b&lt;/sup&gt; (1)</td>
<td>7.4 ± 3.0&lt;sup&gt;c&lt;/sup&gt; (9)</td>
</tr>
<tr>
<td>12</td>
<td>129</td>
<td>0.0 ± 0.0&lt;sup&gt;b&lt;/sup&gt; (0)</td>
<td>0.0 ± 0.0&lt;sup&gt;c&lt;/sup&gt; (0)</td>
</tr>
<tr>
<td>15</td>
<td>125</td>
<td>0.0 ± 0.0&lt;sup&gt;b&lt;/sup&gt; (0)</td>
<td>0.0 ± 0.0&lt;sup&gt;c&lt;/sup&gt; (0)</td>
</tr>
</tbody>
</table>

KS= condensed sperm; DS= decondensed sperm; 2PPN= two prepronuclei; 2PN= two pronuclei; >2PN= more than two pronuclei;<sup>a-c</sup>= in the same column show significant differences (P<0.05).
swelling (Dittmer and Misteli, 2011).

Furthermore, the formation of condensed and decondensed sperm decreased at 9 hours of fertilization and disappeared at 12 hours of incubation period due to the transformation of sperm into pronucleus (49.7%). At the end of 12-15 hours of incubation, polyspermy was significantly increased (P<0.05). Polyspermy significantly increased with the prolongation of fertilization time (P<0.05). Sattar et al., (2011) and Long et al., (1994) confirmed that too long fertilization time leads to the increase in polyspermy. The length of time IVF is associated with the occurrence of aneuploidy (Gould and Griffin, 2018). Liu et al., (2016) reported that the reduction fertilization time will increase the efficiency of fertilization. Reduction of fertilization time also recommended by Enkhmaa et al., (2005) which confirmed that the increase too long fertilization time leads to reactive oxygen species (ROS). ROS have negative effects to increase DNA fragmentation on sperm (Cicare et al., 2014), fertilization rate in IVF and the embryo development (Goncalves et al., 2010; Lopes et al., 2010; Bain et al., 2010). Increased ROS can be caused spermatozoa produce ROS (Olmo et al., 2014; Abreu et al., 2017) therefore, the prolonged exposure sperm-oocytes can add to the accumulation of increased ROS. The other effects, immature (Yamaguchi and Kuroda, 2018) and over matured oocytes reduce the effectiveness of cortical granules, so reducing the incubation time of fertilization reduces the level of polyspermy (Long et al., 1994).

Several reports suggested that the optimum time of fertilization was 4 hours in mice (Enkhmaa et al., 2009), 8 hours in bovine (Long et al., 1994), and 6 hours in porcine (Alminana et al., 2005). The optimum fertilization time leads to increase monospermy, fertilization rate efficiency, and successfull rate of embryo development. In this research also shows the same result that the fertilization more than 12 hours is not recommended due to increase in polyspermy.

CONCLUSION

Penetration of sperm into oocytes has been occurred at 3 hour of fertilization period. The formation of pronuclei was found at six hours after fertilization period and the incidence of polyspermy increased as the fertilization period prolonged.

REFERENCES


Liu, J., X. Zhang, Y. Yang, J. Zhao, D. Hao, J.


Yasmin, C., T. Otoi, M.A. Setiadi and N.W.K. Karja. 2015. Maturation and fertilisation of...
sheep oocytes cultured in serum-free medium containing silk protein sericin.