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Evaluation of Different Techniques in Recovering of Oocytes and Storage Duration of Ovaries on the Quality and Quantity of Bovine *in Vitro* Maturation

Zaituni Udin*, **Masrizal, Hendri and Syafri Nanda**

Department of Animal Technology and Reproduction, Faculty of Animal Science, Andalas University, Padang, 25163, Indonesia

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* Corresponding author:
E-mail: zaituni@ansci.unand.ac.id/ zaituniudin @yahoo.co.id

ABSTRACT

This study aimed to evaluate different recovery techniques of oocytes and the duration time between ovary collection and processing in the laboratory on the quality and quantity of Simmental cross bovine oocytes in vitro maturation. A total of 75 bovine ovaries were divided into three groups. Experiment-1; the storage duration of ovaries were 6, 9 and 12 h. Experiment -2; the techniques for oocytes recovery were slicing, aspiration and slicing + aspiration. The ovaries were collected from slaughterhouse and kept in saline solution during transportation to laboratory. A total of 136 oocytes were used for experiment-1, and 246 oocytes for experiment-2. This study found that the number and quality of oocytes were significantly different ($P<0.05$) as the effect of storage time. The average number of oocytes were 15.90±2.48; 17.10±1.38 and 13.60±3.00, in 6 h; 9 h and 12 h of storage time, respectively. The techniques of oocytes collection significantly ($P<0.05$) affect the quantity of oocytes but no significant ($P>0.05$) on oocytes quality. The average of oocytes of groups slicing, aspiration and slicing plus aspiration were 14.66±2.09; 9.46±2.99 and 16.40±6.86, respectively. Both experiment was significant effect ($P<0.05$) on immaturation rate of bovine. Conclusion of this study the storage time of ovaries was suitable in 9 h and the technique of oocytes collection was in slicing plus aspiration.

Keywords: Collection technique, In vitro maturation, Oocyte, Ovary, Storage time

Introduction

In vitro embryo production (IVP) is using in many developing countries to increase the offspring of cows. The ovary of cows from the slaughterhouse is a cheap source of oocyte for research purposes and is commonly used for the standard of in vitro embryo production (Dey *et al*., 2011). The technique of in vitro maturation (IVM) is a part of assisted reproductive technology (ART) to conduct in vitro embryo production (IVEP) in cattle. In vitro culture (IVC) of the fertilized oocytes up to the blastocyst stage. In vitro embryo development is strongly influenced by the process during oocyte maturation, fertilization and the subsequent development of the fertilized oocytes. In vitro maturation (IVM) is a reproductive technology whereby to collect oocytes from follicles in the ovary and be matured in a laboratory setting. The embryo production is carried out through a combination of techniques of collection of immature oocytes, *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC). However, the significant contributing factors in the success of IVEP are the quality and number of collected oocytes.

Usually, ovaries obtained from the slaughterhouse constitute an economical source of oocytes. The recovery of oocytes with the proper technique is the first and very important step in the in vitro production of oocytes in respect of quality and quantity. In cattle ovary storage at 37ºC for 8 h significantly decreased both rates of cleavage and blastocyst formation after IVM/ IVF oocytes (2). According to Saleh (2017) that period between slaughtering and sample processing significantly affect oocytes collected percentage and quality. Bacelo-Fimbres *et al*. (2015) founded that shipping slaughterhouse derived bovine oocytes either for 18 hours or for 24 hours was superior to conventional maturation system for blastocyst production at day 7 of IVC.

The recovery of oocytes using the slicing and puncture techniques yielded more oocytes per ovary than the other 2 aspiration method (Wang *et al*., 2007). The oocytes quality reflected by the oocyte developmental competence may be affected by several factors, such as individual variability, source of gamete, technique collection,

the time between collection and processing. The methods that are generally used for the collection of immature oocytes from slaughtered animal ovaries include 1) aspiration from surface follicles using 18-20G needle, 2) puncturing of the prominent follicle and 3) slicing of ovaries into small pieces. Among these methods, aspiration is mostly used as it is easier and faster to perform and gives a high yield of oocytes. Depending on the efficiency of aspiration and the oocyte grading system, the oocyte harvest per ovary can range from 0.46 to 3.0, with an average of 1.5 per ovary (Totey *et al*., 1992), but the limitation of oocyte collection from slaughtered ovaries is that no information about pedigree of dam is obtained. According to Saleh (2017), slicing the cow ovaries methods for oocytes collection yields more collected oocytes count with moderate quality while aspiration methods yield fewer oocyte numbers with good quality.

The first and most critical step towards successful in vitro embryo production is the method of oocyte collection for IVM (Hegab *et al*., 2009), because the use of these oocytes makes it feasible to obtain a large number of ovaries at relatively low cost. The previous studied on embryo transfer concluded that equal numbers of embryos and transferable embryo by using GnRH plus progesterone combination of local pesisir cows (Zaituni *et al*., 2018).

This research is an evaluation of the suitable time storage of ovaries and collection methods of oocytes on quality, quantity, and maturation rate of bovine oocytes.

Materials and Methods

Materials

Ovaries of simmental cross from the slaughterhouse, Saline solution (NaCl) for ovaries transportation, PBS solution for evaluation of oocytes, dan medium maturation TCM-199 + BSA + hormone fixation solution to determine the nuclear maturation rate.

Methods

Exp-1: effect of ovary storage time on quantity and quality of oocytes in IVM of crossbreed cows. 30 ovaries were divided into 3 groups of storage time, those are: 6, 9 and 12 h.

Exp-2: effect of collection methods on quantity, quality and IVM of crossbred cows. 45 ovaries were divided into 3 collection methods of oocyte, those are slicing, aspiration and aspiration plus slicing.

Procedure

Collection of ovaries. Crossbred cows ovaries were collected from the local slaughterhouse immediately after slaughter. The collection ovaries were placed into the plastic at 27-30ºC in saline solution and transported to the laboratory and stored into 3 groups of storage time. Group-1 = 10 ovaries were stored for 6 h, Group-2 = 10 ovaries were stored for 9 h, Group-3 = 10 ovaries were stored for 12 h.

Oocytes collected methods. In the laboratory, the oocytes were collected from the ovaries by three methods. The ovaries were placed in the sterile petri dish containing PBS. For aspiration technique, a total of 15 ovaries were aspirated from individual ovaries after removing the tissue and placed in a petri dish containing PBS. For slicing technique, a total of 15 ovaries were held firmly and sliced with the surgical blade. For the aspiration plus slicing technique, a total of 15 collected ovaries were previously subjected for aspiration method followed by slicing to obtain more oocytes

Oocytes evaluation. Oocytes were examined under stereomicroscopes and classified according to their compaction, number of cumulus cell layers and homogeneous cytoplasm. Grade A defines cumulus oocytes- complexes (COCs) with compact cumulus cell (≥3 layers) and homogenous ooplasm. Grade B defines expanded cumulus cell oocytes. Grade C defines denuded oocytes with completely devoid cumulus cell and heterogeneity ooplasm. Grade D defines partial denuded oocytes with cumulus cell present either the oocytes.

***In Vitro* Maturation (IVM).** A total of 159 oocytes of 6 h storage group; 171 oocytes of 9 h storage group and 136 oocytes of 12 h storage group were evaluated. The evaluation also was performed to a total of 220, 142 and 246 oocytes of slicing, aspiration and aspiration plus slicing method, respectively. The oocytes and medium were incubated at 38.5ºC in CO_2 incubator supplied with 5% CO_2 and 95% humidified air for 24 h.

Fixation. After 24 h of maturation period, oocytes were washed 3 times with PBS supplemented with 2% BSA and loads on a slide. Slides were placed 48 h into fixation solution (overnight 3 ethanol; 1 glacial acetic acid), Oocytes were stained with 1 % orcein in 45% acetic acid and examination of maturation rate under microscope into different stage.

Maturation oocytes. The oocytes were classified into GV (denoting chromosomes enclosed within a nuclear membrane), GVBD (an absence of a visible nuclear membrane and chromatin condensation), MI (showing chromosome condensed in pairs without defected polar body) and MII (denoting one large group of chromosomes formed an equatorial plate and the remaining chromosomes are highly condenced or extruded 1st polar body, mature oocyte).

Statistical analysis

The obtained data were analyzed using SPSS 16 followed by chi–square for the mean comparation.

Results and Discussion

Experiment-1. Effect of storage time on oocytes quantity

The average quantity of oocytes per ovary was 15.9 in 6 h, 17.1 oocytes per ovary in 9 h and 13,6 oocytes per ovary and the highest in 9 h (Figure 1). The time storage was no significant effect (P>0.05) on quantity oocytes of the simmental cross. This result was similar to Abdel-Khalek *et al*. (2010) that reported the recovery rate was in significant decrease from 68.1% at 5 h to 78.5% at 9 h of preservation. However, it is better to transport the specimen from slaughterhouse to the place of processing directly after slaugther if preserved well by cool box under 4-8ºC (Saleh, 2017). The result of this study was higher than that of Saleh (2017) who reported a recovery rate of 75% at 2 h and 55% at 24 h, and Abdel-Khalek *et al*. (2010) who reported a range from 9.9 to 12.4 oocyte per ovary. The different of oocytes quantity is due to several factors such as the reproductive status, age and the the collection methods of oocytes. In this research the presented of CL was not separated because the difficulties to select the ovarium in the limited time and specimens. The result can be recommended that the storage time up to 6 h is suitable for oocytes quantity of Simmental cross cows.

The effect of storage time on oocytes quality

The percentage of oocytes quality was higher in B grade for all collection technique of oocytes and the lowest was found in A grade for all collection technique of oocytes. The highest oocyte quality was 69.8% at 6 h then 53.2% at 12 h and the lowest was 40.4% at 9 h (Figure 2).

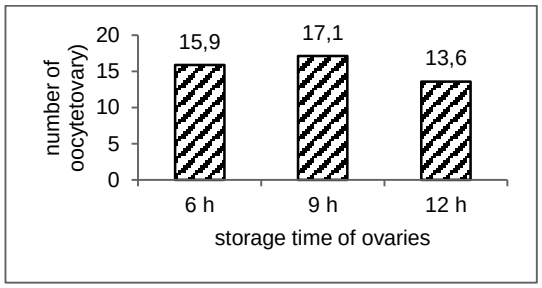

Figure 1. Effect of storage time oocytes quantity of Simmental cross.

The percentage of grade A was range from 7.5 to 9.2% at 6 h. For grade C, the percentage of oocytes quality range from 13.2% to 20.2% and grade D ranged from 9.4% to 21.6%. The results showed that the storage time was significant effected (P<0.05) the oocytes quality of Simmental cross. This result supported by Saleh (2017) that there is the direct effect on elapsed time from the period of slaughtering the donor animals toward time of the speciments processing inside the lab. As this time prolonged, it effects the oocytes quality which interferes with final result which is the oocytes quality. Francesco *et al*. (2007) stated that it is possible to optimize the quality oocyte, by prolonging the tolerance time for collection ovary up to 6 h. In addition, this might be due to many factors affect directly the oocytes quality, time of slaughter is the more dominant factor that influence the quality (Lonergan and Fair, 2016). According to Sonowal *et al*. (2017) that the oocytes having more than 4-5 layers of cumulus cells surrounding the zona pellucida along with homogeneous cytoplasmic appearance are considered as good quality oocytes. Recovery of oocytes with the proper technique is the first and very importance step in the IVP of embryos in respect of quality and quantity. From the result, it can be recommended that the storage time of 6 h produced the best quality of oocytes of Simmental cross cows.

Effect of storage time on maturation rate

The average percentage of maturation rate at stage M-II was 72.4% at 6h, then 67.1% at 9 h and 45.8% at 12 h storage time (Figure 3). The percentage of stage M-I was range from 3.6% at 6 h to 4.9% at 6 h storage time. For stage GV was range from 10.6% at 6 h to 37.3% at 12 h storage time, then the percentage of GVBD was range from 12.2% at 6 h to 16.5% at 9 h storage time. There was significant effect (P<0.05) of storage time on maturation rate of Simmental cross cow, but no significant different (P>0.05) was found between 6 h and 9 h storage time on maturation percentage. This result was supported by Bacelo-Fimbres *et al*. (2015) founded that shipping slaughterhouse derived bovine oocytes either for 18 hours or for 24 hours was superior to conventional maturation system for blastocyst production.

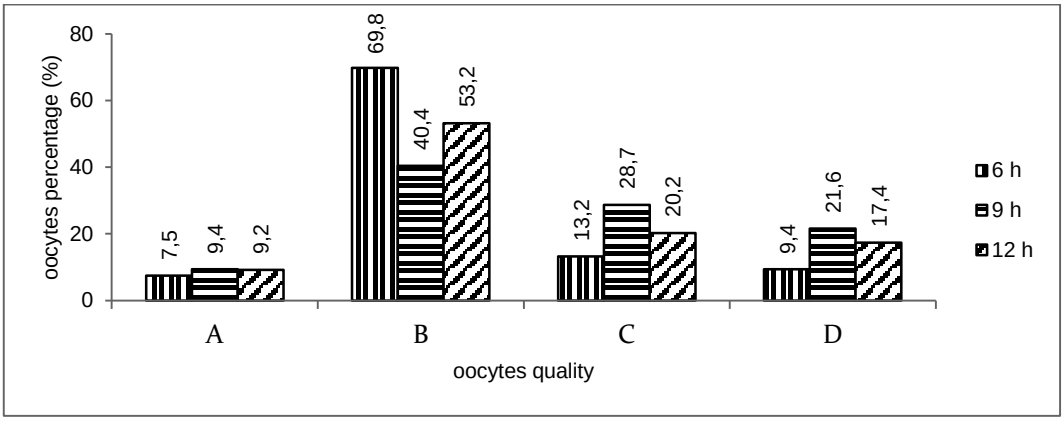

Figure 2. Effect of storage time on oocytes quality.

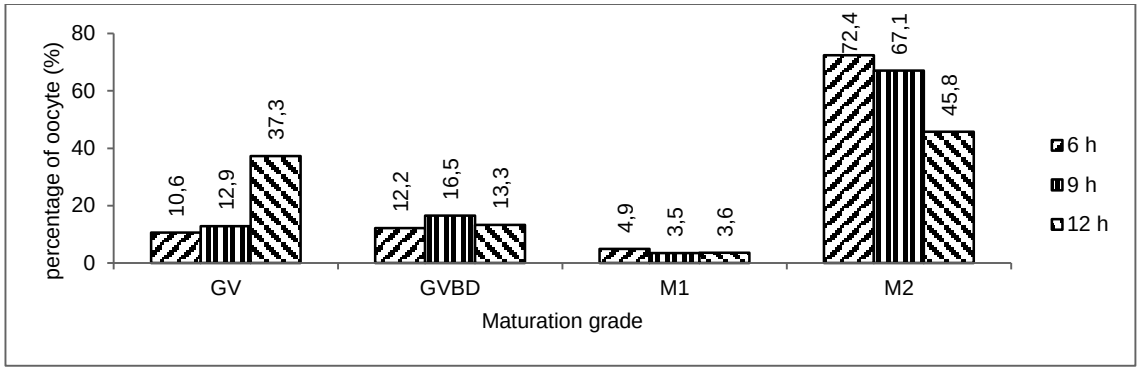

Figure 3. Effect of storage time on maturation rate of Simmental cross.

This result was higher than the finding of Elbaz *et al*. (2019) which found 42.0% in ovaries with CL and 55.6% in ovaries without CL. The quality of oocytes for IVM was compact oocytes complexs (COCs) with the 3 to 4 cumulus layers and homogeneous ooplasm. Only good quality oocytes were chosen for in vitro maturation as an increase in the percentage of good quality oocytes increase the cumulus expansion and maturation rate. According to Mohammed *et al*. (2019), there are differences in follicular fluid compositions due to follicle size, level of nutrition and animal species, which either stimulate or inhibit oocytes maturation and futher embryonic development. The conclusion that the storage time at 6 h was the suitable of IVM of Simmental cross cows oocytes.

Experiment- 2. Effect collection technique on quantity of oocytes

The number of oocytes per ovary was 14.67 in slicing, 9.47 oocytes per ovary in aspiration and 16.4 oocts per ovary in aspiration plus slicing method (Figure 4). It is showed that a higher quantities of oocytes was found in aspiration plus slicing ($P<0.05$) methods than that of aspiration and slicing, but no significant effect between slicing and aspiration plus slicing was found ($P>0.05$). This result supported by Habeed and Hussain (2017) who reported that the slicing method yielded significantly higher number of oocytes, and Wang *et al*. (2007) found that when compared with many collection methods of bovine oocytes, the slicing method yield more oocytes count than the other methods. Result of this study was higher than that of Mamy *et al*. (2016) was reported 10.5±1.5 in ovary with CL and 11.2±1.8 in ovary without CL. Nandi *et al*. (2000) stated that when ovaries had a corpus luteum, the oocytes recovery rate decreases. According to Saleh (2017), slicing methods yield more number of oocytes with a moderate quality and embryo production while aspiration methods yield a moderate oocyte count with an elevated quality and good embryo production. In this research the quantity of oocytes was higher than previous researcher may be caused the ovaries status and estrus cycle of donor cows. So, can be concluded that the slicing and aspiration plus slicing were recommended to collection methods of oocytes for Simmental cross.

Effect of collection technique on oocyte quality

The highest percentage of oocytes quality was found in Grade B which were 51.4% in slicing, 42.30% in aspiration and 54.90% in aspiration plus slicing. The lowest percentage oocytes quality in grade A, which were 10.5% in slicing, 7.70% in aspiration and 8.10% in

aspiration plus slicing (Figure 5). Grade A and grade B were classified as a normal and grade C and grade D were classified as abnormal COCs.

According to Sonowal *et al*. (2017), the oocytes having more than 4-5 layers of cumulus cells surrounding the zona pellucida along with homogeneous cytoplasmic appearance are considered as good quality oocytes. The collection technique of oocytes was no significant effect (P>0.05) on oocytes quality. Similar result and same trend was reported by Mamy *et al*. (2016) that higher percentage of COCs recovery was also recorded in blunt dissection (61.6±4.6%) than aspiration (48.6±2.9%) technique. This result lower than that of Singh *et al*. (2019) who reported the recovery of A+B grade oocyte was 86.01% and 81.76% by aspiration and slicing technique, respectively. Saleh (2017) found recovery percentage of 80%; 45%; and 40%; in aspiration; aspiration plus slicing and slicing, respectively. on the other hand, slicing technique was found to be more efficient than aspiration technique for COCs collection and this technique seems to be more practical in the place where getting slaughterhouse ovaries is difficult (Khandoker *et al*., 2016). Less number of COCs retrieved by aspiration technique might be because aspiration disruptscumulus cells or damages the whole oocytes (Nowshari, 2004). In addition, Shirazi *et al*. (2005) also reported high number of denuded oocytes recovered through aspiration. This study recommended that the sling technique good technique for collection oocytes of Simmental cross.

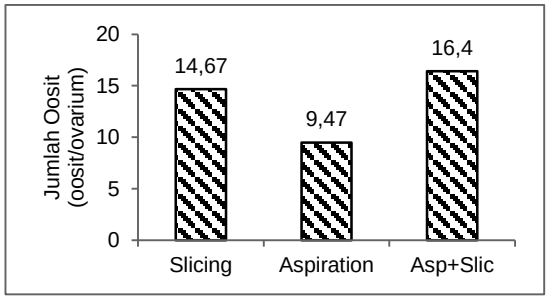

Figure 4. Effect of collection technique of oocytes on quantity of oocytes.

The effect of collection technique on IVM

The percentage of maturation in M-II stage was 61.8% by slicing; 54.9% by aspiration and 64.5% by aspiration plus slicing method (Figure 6). The effect of collection technique was not significant (P>0.05) on maturation percentage of Simmental cross cows. The percentage of M-II was higher than GV stage and GVBD stage. The lowest percentage of maturation was in M-I stage. This result supported by Vivian *et al*. (2018) who reported the average nuclear maturation in bovine was 58.2%, and lower than Deb *et al*. (2016) who reported 74.16% as detected by extrusion of first polar body and Gabr *et al*. (2015) who reported 67% in TCM -199 medium. However this result was higher than Saleh (2017) who reported 45% by aspiration, 31% in slicing and 32% in aspiration plus slicing.

According to Hammad *et al*. (2016) when trying to compare between different methods for oocytes collection in regarding to the oocytes number, quality and degree of maturation, the slicing and slicing after aspiration methods came in earlier methods that yield more oocytes count with good quality that reached to maturity condition in well considered degree. The oocytes collection methods also did not influence subsequent embryonic developmental competence after *in vitro* fertilization with M II stage oocytes (Wang *et al*., 2007). In contrary founded by Jalali *et al*. (2014) that the debris in the culture medium may have an adverse effect the oocytes maturation *in vitro*. Therefore that aspirating the ovarian surface is a better method to recover to recover oocytes for *in vitro* studies. Aspiration technique is the better method for recovery of oocyte for further *(in vitro*) studies (Singh *et al*., 2019).

This result might be considered that the quantity of oocytes depended to collection technique of oocytes and the suitable method was slicing technique. For quality of oocytes was effecting by storage time of ovarium and the best storage time was 6 h and the maturation of oocyte was affected by COCs at collection.

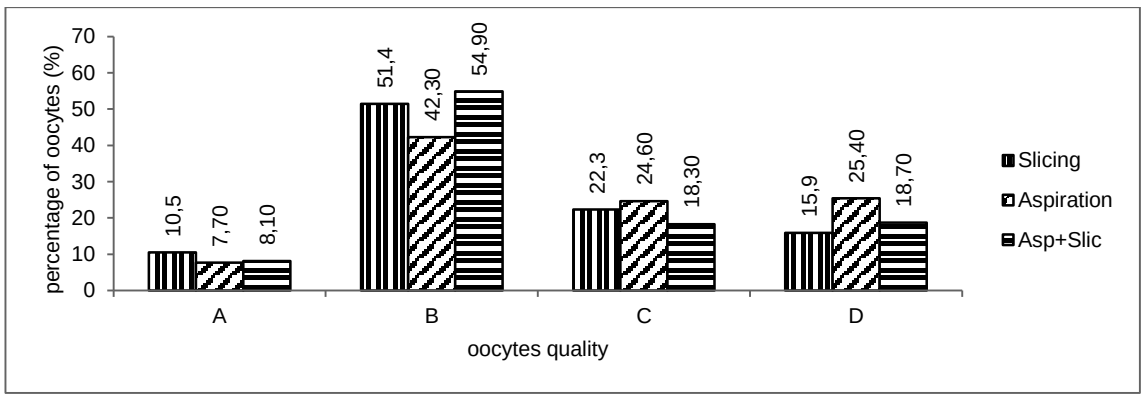

Figure 5. Effect of collection technique on quality of oocytes of Simmental cross.

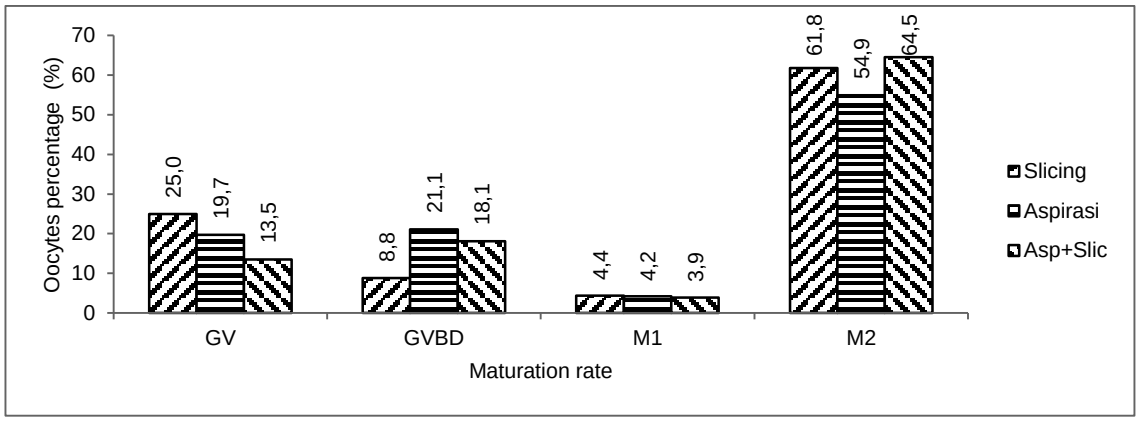

Figure 6. Effect of collection technique on IVM of Simmental cross.

Conclusion

The storage time affected the quality of oocyte ($P<0.01$), maturation rate, but did not affect the quantity of oocyte. The collection method of oocytes was significantly affected the quantity but did not affect the quality and maturation rate. The suitable storage time of ovarian was 6 hours and collection methods of oocyte was aspiration plus slicing.

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