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ASPIRATION, SLICING, AND FLUSHING MEDIUM TECHNIQUES IN COLLECTING OOCYTES OF SHEEP: SEARCHING FOR THE BEST METHOD

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ABSTRACT: The aim of this study was to compare the effect of the techniques of aspiration, slicing, and flushing medium in collecting oocytes on the quantity and quality of oocytes, the average time used for collecting oocytes per ovary, and the volume of the medium used. The material utilized was 274 ovaries from ewes aged 2.5 to 3.5 years and body weight ranging between 25 and 35 kg. This study used a Completely Randomized Design consists of 3 treatments of techniques were aspiration, slicing, and flushing medium. The parameters measured included the average quantity, quality, and weight of oocytes per ovary (right/left), the effect of the techniques of aspiration, slicing, and flushing medium on the quantity and quality of oocytes, and the efficiency of use of medium and time spent to collect oocytes by using aspiration, slicing, and flushing medium techniques. Data were analyzed by one-way analysis of variance. The results showed that the aspiration technique collected the highest percentage (P<0.01) of oocytes quality A (38.49%) compared to the slicing technique (17.93%), and the flushing medium technique (11.71%). In terms of time, the aspiration technique was the fastest (8-10 minutes) compared to the slicing technique (10-12 minutes), and flushing medium technique (13-15 minutes); meanwhile, the aspiration technique was the most efficient technique (1-2 ml) compared to slicing technique (3-5 ml) and flushing medium technique (6-10 ml). In conclusion, the aspiration technique is the best one for oocyte collection from sheep ovaries. This technique proves to be efficient in terms of quantity and quality of the oocytes collected, time to perform, and medium to use.

Keywords: Ewes, Ovaries, Oocytes, Reproductive techniques, Sheep breeding.

INTRODUCTION

In the biotechnology application on animal reproduction, in vitro embryo production requires good quality oocyte base materials. The oocytes in either the left or right ovaries can be collected using an appropriate technique and grown in an oocyte growth medium. In mammals, particularly sheep, the ovary may supply oocytes (primordial follicles) approximately 195,450-200,000 at birth (Sun et al., 2017; Cox and Yakov 2021). Yet only 475 oocytes reach their maturation stage and can be ovulated, while others live briefly and then die (Van Den Hurk, 2005). Some researchers found that the oocytes of cows cultured in an in vitro will still be able to grow and develop to the next stage (Ríos et al., 2015; Telfer et al., 2020). Similar result also found when they are cultured in a medium supplemented with cumulus cell culture (Bruynzeel et al., 1997), Fallopian tube epithelial cells (Barahona et al., 1997), gonadotropin hormone and steroid hormones (MacCallum et al., 1997; Lorenzo et al., 1997; Martínez et al., 2006). According to Sirard (2011), if the oocytes are incubated at a certain time, meiotic division may occur so that the oocytes will reach their optimal maturity level and are ready to be fertilized by spermatozoa. The zygotes formed can be grown into embryos until they reach a certain stage of growth (stages 2, 4, 8, 16, 32 or more than 32 cells). Thus, the ovaries potentially produce a good number of high-quality oocytes; however, collecting techniques used to accumulate the oocytes from the ovaries contribute their quality and quantity.

According to El-Sharawy et al. (2021), the flushing medium technique can collect an average of 4.32 oocytes from pubertal goats and 5.19 oocytes from adult goats. Wang et al. (2007) discovered that the slicing technique produces 6.3 oocytes, the flushing technique produces 5.8 oocytes, and the aspiration technique produces 2.9 oocytes per ovary. In addition, the finding of Martino et al. (1995) showed that slicing, aspiration, and flushing techniques produce an average of 1.71, 1.27, and 6.05 oocytes per ovary, respectively. Meanwhile, Hoque et al. (2011) identified that the technique of flushing, slicing, and aspiration is able to collect good quality oocytes per ovary on average 4.22, 4.14, and 3.28, respectively.

Regarding the review of the literature (Wani et al., 1999; Rodriguez et al., 2006; El-Sharawy et al., 2021), the number of oocytes collected using several techniques may have been considered in limited research-works.Therefore, further investigation in terms of collecting techniques for oocyte collection needs to be conducted. So, this study aimed to

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compare the effect of the techniques of oocyte aspiration, slicing, and flushing medium in collecting oocytes on the quantity and quality of oocytes, the average collection time used per ovary, and the volume of the medium used.

MATERIALS AND METHODS

Ethical regulations

The protocol of the current research was under the standard rule of animal treatment as designated in the Republic of Indonesia's law with number 41, 2014

Material

The materials used were 274 ovaries taken immediately from 137 ewes with range of age was 2.5 – 3.5 years and body weight of in between 25 and 35 kg. The chemical material used was sodium chloride, $CaCl^2$, de-ionized water, penicillin-streptomycin (SIGMA), and Kanamycin (SIGMA). The equipment used were dissecting microscope (Olympus SZ), inverted microscope (Olympus CK2) equipped with photographic equipment, laminar-flow hood (NUAIRE), filter with 0.22 µm (SIGMA) pore diameter, heating place (FISHER), petri dish (CORNING) with diameter 60 mm and 35 mm, disposable Pasteur pipette (VWR SCIENTIFIC), pipette-tip, Erlenmeyer, 25, 50 and 100 ml vial bottles, disposable syringes of various sizes 1, 5, and 10 ml, tweezers, scalpels, scissors, and gloves.

Oocytes collection

The total Oocytes have been collected from each technique was: 96, 88 and 90, respectively for aspiration, slicing and flushing medium techniques. This study used a Completely Randomized Design consists of 3 treatments of techniques were aspiration, slicing, and flushing medium. The aspiration technique was performed by sucking the follicular fluid having a diameter of 1.0 to 5.0 millimeters by using a 21 G syringe (Hashimoto et al., 1999) filled with 1.0 to 1.5 milliliters of Dulbecco's Phosphate Buffered Saline (D-PBS). The sucked follicular fluid was transferred into a petri dish, and then the quantity and quality of the oocytes were counted and observed, respectively using a 40 times magnification microscope. The slicing technique was performed by slicing the ovaries into small and thin parts using a scalpel in a petri dish containing D-PBS solution (Kouamo et al., 2014). The sliced parts were then transferred into another petri dish, counted, and observed for quantity and quality using a 40 times magnification microscope.

The flushing medium technique was done by puncturing repeatedly on the surface of the ovaries. Then rinsing them with the D-PBS medium slowly through punctures repeatedly across the entire surface of the ovaries using a syringe containing 1.0 to 1.5 milliliters of medium with a 21 G needle (Wongtra-ngan et al., 2010).

Parameters observed

Average quantity, quality, and weight of oocyte obtained per ovary (right/left). Effect of the techniques of oocyte collection on the quantity and quality of oocytes. The quantity and quality of the oocytes obtained by the techniques of aspiration, slicing, and flushing medium was statistically tested whether or not there were differences among the three techniques. The collected oocytes were classified according to their quality following Hoshino (2018) and Loos et al. (1992) that (1) Quality A, oocytes which are all surrounded by layers of cumulus cells, have homogeneous ooplasm and look clear and bright; (2) Quality B, oocytes which are mostly surrounded by cumulus cells, have homogeneous ooplasm but look rather dark on the edges; (3) Quality C, oocytes which are surrounded by a small portion of cumulus cells, have irregular ooplasm and dark; and (4) Quality D, oocytes with no cumulus cells around them, and the ooplasm looks very dark and irregular. Data related to time (minutes) needed to perform the techniques of aspiration, slicing, and flushing medium and the volume of collection medium used by all ovaries were tabulated averagely.

Statistical analysis

The length of time and the volume of the medium used reflected the efficiency of the best treatment. Collected data were analyzed by One-way Analysis of Variance with the techniques as independent variable.

RESULTS AND DISCUSSION

The results of the observation showed that the average weight of the right ovary (0.945 g) was heavier than that of the left ovary (0.855 g), the number of oocytes collected from the right ovary (11.7) was higher than that of the left ovary (10.1), and the quality A of oocytes collected from the right ovary (3.9) was better than that of the left ovary (2.2; Table 1). The number of oocytes according to the quality obtained by aspiration, slicing, and flushing medium techniques are presented in Table 2. The result showed that the aspiration technique collected the highest number of oocytes (1,351) from 96 ovaries, the flushing medium technique collected the second best (982) from 88 ovaries, and the slicing technique collected the lowest (853) from 90 ovaries.

Judging from the aspect of using the medium, the aspiration technique was more efficient than the slicing or flushing medium technique (Table 3). The results exhibited that the aspiration technique needs 1-2 ml per ovary, the slicing technique needs 3-5 ml per ovary, and the flushing medium technique needs 6-10 ml per ovary. This experiment showed that among the three treatments, aspiration techniques yielded the highest number of oocytes with quality A and B, respectively. Therefore, the quality of the oocytes by slicing and flushing techniques fell into categories C and D, respectively.

Table 1 - Average of oocytes quality collected from the right and left ovaries of sheep.

Ovary	Average weight of the ovary (gr)	Average oocyte collected per ovary according to its quality [1]				Number of Oocytes
		A	B	C	D	
Right	0.945	3.9^{ns}	3.3^{ns}	2^{ns}	2.5^{ns}	11.7
Left	0.855	2.2^{ns}	3.3^{ns}	2.1^{ns}	2.5^{ns}	10.1
Total	-	6.1	6.6	4.1	5.0	21.8

[2]Di [1]A: oocytes which are all surrounded by layers of cumulus cells, have homogeneous ooplasm and look clear and bright; B: oocytes which are mostly surrounded by cumulus cells, have homogeneous ooplasm but look rather dark on the edges; C: oocytes which are surrounded by a small portion of cumulus cells, have irregular ooplasm and dark; D: oocytes with no cumulus cells around them, and the ooplasm looks very dark and irregular. [2]NS=non-significant differences

Table 2 - The number of oocytes according to the quality obtained by aspiration, slicing, and flushing medium techniques

Oocyte Collection Techniques	Number of ovaries	Number and percentage of oocytes according to their quality [1]								Number of oocytes[2]
		A		B		C		D		
		Σ	%	Σ	%	Σ	%	Σ	%	
Aspiration	96	520	38.50	460	34.04	217	16.06	154	11.40	1351^{a}
Slicing	88	153	17.94	199	23.33	221	25.90	280	32.83	853^{b}
Flushing	90	115	11.71	226	23.01	284	28.92	357	36.36	982^{b}
Total	274	788	24.80	885	26.90	722	22.70	791	24.90	3186

[1]A: oocytes which are all surrounded by layers of cumulus cells, have homogeneous ooplasm and look clear and bright; B: oocytes which are mostly surrounded by cumulus cells, have homogeneous ooplasm but look rather dark on the edges; C: oocytes which are surrounded by a small portion of cumulus cells, have irregular ooplasm and dark; D: oocytes with no cumulus cells around them, and the ooplasm looks very dark and irregular. [2]Different superscripts in the same column show significant differences (P<005).

Table 3 - Efficiency among treatment of aspiration, slicing, and flushing medium in collecting oocytes according to quality, number of mediums, and time required per-ovary in average.

Collection Technique	Average number of oocytes according to quality [1] (per ovary)				Average volume of medium (ml)	Average time (minute)
	A	B	C	D		
Aspiration	5.4^{a}	5.2^{a}	2.5^{b}	1.9^{b}	1-2	8-10
Slicing	1.9^{b}	2.5^{b}	2.8^{b}	3.3^{ab}	3-5	10-12
Flushing	1.6^{b}	2.8^{b}	3.2^{a}	4.0^{a}	6-10	13-15
P-values	*	*	*	**		

[1]A: oocytes which are all surrounded by layers of cumulus cells, have homogeneous ooplasm and look clear and bright; B: oocytes which are mostly surrounded by cumulus cells, have homogeneous ooplasm but look rather dark on the edges; C: oocytes which are surrounded by a small portion of cumulus cells, have irregular ooplasm and dark; D: oocytes with no cumulus cells around them, and the ooplasm looks very dark and irregular. [2]Different superscripts in the same column show significant differences (*=P<0.05;**=P<0.01).

Data exhibited in Table 1 could be interpreted that the right and left ovary being were active. According to Hafez and Hafez (2000 because the right ovary naturally produces more gonadotropin hormone and participates in the oogenesis process, there are more mature oocytes. However, in terms of collection, the number of oocytes collected from the ovaries was also determined by the chosen technique of collection (Wang et al., 2007). The appropriate technique performed in collecting oocytes affects the number of good quality oocytes to be grown as embryos in vitro (Kruip et al., 1994).

The aspiration technique produced the highest percentages of oocytes with quality A (520; 38.50%) and B (460; 34.04%) while the slicing and flushing medium technique produced the highest percentages of oocytes with quality C (221; 25.90% and 284; 28.92%) and D (280; 32.83% and 357; 36.36), respectively. Meanwhile, the percentages of oocytes quality A (38.49%) and B (34.04%) collected by aspiration technique is higher than the average percentage of oocytes collected by the three techniques (22.50% and 26.79%), while the other two techniques collected less than the average. In comparison, the percentages of oocytes quality C and D collected by the slicing technique (25.90% and 32.82%) and the flushing medium technique (28.92% and 36.35%) is higher than the average percentages of oocytes

collected by the three techniques; oocytes quality C (23.62%) and oocytes quality D (26.85%), while the aspiration technique collected less than the average. Therefore, it could be assumed that the aspiration technique could be chosen as the best technique for collecting oocytes quality A and B. This finding is supported by the findings of previous studies, which stating that the aspiration technique in oocyte collection is more basic than other techniques (Marques et al., 2015).

The statistical analysis showed that there was a significant difference ($P < 0.01$) among treatments. Collecting oocytes using the aspiration technique was significantly different ($P<0.01$) compared to slicing and flushing medium techniques, as the last two techniques were not significantly different ($P>0.01$). This finding suggested that the aspiration technique significantly increased the number of oocytes collected. According to Gordon (1994), the total number of oocytes collected is strongly influenced by the oocyte separation technique from the ovary. The aspiration technique was able to collect oocytes precisely and directly on the spot, as the aspirator needle was easy to control and directed to the follicles (both large and small) that appeared on the surface of the ovary (Miller et al., 2004). In addition to the highest quantity and quality of the oocytes collected, this technique does not cause much oocyte damage (Machatkova et al., 1996).

The use of an aspirator needle is an essential factor in collecting good quality oocytes (Healy et al., 2015). In this study, a 21 G size needle was chosen because oocytes having a diameter of approximately 200 µm (Romão et al., 2010) within the ovaries were easily and precisely sucked by the needle of the aspirator. However, this finding was different from a previous study which reported that slicing technique produces more oocytes per ovary compared to the aspiration technique (Sofi et al., 2012). Yet, the aspiration technique performed was different from the one applied in this experiment. They used a vacuum pump aspiration technique to collect oocytes from sheep that had passed their mating season.

In this study, after being observed under a microscope, the number of oocytes collected by the slicing technique was small, as several oocytes were left in the ovary tissue which was caused by not being exposed to the slicing aimed at the follicle. As a result, the follicles with the oocytes remained intact. Moreover, in this technique, the slicing might damage the ovary when it hits the internal part of the follicles (Wang et al., 2007). As the structure of the cumulus cells enveloped the oocytes torn and decayed, the oocyte quality decreased (Lourenço et al., 2014; Xu et al., 2015). A similar result did happen with the flushing medium technique. As the needle randomly punctured the ovaries causing irregular holes, the oocytes were difficult to detach from the follicle when the medium was injected into the ovary. Consequently, the severe damage dropped dramatically in the quantity and quality of the oocytes collected.

The aspiration technique needed 10 minutes, as the position of the follicles was very clearly visible on the ovary surface; therefore, it was easy and fast in conducting aspiration. The slicing technique needed 10-12 minutes because it took time to make ovarian slicing searching for oocytes. Meanwhile, the flushing technique needed 12-15 minutes since puncturing to the ovaries had to be done first followed by 2-3 times rinsing for ovary searching. In terms of time, the aspiration technique was faster than the other two techniques. The findings of this investigation supported those of Martínez et al. (2006), who found that the aspiration approach for oocyte collecting is quick, simple, and effective. Data and analysis previously discussed suggested that the implementation of the aspiration technique was better than that of the other two techniques, as the aspiration technique was able to collect a high quantity and quality of oocytes, was efficient in using the medium and was fast in terms of time.

CONCLUSION

Among the three techniques, the aspiration technique is the best one for oocyte collection from sheep ovaries. This technique proves to be efficient in terms of quantity and quality of the oocytes collected, time to perform, and medium to use. Therefore, this technique is expected to help develop the effort of increasing embryo production in vitro.

DECLARATIONS

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Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Authors' contribution

YS Ondho and Sutiyono: Idea and research design; ET Setiatin, S Sutopo, and D Samsudewa: Data collection; E Kurnianto, and A Setiaji: Data analysis and Writing the manuscript; DA Lestari: Writing the manuscript

Conflict of interests

The authors declare that they have no conflict of interest.

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