Original Article

Interaction of cumulus-oocyte complex (coc) number, oocyte quality, and blastocyst numbers by repeated ovum pick-up (opu) in in vitro embryo production

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Abstract

Ovum pick-up (OPU) is a repeatable technique that is used for the retrieval of large numbers of immature oocytes from the antral follicles of live animals. The aim of the present study was to determine the effect of repeated OPU application on cumulus-oocyte complex (COC) number, oocyte quality, and blastocyst rates. The animal material for the study consisted of 10 Holstein heifers. Heifers selected as donors were administered OPU a total of 9 times on random days of the cycle at 1-week intervals. All antral follicles with a diameter of 2-8 mm were aspirated during the OPU procedure. *In vitro* embryo production (IVEP) was performed on the oocytes obtained after quality assessment. Various degrees of adhesion and connective tissue thickening were found in the ovaries of the animals after 9 repeated applications of OPU. It was found that the average number of A-quality oocytes was higher than the number of B, C, and D-quality oocytes. The number of cleavages per OPU was 2.69 (cleaved oocytes/opu session), and the number of blastocysts was 0.72 (number of blastocysts/opu session). It was determined that the general average of cleavage ratios was 73.62%, and blastocyst rates were 18.97%. It was observed that the number of oocytes, cleavage, and blastocysts obtained as a result of repeated OPU applications was not affected by repeated OPU. It was concluded that in order to achieve the target number of oocytes and blastocysts with recurrent OPU, experiments should be carried out with varying frequencies of OPU, and prophylactic measures should be taken to prevent adhesions.

Keywords: Blastocyst, Holstein heifer, In Vitro Fertilization, Repeated Ovum Pick-Up

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Introduction

The interaction of repeated oocyte retrieval with cumulus-oocyte complex (COC) count, oocyte quality, and blastocyst count is very important in vitro embryo production. Biotechnological methods such as *in vitro* embryo production (IVEP) have been widely used in recent years for genetic improvement (Guerreiro *et al*., 2014; Vieira, 2014). In 2019, IVEP accounted for 72.68% (1,031,567) of the total embryo production (1,419,336) in cattle breeding worldwide. The vast majority of embryos (1,010,680) were produced using OPU, while very few embryos (20.887) came from slaughterhouses (Viana, 2020). IVEP is, therefore, one of the most important methods for supporting genetic improvement (Vernunft *et al*., 2015).

The OPU is a repeatable technique that is used to obtain a large number of immature oocytes from the antral follicles of live animals (Choudhary *et al*., 2016). It is a less invasive method than multiple ovulation embryo transfer (MOET) and can be repeated at short intervals (1-2 weeks). It also causes minimal stress and side effects to the animals (Purohit *et al*., 2003; Boni, 2012; Choudhary *et al*., 2016). OPU can be applied in prepubertal calves (2 weeks - 6 months), heifers, healthy animals, clinically sick animals not responding to ET, and pregnant donors up to the first 3 months of pregnancy. There are many factors that affect the quantity and quality of oocyte and transferable embryos produced using the OPU/IVEP method. Follicle-stimulating hormone (FSH) application before OPU, donor, race, semen, frequency of OPU, donor parity, embryo stage, oocyte quality, embryo production method, temperature-humidity index, birth problems, and metritis can be given as examples (De Roover *et al*., 2005; Pontes *et al*., 2011; Abd El-Aziz *et al*., 2016; Baruselli *et al*., 2016 Ferraz *et al*., 2016).

In comparison with MOET, more transferable embryos can be obtained monthly from each donor with OPU (Boni, 2012). For example, while in superovulated cows, a single classical wash can be done in a period of 40-60 days, oocytes can be collected 4 times with OPU/IVEP during this period, and significantly more embryos can be produced (Blondin, 2015). While ET can produce 10 times the offspring from an animal during its life cycle, it is possible to produce 30-40 times the number of offspring with IVEP (Qi *et al*., 2013). The number of embryos produced from oocytes retrieved using OPU varies according to age, season, and FSH use, and it is stated that at least 1-3 transferable embryos can be obtained from each application (Verma *et al*., 2012). The aim of this study was to determine the effect of repeated OPU application on COC number, oocyte quality, and blastocyst rates.

Materials and Methods

Ethic Statement: The study was conducted with the approval of the Selcuk University Veterinary Faculty Experimental Animals Production and Research Center Ethics Committee (SÜVDAMEK) (2019/89).

Animal Materials: The animals in the study consisted of 10 Holstein heifers. The heifers were 13-15 months old at the beginning of the study. They were fed with a TMR (Total Mixed Ration) prepared at a survival rate in a semi-open barn.

Experiment design: The heifers selected as donors were administered OPU 9 times in total on random days of the cycle at weekly intervals. No hormones for superovulation or synchronization were administered prior to the OPU applications. OPU applications were made between November and April.

OPU Applications: An Esaote MyLab TwiceVet ultrasonography device and a compatible intravaginal OPU probe, catheter, and aspiration device were used for the OPU application (Esaote 5001, Italy). For OPU, the animal was put into labor and anesthetized by the lower epidural route (4–6 mL of local anesthetic, Adokain, Sanovel, Istanbul, Turkey). All antral follicles with a diameter of 3-8 mm in the ovaries were aspirated using a special convex vaginal probe (4.0-9.0 MHz, combined with the probe and the catheter with the 18-gauge needle at the tip) using 80-90 mmHg aspiration pressure. The aspiration line was flushed continuously with Ovum Pick Up solution (IVF Bioscience, Denmark) during follicular aspiration. During the application, aspirated follicular fluid and COCs were kept at 37 °C in a special section connected to the aspiration device.

IVEP stages: Commercial kits (Bioscience, IVF Bioscience, Denmark) were used during OPU and IVEP applications. The commercial kits used include mineral oil, OPU, oocyte and embryo washing, semen preparation, maturation, fertilization, and culture media. Collected follicle fluids were transferred to Petri dishes and scanned under a stereomicroscope (Leica S8 APO, Germany, 1.6X magnification), and oocytes were collected and transferred to the washing solution.

Collected COCs were evaluated according to:

A Quality: It has > 5 compact cell layers and homogeneous cytoplasm,

B Quality: It has 3-5 layers of compact cells and few inhomogeneous areas in the cytoplasm or >5 layers of compact cells and dense inhomogeneous areas,

C Quality: It has several cell layers (>3) and few inhomogeneous areas in the cytoplasm or no regional cumulus and has a little homogeneous area.

D Quality: It has completely bare, small, granular, inhomogeneous cytoplasm (Petyim *et al*. 2003).

After evaluation, A, B, and C quality oocytes were separated to be transferred to the maturation stage. After being removed from the washing solution, they were transferred to the maturation medium that had been equilibrated for at least 4 hours in the incubator. Oocytes transferred to the maturation medium were incubated at 38.8 °C for 22-24 hours in an incubator providing 5% CO2 and 95% humidity. After the maturation procedure, mature oocytes were transferred to the equilibrated fertilization medium and incubated again for 20-22 hours. The semen to be used for fertilization was thawed at 37 °C. After centrifuging twice at 1,200 rpm for 5 minutes with the semen preparation solution, 100,000 spermatozoa per oocyte were added to the fertilization drops. After the

fertilization controls were made, the zygotes were cleaned from the cumulus cells and put into the culture media: then they were transferred to an incubator containing % 6 CO^2, 6% O^2, and % 95 at 38.8 °C and incubated for 6-7 days.

Statistical Analysis: The values are presented as means±standard deviation (SD). Probability values $p<0.05$ were considered statistically significant. Mean±SD for COCs quality, embryo cleavage, and development rates were calculated from the data recorded for all cows during each OPU session. For these endpoints, the effect of time was determined with ANOVA, utilizing a repeated measures model (SPSS Version 22, SPSS Inc., Chicago, IL, USA). Percentage data (maturation, cleavage, and blastocyst rates) were analyzed using Chi-square.

Table 1 Oocyte, cleavage, and blastocyst counts, rates of viable oocyte, maturation, cleavage, and blastocyst.

OPU weeks	Animal (n)	A quality	B quality	C quality	D quality	Total	Maturation rate %	Cleavage rate %	Blastocyst rate %
1	10	1,80^a	1,10^a	1,90^a	2,50^a	7,30a	93,75^a	65,79^a	11,11
2	10	0,60^b	0,80^b	1,90^a	1,10^a	4,40^b	72,73^a	54,17^b	8,33
3	10	1,30^a	1,90^a	1,70^a	2,10^a	7,00^a	85,71^a	64,29^a	28,57
4	10	1,80^a	1,00^a	1,00^a	1,10^a	4,90^b	78,95^a	70,00^a	23,33
5	10	1,80^a	1,50^a	0,60^b	0,90^b	4,80^b	41,03^b	81,25^c	18,75
6	10	3,00^c	2,20^a	1,40^a	2,90^c	9,50^c	39,39^b	80,77^c	15,38
7	10	2,90^c	3,30^c	1,30^a	1,30^a	8,80^c	73,33^a	88,89^c	21,82
8	10	2,10^a	0,80^b	1,70^a	2,60^a	7,20^a	93,48^a	84,09^c	27,91
9	9	2,1^a	2,11^a	1,89^a	1,56^a	7,67^a	81,82^a	73,33^a	15,56
Total and mean	89	1,93	1,63	1,49	1,78	6,84	73,35	73,62^a	18,97
	SD	0,74	0,83	0,46	0,75	1,80	20,23	11,14	7,06
	SE	0,25	0,28	0,15	0,25	0,60	6,74	3,71	2,35

Probability values $P<0.05$ were considered statistically significant

Result

In this study, it was determined that the manipulation of the ovaries became more difficult over time, depending on the number of punctures performed after repeated OPU procedures. Although adhesions made it difficult to manipulate the ovary, it was determined that they did not have a significant effect on follicle aspiration.

As a result of repeated OPU applications, In the statistical analysis of the change in oocyte quality by weeks, It was determined that the statistical difference was significant in all qualities and in the total number of oocytes ($p<0.05$). It was observed that significance did not show a regular distribution between weeks, and the difference in the total number of oocytes was significant at weeks 2nd, 4th, 5th, 6th, and 7th. Between weeks 2nd, 6th, and 7th, the statistical difference in the number of A-quality oocytes was significant. At weeks 2nd, 7th, and 8th, the difference was significant in B-quality oocytes, while the difference was significant in C-quality oocytes at week 5th and in D-quality oocytes at weeks 5th and 6th. Oocyte quality and number according to weeks are given in Table 1. Additionally, data on the total number of oocytes per OPU and the quality and quantity of oocytes per OPU are given in Figure 1 and Figure 2.

As a result of OPU applications, the number of cleavages per OPU was 2.69, and the number of blastocysts was 0.72. The average viable oocyte ratio was approximately 75%, and there was no significant increase or decrease regardless of the weeks. As a result of the in vitro production stages, the average maturation rate was 73.35%. However, the results of the 5th and 6th weeks were below the average. It was determined that the general average of the cleavage rates was 73.62%, the cleavage rates were above the average in the 5th, 6th, 7th, and 8th weeks, and the cleavage rates were well below the average in the 2nd week. It was observed that the average blastocyst rate was 18.97% and remained (Figure 3).

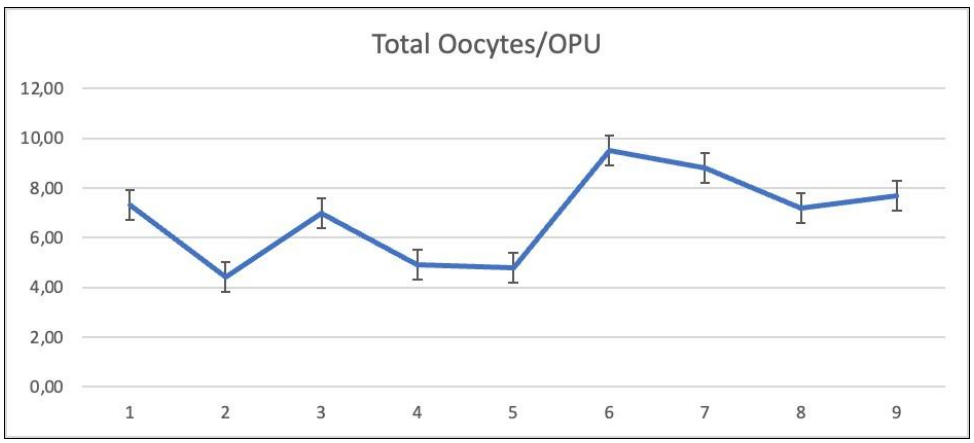

Figure 1 Total oocytes number per OPU.

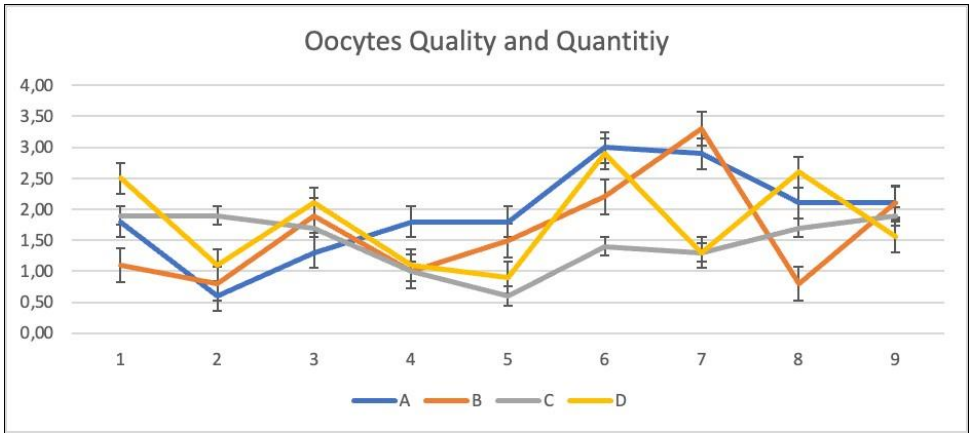

Figure 2 Oocytes quality and quantity per OPU.

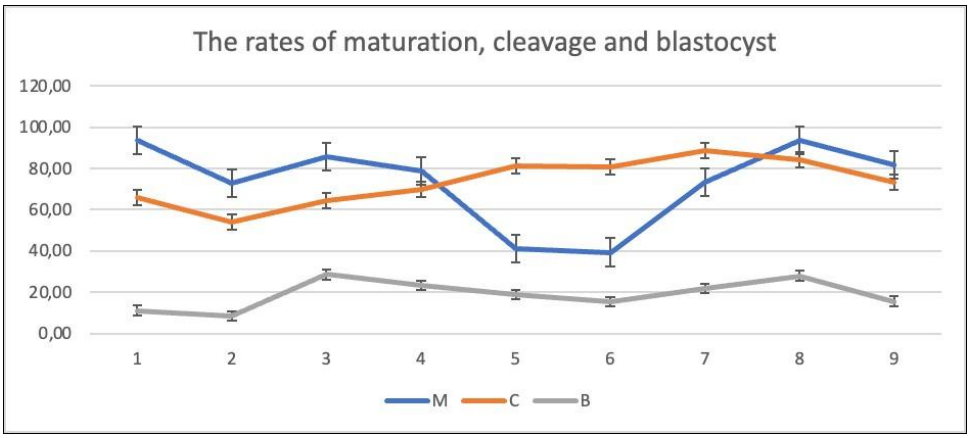

Figure 3 The rates of maturation, cleavage, and blastocyst (M: maturation, C: cleavage, B: blastocyst).

Discussion

Physical damage such as bleeding, immune cell infiltration, and fibrosis can occur in the ovaries during and after OPU sessions. In addition, it has been reported that some follicular cells may luteinize during the aspiration of follicles and subsequently produce progesterone (Viana *et al*., 2003). Other disadvantages include thickening and hardening of the connective tissue around the ovaries as a result of manipulations (Petyim *et al*., 2001). However, the most important problem may be the adhesion of the ovaries to the surrounding tissues and organs during the OPU process (Viana *et al*., 2003). These effects should be at a level that does not interfere with the normal functioning of the ovaries so that the success of OPU procedures is not affected. However, the number and continuity of OPU procedures seem to be directly related to the extent of ovarian damage. In this study, rectal and ultrasonographic examinations revealed that the ovaries had adhesions that increased with the number of OPU procedures and varied individually, making manipulation difficult due to the difficulty in moving the ovary. As the number of procedures increased, there were changes in the resistance felt by the operator during puncture during the entry and exit of the needle, suggesting that a different tissue than the normal ovarian tissue was formed and that this may be an increase in connective tissue. Although adhesions made the ovary difficult to manipulate, they did not have a significant effect on follicle aspiration. Considering the number and quality of oocytes obtained, neither adhesions nor connective tissue development seemed to affect the number and quality of oocytes produced.

In a study in Holstein cows in which three OPU applications were performed during a cycle (approximately once a week), it was reported that an average of 12.6 follicles were aspirated and 6.9 oocytes were collected per cow. However, the researchers reported that the number of follicles and oocytes obtained in OPU applications performed once a week was lower than twice a week (Pieterse *et al*., 1991). In many studies, it was reported that the number of oocytes was between 4.7 and 13.2, and the percentage of blastocysts was between 12.4% and 32.5% after two OPU applications per week in Holstein heifers. In cows, these values were reported to be between 2.4-17.2% and 8.1-39.5%, and when FSH was applied to donors, the values increased to 5.6-11% and 42-43% (Blondin *et al*., 2002; Rizos *et al*., 2002; Roth *et al*., 2002; Argov *et al*. 2004; De Roover *et al*., 2005).

In a study conducted on 18 podolic cows, it was reported that the average number of oocytes was 3.4, the number of divisions was 1.7 (49.4%), and the number of blastocysts was 0.9 (23%) in the non-eCG group, whereas these rates were 2.3, 1.7 (72.9%) and 0.9 (41.1%), respectively, in eCG-treated animals (Presicce *et al*., 2020). In a 3-year study conducted on domestic cattle in Korea, oocyte counts were 9.76 (first year),

10.68 (second year), and 14.03 (third year), cleavage counts were 4.77, 5.27, and 7.43, and blastocyst counts were 3.89, 3.29 and 3.51 after OPU treatments at 4-day intervals. The researchers also reported that repeated OPU of donors for up to three years is possible for in vitro embryo production (Choi *et al*., 2018). In Nelore (Bos Indicus) cows, it was reported that the average number of oocytes was 16.0, and the number of blastocysts was 5.4 after 12 repeated OPU applications at 30-day intervals. Researchers reported that in donors with more COCs, the number of COCs collected in repeated OPU applications decreased, but the number of blastocysts per OPU was not affected (Monteiro *et al*., 2017). In Angus cows, it was reported that the average number of oocytes per animal was 4.6, and the number of blastocysts was 0.9 in the group that received OPU once a week without any hormone application. It was reported that the same values were 3.9 and 0.8, respectively, in biweekly applications. In the same study, it was reported that the same parameters were 10.6 and 2.4, respectively, in animals in which OPU was performed by giving FSH once a week (Chaubal *et al*., 2006).

Repeated OPU treatments every two weeks were reported to have a significant negative effect on follicle number in Bos Indicus heifers compared to Bos Taurus heifers (Kruip *et al*., 1994). Furthermore, in animals with more follicles, more punctures per OPU session resulted in more damage and scar tissue in the ovarian cortex and stroma. A positive correlation between the antral follicle population and the total number of COCs collected per OPU has been reported (Batista *et al*., 2014; Guerreiro *et al*., 2014). However, no positive correlation has also been reported for variables related to in vitro developmental competence (cleavage and blastocyst rates) (Guerreiro *et al*., 2014; Gimenes *et al*., 2015).

The present study determined that the average number of total oocytes was 6.84, the number of viable oocytes was 5.06, the number of divisions per OPU was 2.69, and the number of blastocysts was 0.72. All the data obtained were found to be in parallel with the results obtained in previous studies on Holstein breed animals. When compared with the results of studies conducted on Bos Indicus breeds, it was observed that fewer oocytes could be collected, and fewer blastocysts could be produced in parallel with the breed characteristics. Still, the number of divisions was similar to those in these breeds.

An important aspect of optimizing OPU is the sampling frequency. When determining the donor's usage time for oocyte retrieval, the duration of rest periods and the time of repeated administration should be taken into account (Choi *et al*., 2018). It has been reported that the once-weekly administration of OPU does not provide sufficient numbers and quality of oocytes for IVEP. Researchers reported that twice-weekly administration of OPU instead of once-weekly administration increases the number and quality of oocytes collected (Van der Schans *et al*., 1991). In addition, when the OPU program is used twice a week, the follicular wave frequency is improved, and the estrous cycle and follicular development are regulated (Kruip *et al*., 1994).

In living animals, the bonds between the follicle and the COC are strong, and consequently, the cumulus layers of oocytes obtained from OPU material are irregular (Mullaart *et al*., 1999). Quality oocytes bind more tightly to the stratum granulosum. This may result in these high-quality oocytes not being retrieved or being assessed as poor quality due to the disruption of the cumulus integrity during retrieval (Gordon, 2003). The low quality and quantity of oocytes obtained with OPU may also be due to the aspiration of atretic and early atretic follicles (Karadjole *et al*., 2010).In addition, it has been reported that extrinsic factors such as vacuum pressure and needle diameter used during aspiration in the OPU method affect the morphological characteristics of retrieved oocytes (Hashimoto *et al*., 1999). COCs obtained from some follicles, especially atretic follicles, may appear to be of high quality but may fail to develop blastocysts. In the OPU procedure, follicles that have not started atresia and are developing before the dominant follicle selection, i.e., 3-8 mm in diameter, are aspirated. However, follicles with atresia due to different reasons may also be encountered in these follicles. This may explain the similar blastocyst rate despite different oocyte quality (Merton, 2014).

A good ratio between collection efficiency and the quality of the COCs must be established. In fact, the mechanical damage caused during needle transport of COCs depends on the needle size and length as well as the vacuum pressure. However, the first two parameters are chosen by the operator, while the latter depends on factors such as needle size and tilt, as well as the negative pressure present in the vagina and peritoneum (Boni., 2012). Aspiration pressure affects the number and quality of collected oocytes. Aspiration pressure can be used between 70-130 mmHg (López, 2020). An increase in pressure may cause the COC structure to deteriorate, and a decrease in pressure may cause the oocytes not to be collected from the follicle (Bols *et al*., 1996). It is reported that if the pressure exceeds 50 mmHg, the quality of the collected oocytes decreases significantly, and approximately 50% of the oocytes obtained are grade 3 (Ward *et al*., 2000). In the current study, oocytes were aspirated at 80-90 mmHg during OPU application. It was thought that this pressure value may have changed the quality of oocyte retrieval. It was thought that the studies could be repeated with higher or lower pressure values , and the effect of pressure could be determined.

In addition, it was thought that better quality and more number of oocytes could be collected by performing OPU on certain days of the cycle (by applying synchronization and superovulation protocols twice a week). A 7-day interval between two OPU treatments has been shown to collect more COCs than 3-4 days. However, a 3-day interval has also been shown to yield better quality COCs and a higher blastocyst rate. This is because the dominant follicle (DF), which appears approximately three days after OPU treatment, suppresses the development of other follicles. The number of oocytes collected per session does not differ between the commonly used OPU schedule of 3- and 4-day intervals and the OPU schedule of 2- and 5-day intervals (Sirard, 2012; López,

2020). In various OPU/IVEP protocols, follicles above 2 mm are aspirated on random days of the estrous cycle (Dos Santos *et al*., 2016). Oocyte growth rate, oocyte quality, and in vitro embryo production success are influenced by the duration of the estrous cycle during follicle aspiration (Camargo *et al*., 2006). It has been reported that more blastocysts can be produced from oocytes collected on the 4th, 14th, and 18th days after ovulation of the estrous cycle (Gonjalves *et al*., 2022). It was determined that more and better quality oocytes were collected during the period when the dominant follicle was absent due to follicular atresia. It is also reported that oocytes collected at this stage have a higher rate of reaching blastocyst (Bacelar *et al*., 2010).

During OPU application, removal of all follicles with a diameter of ≥3 mm causes the start of a new follicular wave in the ovaries (Adams *et al*., 1992; Viana *et al*., 2010; Surjus *et al*., 2014). It has been reported that at least 2 days are needed for the start of a new follicular wave and the growth of new follicles, and sometimes this period may exceed 3 days (Boni *et al*., 1997). It is thought that the differences that occur after OPU applications once a week may be shaped by individual differences in the animals, the duration of follicle development, and the aspiration of atretic follicles together with new follicles. For these reasons, there may be changes in oocyte quality and quantity.

In the presented study, it was thought that there may be atretic follicles among the follicles smaller than 2 mm collected during OPU, that there may be individual cycle differences on the day of application, and that these reasons may have negatively affected oocyte quality.

It was observed that the oocyte quality, maturation, cleavage, and blastocyst rates obtained as a result of repeated OPU applications were affected by OPU with a long repetition interval. It was determined that adhesions were formed at different levels depending on the repetitions and the temperament of the animal, that aggressive animals moved more during the application, and that these movements were sudden and harsh, causing more damage to the ovary and more adhesion during manipulation. It was thought that this situation also triggered some side effects, such as increased connective tissue and bleeding. It was concluded that trials should be conducted by changing the OPU frequency to reach the targeted oocyte and blastocyst numbers in repeated OPU applications and that prophylactic measures could be taken to prevent adhesions.

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