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Ovum Pick Up: Which Day of the Estrous Cycle Should be Performed in Cattle?

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ABSTRACT

***Backgraund*:** The first step in the *in vitro* embryo production (IVEP) procedure is to obtain high-quality and viable oocytes. Therefore, the ovum pick-up (OPU) method was developed in cattle. Ovum pick up in cattle is usually performed once or twice a week. However, when a fixed time follicular aspiration procedure is planned, the OPU application is conducted on different days of the donor's estrous cycle. Furthermore, there is no recommendation as to the optimum day within the estrous cycle for OPU. Therefore, the main objective of the present study was to determine the effect of OPU application on oocyte yield, quality and blastocyst rates on different days of the estrous cycle.

***Materials, Methods & Results*:** In the study, 18 Holstein heifers were administered OPU on days 2-3, 4-7, 8-12 and 13-16 of the estrous cycle. The OPU application was performed once in one oestrus cycle for all donors. The following OPU application waited at least one estrous cycle. Grade A quality oocytes were obtained in greater numbers in D 2-3 and D 4-7 groups than in D 13-16 group ($P < 0.05$). There was no difference in other oocyte quality and total number of oocytes between the groups. The number of viable oocytes was similar between D 2-3 and D 4-7 groups, but greater than D 13-16 group ($P < 0.05$). Concurrently, the number of viable oocytes was greater in D 4-7 group than in D 8-12 group ($P < 0.05$). There was no difference between D 2-3 and D 8-12 groups in terms of viable oocyte number. The number of blastocysts in D 2-3 and D 4-7 groups was greater than D 8-12 and similar to D 13-16 ($P < 0.05$).

***Discussion*:** A follicle may be in 1 of 4 phases during the follicular wave: growth, dominance, static, or regression. In present study, the greater number of oocytes with greater quality and viability rate in D 2-3 and D 4-7 groups is thought to be due to the absence of follicles in the dominant stage in these two groups. In OPU/IVEP method, a greater number of oocytes per donor increases the rate of blastocysts and viable embryos and accordingly positively affects the post-transfer pregnancy rate. In current study, the number of Grade A oocytes and viable oocytes were greater in D 2-3 and D 4-7 groups of the cycle compared to D 13-16 group of the cycle. The reason for this is that when OPU is used on days 13-16 of the cycle, different follicular groups can be found on the ovary. In addition, the present study demonstrated that the number of viable oocytes and blastocysts was greater in the D 4-7 group compared to the D 8-12 group. This may be due to the greater number of atresia follicles (subordinal follicles) between days 8-12 of the cycle (compared to days 4-7) and the lower developmental competence of oocytes in the subordinal follicle. The most significant factors influencing the success of an *in vitro* embryo production process are the quality and viability of the oocyte. In the present study, the number of Grade A oocytes was greater in the D 2-3 and D 4-7 groups than in the D 13-16 group, but there was no difference in the number of blastocysts. However, in parallel with the number of viable oocytes, the number of blastocysts obtained in groups D 2-3 and D 4-7 was greater than in group D 8-12. In conclusion, the determination of the appropriate day of the cycle for OPU will rule out synchronisation or stimulation of the follicular wave and ablation of the dominant follicle. Therefore, the oocyte quality, number and most importantly the number of blastocysts can be increased by performing OPU between 2-3 or 4-7 days of the estrous cycle.

Keywords: blastocyst, holstein heifer, luteal phase, oocyte quality, ovum pick up.

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INTRODUCTION

The 1st step in the IVEP procedure is to obtain high-quality and viable oocytes [6]. Therefore, the transvaginal ultrasound-guided oocyte collection (OPU, ovum pick-up) method was developed in cattle [18]. The most important advantage of OPU is that it makes it possible to obtain oocytes more than once at regular intervals without harming donors of high genetic value (minimally invasive technique) [2,14,26].

The objective of the ovum pick-up method is to procure a substantial number of high-quality oocytes from donors. Therefore, many studies have recently been carried out aiming to increase the quality and number of oocytes by methods such as synchronisation, stimulation (FSH, long-releasing FSH) and dominant follicle removal (DFR) in donors before OPU [3,5,23]. In contrast, there is no consensus on the use of synchronisation or stimulation prior to OPU in cattle.

Considering the studies without any stimulation, OPU application is generally scheduled once or twice a week [20]. However, when a fixed time follicular aspiration procedure is planned, the OPU application is conducted on different days of the donor's estrous cycle. Therefore, oocytes are collected from follicles at different stages of development. Furthermore, there is no recommendation on which day of the estrous cycle OPU should be performed without synchronisation or stimulation. The aim of the present study was to determine the effect of OPU application without any manipulation (estrous synchronisation, stimulation and DFR) on oocyte yield, quality and blastocyst rate on different days of the estrous cycle.

MATERIALS AND METHODS

Animals

The study was conducted in Konya province (37°34′30″N/ 32°46′29''E) between June 2020 and January 2022. The animals in this study were selected from a farm using genomic selection. These animals in the present study consisted of 18 Holstein heifers aged 14-16 months (rectal palpation/ultrasound examinations showed no problems in reproductive function and genital system). The body condition score of the heifers in the study was between 2.50-3.25 and the animals were used in repeated OPU applications. The ration consisted of corn silage, alfalfa silage, alfalfa, hay, concentrate feed, vitamin and mineral additives.

Experimental setup

In the study, the donors' estrous detection was determined and recorded using a pedometer system[1]. All donors were subjected to ovum pick up at 4 different stage of the luteal phase. The OPU application was performed once in 1 oestrus cycle for all donors. The following OPU application waited at least 1 estrous cycle. D 2-3 (n= 18): Early luteal phase includes animals treated with OPU on days 2-3 of the estrous cycle, D 4-7 (n= 18): Early to mid-luteal phase includes animals treated with OPU on days 4-7 of the estrous cycle, D 8-12 (n= 18): Mid-luteal phase includes animals treated with OPU on days 8-12 of the estrous cycle, D 13-16 (n= 18): Late luteal phase includes animals treated with OPU on days 13-16 of the estrous cycle.

Follicular aspiration - Follicle and oocyte evaluation

A combination of real-time ultrasonography device[2], 4.0-9.0 MHz micro-convex probe catheter and aspiration device[3] were used for ovum pick up. For OPU, the animal was put into anesthetized via the lower epidural route (4-6 cc of local anesthetic)[4]. The ovaries were scanned by ultrasound prior to aspiration and number of follicles present on the ovary was counted. All antral follicles with diameters of 2-8 mm in the ovaries were aspirated. The follicles were aspirated using a 20 gauge needle catheter and a special convex vaginal probe. The aspiration line was rinsed continuously with an ovum pick-up (OPU) solution during follicular aspiration. During the procedure, the aspirated follicular fluid and COCs were maintained at 37°C in a special section connected to the pump.

Classification of cumulus oocyte complexes

A stereomicroscope[5] was used to scan the aspiration fluid collected after follicular aspiration. The obtained cumulus-oocyte complexes (COCs) were classified as very good quality (grade A), good quality (grade B), fair quality (grade C) or poor quality (grade D) based on morphological characteristics, including cytoplasmic homogeneity and cumulus characteristics

[24]. In this study, oocytes graded A, B or C were selected for further *in vitro* embryo production.

Oocyte maturation, fertilization and culture

Grade A, B and C cumulus-oocyte complexes obtained from heifers by the ovum pick-up method were washed 3 times in pre-warmed microdrops (100 µL) of BO-Wash medium[6]. Subsequently, COCs were transferred to 100 µL BO-IVM droplets for *in vitro* maturation, and were covered with mineral oil. The COCs were then placed in the maturation medium and incubated for 22-24 h at 38.8°C in a mono gas incubator with 5% CO_2 and 95% humidity. Following *in vitro* maturation, cumulus expansion and the presence of a 2nd polar body were assessed in COCs. *In vitro* fertilisation was conducted by transferring the COCs to BO-IVF medium supplemented with semen and incubating them for 21-24 h at 38.8°C in a mono gas incubator[7], which provided 5% CO_2 and 95% humidity conditions. Following the completion of the *in vitro* fertilisation process, the cumulus cells were carefully and gently pipetted in order to separate them from the oocytes. The oocytes were separated from the cumulus cells and transferred to BO-IVC medium in 100 µL drops, after which they were covered with a layer of mineral oil. *In vitro* culture was conducted in a tri-gas incubator at a temperature of 38.8°C, with a CO_2 concentration of 6% and an O_2 concentration of 6%. The humidity was maintained at 95% throughout the incubation period. The cleavage and blastocyst rates of oocytes were assessed under a microscope on days 7 following fertilisation [21].

Evaluation of embryos

After *in vitro* culture, embryos were scanned under stereomicroscope and recorded according to their quality and developmental stages. According to IETS criteria, the quality of embryos is evaluated with codes ranging from 1-4 according to their morphological appearance. Code 1 (Excellent or Very good), blastomeres forming the embryo appear uniform in size, colour and density. The embryo is characterised by a symmetrical and spherical cellular mass. At least 85% of the blastomeres forming the embryo are viable and there is little or no irregularity between them. The zona pellucida is smooth and spherical. In Code 2 (Good), the blastomeres of the embryo are inconsistent in size, colour and density. At least 50% of the blastomeres forming the embryo are viable and there is moderately irregular between them. The zona pellucida is intact and partially retains its spherical structure. Code 3 (Poor), the cellular mass of the embryo and the blastomeres are severely irregular in size, colour and density. At least 25% of the embryo cell mass is viable. The zona pellucida is intact, but its spherical structure is distorted and there are concave or flat structures on it. Code 4 includes dead or degenerated embryos and unfertilised oocytes. These embryos are not used for embryo transfer [1]. In this study, Code 1 and Code 2 embryos were considered as transferable and Code 1 embryos were considered as freezable quality.

Statistical analysis

The statistical analysis of the data was conducted using the statistical software package SPSS 25.08. The Kolmogorov-Smirnov test was used to check the homogeneity of variances and the normality assumptions of the variables. Variables that do not have a normal distribution are presented as median (min/max), and the Kruskal-Wallis test was employed for analysis of variance. Normally, distributed variables were presented as mean ± standard deviation (SD), and ANOVA with post-hoc Duncan test was used to evaluate the variables. The significance level of the tests was accepted as $P < 0.05$.

RESULTS

As a result, Grade A quality oocytes were obtained in greater numbers in D 2-3 and D 4-7 groups than in D 13-16 group ($P < 0.05$). There was no difference between the groups in other oocyte grades and total number of oocytes (Table 1). The number of viable oocytes was similar between D 2-3 and D 4-7 groups, but greater than D 13-16 group ($P < 0.05$). Concurrently, the number of viable oocytes was greater in D 4-7 group than in D 8-12 group ($P < 0.05$). There was no difference between D 2-3 and D 8-12 groups in terms of viable oocyte number. There was no difference between all groups in terms of cleavage rate. The number of blastocysts in D 2-3 and D 4-7 groups was greater than D 8-12 ($P < 0.05$) and similar to D 13-16. In addition, there was no difference between D 8-12 and D13-16 groups in terms of blastocyst number (Table 2).

Table 1. Median (min-max) values of oocyte quality and total number of oocytes in 4 different luteal phases of the estrous cycle (D 2-3, D 4-7, D 8-12 and D 13-16).

Parameter	Groups				Total
	D 2-3	D 4-7	D 8-12	D 13-16	
Grade A	2 (0-13)[a]	3 (0-12)[a]	2 (0-6)	1(0-4)[b]	2 (0-13)
Grade B	1 (0-9)	1 (0-6)	1 (0-4)	1 (0-4)	1 (0-9)
Grade C	1 (0-4)	2 (0-6)	1 (0-4)	1 (0-6)	1 (0-6)
Grade D	2 (0-16)	1 (0-7)	2 (0-4)	2 (0-8)	2 (0-16)
Total	7 (2-42)	9 (5-21)	6 (2-12)	7 (1-12)	7 (1-42)

[a-b]The difference between groups in the same row ($P < 0.05$).

Table 2. Mean (±S.E.M.) values of the number of viable oocytes, cleaved embryos and blastocysts in 4 different luteal phases of the estrous cycle (D 2-3, D 4-7, D 8-12 and D 13-16).

Parameter	Groups				Total
	D 2-3	D 4-7	D 8-12	D 13-16	
Number of viable oocytes	5.83 ± 5.82[ae]	6.88 ± 2.76[ac]	3.94 ± 2.68[de]	3.83 ± 2.50[b]	5.12 ± 3.85
Number of cleaved embryos	4.00 ± 3.23	4.16 ± 3.32	2.72 ± 2.05	2.27 ± 1.93	3.29 ± 2.78
Number of blastocysts on day 7	1.44 ± 1.54[ac]	2.38 ± 2.32[ac]	0.61 ± 0.91[bd]	1.22 ± 1.76[cd]	1.41 ± 1.79

[a-e]The difference between groups in the same row ($P < 0.05$).

DISCUSSION

The objective of present study was to determine the optimal day of the estrous cycle for OPU application, without the use of synchronisation or stimulation prior to OPU. In studies without synchronisation/stimulation, it has been reported that twice-weekly OPU application yields a greater number and quality oocytes [14,17]. The reason for it is reported to be the lack of development of the dominant follicle, which causes atresia in follicles in the growth stage [15]. In other words, it is stated that a 7-day interval between OPU applications is due to the emergence of a dominant follicle that will have a negative effect on the oocytes in the follicles in the 2nd OPU application [4]. In another study, in which OPU was performed at regular intervals (i.e., at 7 and 14 days after the ablation of the dominant follicle), the number of grade I and II oocytes was greater in the group in which OPU was performed at 14 days. The reason for it is reported to be that more follicles of 3-8 mm in size were observed in the group in which OPU was performed at 14 days [11]. In cows, 2 or 3 follicular waves occur during estrous cycle. In a follicular wave, it is reported that recruitment begins between days 1 and 3 of the cycle, selection occurs between days 3 and 6, and the dominant phase occurs between days 6 and 8 [10]. The dominant follicle of the 1st follicular wave continues its dominant effect between days 8-11 of the cycle [16]. A follicle may be in 1 of 4 phases during the follicular wave: growth, dominance, static, or regression. Therefore, it has been reported that the stage of the follicle during OPU may affect oocyte number, quality, cleavage and blastocyst rate [8]. In present study, the greater number of oocytes with greater quality and viability rate in D 2-3 and D 4-7 groups is thought to be due to the absence of follicles in the dominant stage in these 2 groups. Based on the findings of present study, OPU application in D 2-3 and D 4-7 groups is estimated to be in the recruitment phase (days 2-3 of the cycle) and selection phase (days 4-7 of the cycle) of the 1st follicular wave. Similar to current study, Pieterse *et al*. [19] reported that when OPU was applied on days 3-4, 9-10 and 15-16 of the estrous cycle, significantly more antral follicles were observed on days 3-4 of

the cycle compared to the other groups. In the present study, there was no difference between D 2-3 and D 4-7 groups in terms of oocyte number, oocyte quality, number of viable oocytes and number of blastocysts. Similar to current study, Gimenes *et al*. [8] reported that there was no difference in the number of oocytes, number of live oocytes and number of blastocysts on the 1st, 3rd and 5th days of follicular wave emergence in OPU application in cattle.

In OPU/IVEP method, a greater number of oocytes per donor increases the rate of blastocysts and viable embryos and accordingly positively affects the post-transfer pregnancy rate [25]. In current study, the number of Grade A oocytes and viable oocytes were greater in D 2-3 and D 4-7 groups of the cycle compared to D 13-16 group of the cycle. The reason for this is that when OPU is used on days 13-16 of the cycle, different follicular groups can be found on the ovary. In the 1st days of the cycle after ovulation, there is a follicular cohort forming the 1st follicular wave. However, after the 8th day of the cycle, atresia follicles can be found in the ovary in the secondary or tertiary follicular wave. The OPU procedure, antral follicles with a diameter of 3-8 mm are aspirated from the ovary under ultrasound guidance. Furthermore, it is not clear whether the follicle detected by ultrasound is a newly developing follicle or an atresia follicle (subordinal follicle of the previous follicular wave) [10].

In a study in which OPU was performed on the 3rd and 7th day of the estrous cycle, despite no difference in the number of oocytes between the groups, a reduction in the number of blastocysts was observed on the 7th day [13]. The present study demonstrated that the number of viable oocytes and blastocysts was greater in the D 4-7 group compared to the D 8-12 group. This may be due to the greater number of atresia follicles (subordinal follicles) between days 8-12 of the cycle (compared to days 4-7) and the lower developmental competence of oocytes in the subordinal follicle. In addition, blastocyst yield may be variable for oocytes obtained from follicles of similar diameter [22]. Actually, it is associated with the phase of the follicular wave during the OPU application [8]. Oocytes obtained from atresia follicles are severely damaged, which has been demonstrated to reduce their developmental competence [9,14]. Feng *et al*. [7] reported that the blastocyst rate in early atresia follicles is similar to non-atresia follicles, but it is lower in late atresia follicles. This can be attributed to the loss of developmental competence of the oocyte [7]. In addition, in a different opinion, it is reported that OPU application on the 3rd day of the cycle during the growth phase of the follicles (before the oocyte loses its developmental competence) will be more effective in terms of blastocyst yield [13].

The most significant factors influencing the success of an *in vitro* embryo production process are the quality and viability of the oocyte [12]. In the present study, the number of Grade A oocytes was greater in the D 2-3 and D 4-7 groups than in the D 13-16 group, but there was no difference in the number of blastocysts. However, in parallel with the number of viable oocytes, the number ofblastocysts obtained in groups D 2-3 and D 4-7 was greater than in group D 8-12. Based on the result, it can be assumed that the viability rate is just as important as the quality of the oocyte in reaching the blastocyst stage.

CONCLUSION

In conclusion, the determination of the appropriate day of the cycle for OPU will rule out synchronisation or stimulation of the follicular wave and ablation of the dominant follicle. Therefore, the oocyte quality, number and most importantly the number of blastocysts can be increased by performing OPU between 2-3 or 4-7 days of the estrous cycle.

MANUFACTURERS

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Ethical approval. This study was approved by the Local Ethics Committee (Selcuk University Local Ethics Committee for Animal Experiments, Approval Number: 2024/01/024). The informed consent was obtained from the all owner for the use of cattle. All applied methods are in accordance with the ARRIVE guidelines.

Declaration of interest. The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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