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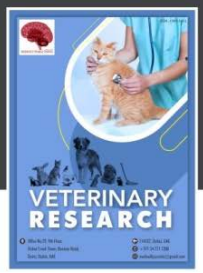

Evaluation of Blastocyst Rates of Immature Bovine Oocytes Collected by Ovum Pick up (OPU) and Slaughterhouse

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Abstract: This study was aimed to produce *in-vitro* embryos massively and at a lower cost. For this purpose, it was aimed to determine and compare the blastocyst growth rates of oocytes collected from slaughterhouse and by Ovum-Pick up (OPU) method. The animal material to be used in the OPU application of the study consisted of 10 Holstein breed heifers 15-25 months-old. A total of 51 times OPU was applied to the animals. In the postmortem study, 52 ovaries belonging to 26 Holstein cows obtained from local slaughterhouses were used. After the obtained oocytes were evaluated according to their quality, they were taken to maturation, fertilization and culture stages respectively. In the presented study, total of 359 oocytes were collected in 51 OPU applications. The cleavage rate was 85.07% and the rate of blastocyst growth was 24,63%. Collected from the slaughterhouse, 325 oocytes were obtained from 52 ovaries. The cleavage rate was determined as 94.19% and the rate of reaching blastocyst was determined as 25.73%. As a result, it was observed that there was no statistical difference in blastocyst development rates ($p>0.05$). In the production of *in vitro* embryos, it has been concluded that by obtaining post-slaughtering ovaries of females with high genetic value, the loss of genetic material can be reduced by producing their embryos at cheaper costs (cost-effective). It is suggested that *in-vitro* embryo production can be carried out massively and at a cheaper cost from slaughtered female animals and material with average or above genetic characteristics can be evaluated.

INTRODUCTION

Assisted reproductive technologies such as In Vitro Embryo Production (IVEP) have been widely used in recent years to ensure genetic advancement[1]. IVEP has increased significantly in the last decade. In the world, 72.68% (1.031.567) of the total embryo production (1.419.336) in cattle in 2019 was produced *in vitro*. 1.010.680 embryos were produced from OPU and 20.887 from slaughterhouse material[2].

Oocytes differ in their ability to support maturation and embryonic development. This ability is called developmental competence or oocyte quality. The capacity of an oocyte to maintain meiosis, maturation, successful fertilization and support normal embryo development is gradually gained as the oocyte grows[3, 4]. During the growth from the primordial follicle stage to ovulation, cytoplasmic organelles proliferate and protein and RNA reserves required for post-fertilization development are stored[5, 6]. Growing oocytes are affected by factors that regulate follicular growth and the surrounding granulosa cells and follicular fluid. Although, the spermatozoid is essential for successful fertilization and embryo development, most of the cellular and molecular mechanisms required for fertilization and early embryo development are inherent in the oocyte. Therefore, insufficient oocyte growth and maturation negatively affect fertilization and subsequent embryo development[7, 8].

In vitro embryo culture of bovine embryos is the last step in embryo production and involves about 6 days of culture time after the zygote is formed. Several important developmental events occur in the embryo, including fertilization, cleavage, activation of the embryonic genome, compaction of the morula and formation of the blastocyst[9].

OPU is a non-invasive and reproducible technique used to harvest large numbers of oocytes from the antral follicles of living animals. Oocyte retrieval and embryo production are affected by age, season and stimulation and an average of 1-3 embryos may develop from oocytes collected per session[10]. Repeated OPU can be performed in cattle without causing adverse effects on the donor[11,12]. This method not only improves the lifetime reproductive efficiency but can also be used for follicle ablation to initiate the follicular wave during the embryo transfer protocol. Follicular aspiration also allows examining the molecular complexity and role of various cytokines during folliculogenesis. One of the limitations of this technique is the low number of quality oocytes collected per ovary[12-14]. Another limitation is that for OPU/IVEP, technical experience and skills are required for the purpose of oocyte retrieval, maturation, fertilization and culture[15].

IVEP was initially tested for research purposes and was developed to produce embryos from oocytes obtained from slaughterhouse material. However currently, >70% of embryo production is currently produced this way. In cases where it is not necessary to know the origin of the embryo, a large number of embryos can be produced from oocytes collected from the ovaries of donors slaughtered at the slaughterhouse. In this case, oocytes collected from donors of the same breed can be processed together. Bulk processing of oocytes reduces labor and embryo production costs. This method is successfully applied in beef animals in countries such as Italy and Japan. Moreover, this mass production can also be used to produce embryos of dairy animals with average genetic characteristics for developing countries[16].

IVEP production trend in the world is shifting to OPU method.However, the ability to produce a new creature by taking only the ovaries of the slaughtered female animals is again that should not be ignored. Especially collecting the ovaries of animals with high genetic value and higher yield characteristics will be an important step in reducing the loss of genetic material and increasing the number of high-yielding animals. Based on this idea, the study was planned to contribute to the mass production of embryos produced *in vitro* at a lower cost. Thus, it was aimed to determine and compare the blastocyst growth rates of oocytes collected from slaughterhouse material and oocytes collected by Ovum-Pick Up (OPU) method.

MATERIALS AND METHODS

In the present study, 10 Holstein heifers aged 15-25 months constituted the animal material to be used in OPU application. A total of 51 times OPU was applied to the animals. OPU application was performed without stimulation and on random days of the estrus cycle (in terms of overlapping with the slaughterhouse material) every animal two weeks apart. Chemicals and IVF Bioscience kits were used in the study (OPU, WASH, BO-IVM, BO-SEMENPREP, BO-IVF-BO-IVC, OIL).

OPU applications: For OPU application, Esaote MyLab TwiceVet ultrasonography device and a combination of intravaginal OPU probe, catheter and aspiration device compatible with this device were used (Esaote 5001). Teflon tube and embryo collection tube (50 mL Falcon tube) were connected to the end and negative pressure was created for aspirating. For OPU, upper epidural anesthesia was applied to the animal (4-6 cc local anesthetic, Lidocaine, Vilcain®, Vilsan, Ankara). After cleaning the rectum, the perineal area was cleaned and disinfected. The ovary was held with the hand in the rectum. Special Convex vaginal probe (4.0-9.0 MHz,

combined with probe and catheter with 18 numbered needle at the tip) was inserted into the vagina and directed to the right fornix vagina for aspiration from the right ovary. Follicles of 3-10 mm diameter determined on the ovary were punched and aspirated. After the aspiration process was completed, the needle apparatus of the probe was removed and washed with OPU medium, allowing oocytes that might remain in the system to be taken into the medium. The same procedure was repeated for the other (left) ovary. Cumulus Oocyte Complexes (COC) in the aspirated follicular fluid were collected under a stereomicroscope.

Post-mortem study: In the postmortem study, 52 ovaries belonging to 26 Holstein cows obtained from local slaughterhouses were used. The ovaries were brought to the IVF laboratory within 2-3 hours in a lactated ringer containing antibiotics (Gentavet 10%, Vetaş, Istanbul) and a thermos providing a constant temperature of 30-35°C, immediately after slaughter. Oocyte Complexes (COC) were collected under a stereomicroscope from follicle fluids picked up by aspiration of follicles with a diameter of 3-10 mm in the ovary. OPU and slaughterhouse applications were carried out in parallel on the same days, 5 times (10 OPU, 10 ovaries) with an interval of two weeks.

IVF procedures: Oocytes classified according to the structure of the cumulus-oocyte complex (A-B quality cumulus oocyte complexes) were taken into the *in vitro* embryo production process. While evaluating the quality of the embryos, the order number and order of the cumulus cells on the oocyte were taken into account. After washing the oocytes in BO WASH solution, they were transferred to equilibrated maturation (IVM) medium for at least 4 hours and matured for 22 hours at 38,5-38,8°C (Hera Cell) in a mono gas incubator. After maturation, the semen in 2 dissolved straws were diluted with 2 mL of semenprep medium and centrifuged at 2000 rpm for 4 min. The process was repeated twice and the supernatants were removed. After the removal process, fertilization (IVF) medium was added to the pellet so that the final concentration would be 2×106/mL sperm cells, the homogeneous mixture was provided. Fifteen microliters of semen were added to drops (70 microliters) of this mixture prepared with IVF medium which was prepared overnight and holded in the incubator. Then mature oocytes were transferred to the drops. It was kept together in an incubator for 18 h for fertilization.

After the fertilization step was completed, oocytes were transferred to 100 microliter drops prepared from IVC medium gassed in a tri-gas incubator to remove them from the cumulus cells and left to culture in tri gas incubator (Hera Cell-6%O_2, 6% CO_2, % 88N, 38.5-38.8°C). Culture drops contained 3-8 oocytes from OPU or 15-20 oocytes from slaughterhouse. Blastocyst rates and embryo quality were evaluated on the 6th and 7th days in the culture medium (IVC).

Statistical analyses: In the statistical analysis of Maturation, Cleavage and Blastocyst rates, SPSS-Statistics-22 and ANSYS-Release-19 programs were used and the Chi-square test was applied.

RESULTS

In the presented study, it was observed that the maturation and blastocyte ratios of oocytes collected from OPU and slaughterhouse material were similar and the statistical difference was insignificant. Cleavaged rates were found to be statistically different ($p<0.05$) and there was more cleavage in oocytes collected from slaughterhouse material. The results was summarized in Table 1 and 2.

Table 1: Developmental data of collected oocytes from OPU and slaughterhouse

Parameters	OPU	Slaughterhouse
Number of animals/ovarium	51	52 (26 animals)
Total oocyte (n)	359	325
A quality oocyte	97	187
B quality oocyte	198	73
C-D quality oocyte	64	65
Average number of oocyte (per animal)	7.04	12.50
Left to maturation	295	260
Matured	268	241
Cleavaged	228	227
Blastosist	66	62
≤16 cells	157	157

Table 2: Maturation, cleavaged and blastocyst rates

Parameters	Maturation rates	Cleavaged rates	Blastocyst rates
OPU	90.85% (295/268)	85.07%[b] (228/268)	24.63% (66/268)
Slaughterhouse	92.69% (260/241)	94.19%[a] (227/241)	25.73% (62/241)

$p<0.05$ was considered statistically significant

DISCUSSION

Assisted Reproductive Technologies (ART) are used to overcome infertility in many living things including humans. Oocytes can be collected from females with high genetic value in farm animals before puberty, during pregnancy or post mortem. Techniques such as In Vitro Embryo Production (IVEP), embryo transfer and cryopreservation can increase the rate of genetic gain and protect endangered species[17]. In cattle, approximately 20-40% of oocytes collected and fertilized *in vitro* turn into transferable embryos and 50% of transferred embryos result in pregnancy[8, 13, 18, 19].

The cattle industry needs to more from artificial insemination that has been routinely used for the last 75 years to more advanced technologies that support genetic progress. Superovulation, synchronization of estrus, Ovum Pick Up (OPU) and Embryo Transfer (ET) technology is the primary means of achieving elite bovine embryos with rapid genetic advancement. OPU-IVEP and embryo transfer are methods that can be routinely used to improve genetic progress in the cattle industry[20,21]. IVEP in cattle is now an established and highly efficient procedure. Moreover, the importance of frequent oocyte retrieval (OPU) in combination with In Vitro Fertilization (IVF) in improving or increasing the yield of embryos from designated donors has been proven[22, 23].

The differences between the quality of oocytes obtained from slaughterhouse and OPU are reported with controversial results. It is stated that oocytes obtained from slaughterhouse material have a higher rate of blastocyst development than oocytes obtained with OPU. Post-mortem cumulus-oocyte complexes have been reported to reduce the bond with the follicle and consequently, the cumulus layers of oocytes obtained from slaughterhouse material are smoother[24]. In addition, quality oocytes bind more tightly to stratum granulosum. This may cause these high-quality oocytes to not be collected or to be evaluated as poor quality due to the disruption of the cumulus integrity while collecting[25]. 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 properties of recovered oocytes[26]. Additionally, the low number of oocytes obtained with OPU causes the use of poorer quality oocytes. While only A and B quality oocytes used in maturation obtained from slaughterhouse material. In this case, it was reported that oocytes obtained from slaughterhouse material will contribute to a better blastocyst rate[27]. In the study, it was determined that the quality of oocytes obtained from slaughterhouse material was better than those collected with OPU and the number of A quality oocytes was in the majority. It was concluded that this quality contributed to the increase of cleavaged rates, but could not sufficiently affect blastocyst formation. This is thought to be due to the possibility to aspirate atretic and early atretic follicles, unlike OPU while the follicles are aspirated in the slaughterhouse material. COCs collected from these follicles may appear high quality but may fail to development blastocyst. In OPU application, follicles that develop and have not started to atresia have aspired before the dominant follicle is selected by aspirating follicles with a diameter of 3-8 mm. This case may explain the similar rate of blastocyst despite different oocyte quality[28].

Some researchers report that they obtain higher rates of blastocysts in B quality oocytes compared to A quality oocytes, except for oocytes obtained from late atretic follicles[29]. They also reported that *in vitro* development was significantly better in C quality COCs (showing signs of early expansion in the outer cumulus layer and showing slightly granulated ooplasma) in oocytes that are morphologically classified as better (A and B quality) or worse (C quality). This supports the suggestion that early signs of atresia have a positive effect on oocyte developmental competence[30].

In a study conducted on Holstein and Angus breeds using the OPU method; The maturation rate in the Holstein breed was reported as 63.5%, the cleavage rate was 70,7% and the blastocyst development rate was 28.9%[31]. In another study that determined the blastocyst rate of oocytes collected by OPU, they reported that they obtained blastocyst at a rate of 34.76%[32]. In a study where OPU was applied for three years, have been reported that the cleavage rates were 48.7, 49.3, 52.9 and blastocyst rates were 39.3, 30.7, 25[33].

In a study in which oocytes collected from a slaughterhouse were cultured, it was reported that the maturation rate was 53%, the cleavage rate was 77% and the blastocyst rate was 26%[34]. In a study in which oocytes obtained from slaughterhouse material were tested with different culture additives, it was stated that the rate of blastocyst in the group added to Bovine Serum Albumin (BSA) was 26.7%, while it was 25.9% in the group with Synthetic Oviduct liquid (SOF)[35]. In a study comparing the addition of Intermedin / Adrenomedulin-2 (IMD/ADM21-47) to culture media with a control group In oocytes obtained from slaughterhouse material, blastocyst rates were reported to be 38 and 18%, respectively[36]. In the study where introstoplasmic sperm injection and normal IVF method of oocytes obtained from slaughterhouse material were tested, the cleavage rates were 60.6 and 59.6%, respectively; Blastocyst rates are reported to be 27.1 and 39.1%, respectively[37]. In the study comparing oocytes obtained with OPU with oocytes obtained from slaughterhouse, it was reported that the rate of reaching blastocyst in oocytes collected with OPU was 44.7%, while this rate was found to be 29.9% in oocytes obtained from slaughterhouse. In this study, the

researchers explain the high rate of blastocysts obtained with OPU with the possibility that the time elapsed until oocytes are collected in the slaughterhouse material and transferred to the maturation medium may have negatively affected oocyte development[27]. In another study, the cleavege and blastocyst ratios of oocytes obtained by OPU were; While it is 64.3 and 13.9, it is reported to be 74.0% and 30.8% in oocytes obtained from slaughterhouse material[38].

In the presented study, it was determined that the maturation rate was close to each other and around 90% in both applications and the result was statistically insignificant. It was thought that the maturation rates were significantly higher than other studies and the medium used gave successful results. When the cleavage rates were examined, it was determined that the difference was statistically significant in oocytes obtained from the slaughterhouse material. On the other hand, it was observed that a higher rate was obtained in cleavage rates compared to the results of existing studies and this was achieved with oocyte quality, sperm fertilization capacity and the suitability of the IVF medium. It was concluded that the usability of the medium and semen used in the fertilization phase was also good. It was observed that there was no statistical difference between the groups in the rates of blastocyst access and blastocysts were reached at similar rates with the studies. It is seen that there are very serious differences between studies in the rate of reaching blastocyst. It is not known exactly which internal and external factors affect positively or negatively in the process until the blastocyst stage is reached. In the presented study, it was thought that a result was obtained in the average of other studies and that the blastocyst rates remained at 25% may be due to the emergence of free oxygen radicals within 6-7 days in the IVC medium[39, 40]. In addition, it was thought that as the embryo develops, the increase in energy need and the change in energy source preference may be another reason[41].

Simple summary: In the presented study, it is aimed to compare the blastocyst growth rates from Ovum Pick Up (OPU) and oocytes obtained from slaughterhouse materials. Reasons such as the acceleration of the development of assisted reproduction techniques and the preservation and development of quality genetic material reveal the necessity of *in vitro* embryo production. Collecting the oocytes of cows with high quality genetic characteristics and producing their embryos for the last time will help prevent the current genetic loss.

CONCLUSION

Although, each ovary contains hundreds of thousands of oocytes at birth, most of them are lost due to atresia. This huge loss of genetic material can be reduced by collecting oocytes from ovaries and using In Vitro Production (IVEP) techniques. Considering that an average of one-fourth of the oocytes obtained from the collected ovaries can be produced, an animal will continue to contribute to the economy and transfer its genetic heritage even when its life ends. Considering the number of female animals slaughtered per year, obtaining an embryo from even half of these animals is a value that should not be wasted on behalf of animal husbandry.

In a simple economic calculation to be made, especially in terms of slaughtered animals; 62 embryos were obtained from 26 animals. This corresponds to about 2.4 embryos per animal. Considering that 3 calves are taken from each of these 26 animals, embryos close to the number of offspring taken from the animal during its life can be taken when slaughtered. If 50% pregnancy is achieved after the transfer of these embryos, it means that a young can be obtained from each animal close to its genetic characteristics after post-mortem. It is a method that can be used in the advancement of genetics in countries with low number of high-yielding animals in animal husbandry. By means of a notification condition that can be brought to the slaughter of high-yielding animals, genetic progress will be accelerated by transferring the embryos obtained from these animals to low-yielding animals.

In an application where external factors such as time, temperature, humidity, medium, semen and work team were completely equal, it was observed that the embryo production rates from oocytes obtained from OPU and slaughterhouse material were similar. As important it is to make OPU from high-genetically high females, *in-vitro* embryo production can be carried out massively and at a cheaper cost from slaughtered female animals. So that material with average or above genetic characteristics can be evaluated in the economy.

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