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Different Harvesting Techniques Used in Ovine in vitro Embryo Production

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Background: To current knowledge, different oocytes recovery methods have high impact on in vitro embryo production (IVEP).
Objectives: The aim of this study was to define an efficient recovery method for oocytes harvesting from slaughterhouse material, and its effect on IVEP yield.
Materials and Methods: Ovaries were obtained from a local slaughterhouse and transported to laboratory in an insulate container physiological saline solution at 37°C. Oocytes were recovered via an aspiration pump (set at a flow rate of 10 mL H2O / min) with a disposable 20 G needle attached, oocyte recovery with centrifugation (ORC procedure), puncture and slicing. Oocytes were categorized into three classes (class I; oocytes with more than 3 layers of cumulus cell, class II less than 3 layers with damaged cumulus cells, and class III; denuded oocytes) and cultured in tissue culture medium (TCM) 199 medium for a period of 24 h in separate groups. Subsequently matured oocytes were subjected to in vitro fertilization (IVF) and afterwards, presumptive zygotes proceed to subsequent in vitro culture (IVC) for 6 days to complete blastocysts development.
Results: The present study showed that the oocyte recovery rate using ORC was significantly higher ($P < 0.05$) compared to other recovery methods. While no significant differences in the proportion of oocytes reaching M-II stage were recorded when using different oocyte recovery methods. Using aspiration for oocyte recovery led to lower proportion of class-I oocytes (60.0 ± 2.1) compared to ORC (82.0 ± 1.2), slicing (80.0 ± 2.1) and puncture (80.0 ± 1.5) methods.
Conclusions: The results revealed that oocytes harvesting techniques are effective in the rate of cleavage and blastocysts development, and despite same meiotic resumption rate in all treatments, it would be better to use ORC for oocytes harvesting.

Keywords: In Vitro Oocyte Maturation Techniques; in Vitro Fertilization; Embryo Culture Techniques

1. Background

In ovine, only a limited number of mature oocytes can be collected via superovulation and subsequent ovum pick up (OPU) procedure. Ovaries obtained from slaughtered animals are the most abundant and inexpensive sources of oocytes for in vitro embryo production (IVEP) (1). Therefore, using slaughterhouse material has been considered as an invaluable source for IVEP purposes. Several factors affecting the IVEP and the optimization of in vitro techniques could improve the IVEP efficiency (2). Higher percentages of blastocysts are obtained via in vivo maturation of oocytes compared to in vitro maturation (IVM). These kinds of evidences suggest that in vitro milieu is not sufficient for supporting development and early embryo competence (3, 4). Thus the in vitro embryo production yield may increase by optimizing the IVM system (4).

One of the most important factors that can influence a successful IVEP is the efficient recovery of the cumulus-oocyte complexes (COC's) (5). A large number of good quality oocytes are needed for successful embryo production, as approximately only 30% of oocytes which are recovered may develop into blastocysts (6). Aspiration, slicing and puncture of whole ovarian surface have served as conventional method for oocytes harvesting. Each has its advantages and disadvantages (7). In this study a new oocytes harvesting technique oocyte recovery with centrifugation (ORC) that has recently been established (8) along with other conventional harvesting methods was used for oocytes recovery. In the production of IVM-IVF embryos the yield of oocytes recovered per ovary and the oocytes competence are of important considerations (9).

2. Objectives

This study was aimed to assess the possible influence

Implication for health policy/practice/research/medical education:
To current knowledge oocyte recovery method has a profound influence on embryo production. Therefore the aim of this study was to evaluate a new oocyte recovery method which has introduced by our laboratory recently.

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of oocyte harvesting technique on oocytes competence and subsequent IVF and in vitro culture (IVC) efficiency, using sheep as a model.

3. Materials and Methods

All media used for IVM and IVF were incubated at 38.5-39°C and 5% CO_2 with maximum humidity for 2h prior to use. Ovine ovaries (n = 1635) were collected from animals with unknown breeds and ages from a local abattoir in Karaj, Iran (Coordinates: 35 ° 43' 40" N – 51 ° 1' 7" E). Ovaries were placed in an insulated container with physiological saline (0.9% NaCl) and penicillin/streptomycin (50 μg / mL), at 37 °C. The ovaries were transported to laboratory within an hour. All the superfluous tissue and bursa were removed from ovaries surface. Each ovary was subjected to three washings in Dulbecco's phosphate buffered saline (DPBS) and two washings in oocyte harvesting medium at 37 °C (DPBS + 4 mg / mL, BSA + 50 IU / mL penicillin), prior to processing (10). All chemicals were purchased from sigma, unless otherwise indicated.

3.1. Oocytes Recovery

Each ovary was handled individually and oocytes were recovered by one of the following methods:

1. Ovarian puncture: in this technique an 18 gauge hypodermic needle was used to puncture the ovarian surface.

2. Aspiration technique: oocytes were recovered using an aspiration pump which was set at the flow rate of 10 mL H2O / min (the 20 G needle attached to the apparatus).

3. Slicing: the whole ovarian surface was incised by a sterile scalpel blade.

4. Oocyte recovery via centrifugation "ORC" as described by Dadashpour Davachi et al. (8).

During oocytes recovery via puncture and slicing techniques, the ovary was kept completely submerged in medium in a 35 mm petridish. When harvesting the oocytes by aspiration only the visible follicles were aspirated (7). Prior to applying ORC technique, modified Falcon tubes were prepared as described by Dadashpour Davachi (8).

3.2. In Vitro Maturation

Groups of 10 COC's were added to 50-pl droplets of maturation medium consisting of tissue culture medium (TCM)-199 with Earle's salts and bicarbonate supplemented with 10% heattreted FCS, 0.5 pg/mL FSH, 5ug/mL gentamicin. The droplets, covered with mineral oil, were preincubated under maturation conditions for a minimum of 2 h (38.5°C, 5% CO_2, and 95% air atmosphere with maximum humidity) (11).

3.3. Preparation of Sperm and in Vitro Fertilization

In vitro fertilization was performed based on the method published by Ushijima et al. (12). Frozen-thawed spermatozoa (Lori Bakhtiari breed) were diluted in TCM 199 with Earle's salts (Gibco, Grand Island, NY, USA) the pH was adjusted to 7.8 and then centrifuged at 200 × g for 2 min. Subsequently, the supernatant was removed and the pellet containing the sperm was preincubated for 15 min at 38.5 °C prior to fertilization. Drop of fertilization medium (90 - μl) which contained in vitro matured COCs (n = 10 - 20) was mixed with 10 μ l of preincubated spermatozoa. The final concentration of spermatozoa in the fertilization droplets was 1×10^6. The fertilization medium included 12 mM KCL, 25 mM $NaHCO_3$, 90 mM NaCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 10 mM sodium lactate, 3 mg / ml bovine serum albumin (BSA; fatty acid free, Sigma), 50 μg / ml gentamicin and 5 mM caffeine (Sigma). Following a period of 12 h coincubation of oocytes with spermatozoa, by performing mechanical forces (gentle pipetting) the cumulus investment and the attached spermatozoa were dissociated from the inseminated oocytes, and then these fertilized oocytes were transferred to culture medium. Presumptive zygotes were cultured in NCSU - 37 supplemented with 4 mg / ml BSA, 0.17 mM sodium pyruvate, 2.73 mM sodium lactate and 50 μg / ml gentamicin. Seventy two h after insemination, all cleaved embryos were transferred into fresh culture medium: NCSU-37 supplemented with 4 mg / ml BSA, 5.55 mM Dglucose, and 50 μg / ml gentamicin. The cleaved embryos were cultured for 7 days to evaluate their ability to develop into morula and blastocyst stages. Data was expressed as Mean ± SEM. After in vitro fertilization (IVF) the proportion of oocytes reaching each stage of meiosis, oocytes fertilized and embryos were subjected to arc-sin transformation before the analysis of variance (ANOVA). The transformed data was analyzed, using ANOVA followed by a post hoc, Fisher's protected least significant difference test (PLSD test) using the Statview software (Abacus Concepts, Inc., Berkeley, CA). A P value ≤ 0.05 was considered significant.

4. Results

Effects of different oocytes harvesting methods on oocytes recovery rate and oocytes quality are set out in Table 1. The oocyte recovery rate using the ORC technique and slicing were significantly ($P < 0.05$) higher than the oocyte recovery rates via the puncture and aspiration (Table 1).

The proportion of fertilized oocytes exhibited monospermic was significantly lower for those oocytes recovered via aspiration (48.2 ± 1.6). Polyspermic was significantly higher ($P < 0.05$) following IVF for those oocytes recovered using aspiration (18.4 ± 1.7) compared to the other methods (Table 2).

Also the results of this experiment showed that the cleavage and the blastocysts formation rate were significantly ($P < 0.05$) affected by harvesting techniques (Table 3).

Table 1. The (Mean ± SEM) Recovered Ovine Oocytes per Ovary per Class

Recovery Technique	Ovary No.	Recovered Oocytes of Each Class, Mean ± SEM[a]		
		I	II	III
ORC	100	82.0 ± 1.2[b]	9.1 ± 1.4[b]	9.0 ± 1.6[b]
Slicing	100	80.0 ± 2.1[b]	11.2 ± 2.0[b]	8.8 ± 1.7[b]
Puncture	100	80.0 ± 1.5[b]	12.0 ± 1.8[b]	8.2 ± 1.5[b]
Aspiration	100	60.0 ± 2.1[c]	15.2 ± 1.7[b]	25.0 ± 1.2[c]

[a] Different superscripts ([b, c]) in the same column differ significantly ($P < 0.05$)

Table 2. The (Mean ± SEM) Monospermic and Polyspermic Rate

	Oocytes Fertilized		
Recovery Technique	**Oocytes Examined, No.**	**Monospermic, Mean ± SEM[a]**	**Polyspermic, Mean ± SEM[a]**
ORC	100	58.4 ± 1.1[b]	11.6 ± 2.1[b]
Slicing	104	57.6 ± 1.0[b]	11.4 ± 1.5[b]
Puncture	100	58.3 ± 1.3[b]	12.3 + 2.1[b]
Aspiration	110	48.2 ± 1.6[c]	18.4 ± 1.7[c]

[a] Different superscripts ([b, c]) in the same row differ significantly ($P < 0.05$)

Table 3. The (Mean ± SEM) Cleaved Oocytes Rate and Those Reaching the Blastocyst Stage

	Oocytes Fertilized		
Recovery Technique	**No. of Zygotes Examined**	**Cleaved, Mean ± SEM[a]**	**Developed to Blastocyst, Mean ± SEM[a]**
ORC	320	72.4 ± 1.6[b]	29.2 ± 1.1[b]
Slicing	310	61.3 ± 1.2[c]	20.1 ± 1.3[c]
Puncture	310	62.0 ± 1.2[c]	19.1 + 1.8[c]
Aspiration	315	51.4 ± 1.3[d]	17.8 ± 1.5[d]

[a] Different superscripts ([b, c, d]) in the same row differ significantly ($P < 0.05$)

5. Discussion

The results demonstrated that the oocyte recovery technique has a great influence on the oocyte recovery rates, cleavage and blastocysts development. By using the aspiration technique and puncture for oocyte recovery from slaughterhouse material, only the visible follicles were used for oocyte retrieving. It seemed that via the ORC and slicing technique, the follicles embedded deep within the cortex can be used in these oocyte retrieving procedures (8). Additionally, it can be concluded from the results obtained via the ORC and slicing techniques that the centrifugation force applied in ORC procedure was the only advantages of this new method when compared with slicing technique. Thus the higher oocyte recovery rate using the ORC technique can be explained. In the present study the proportion of oocytes recovered via the ORC technique (9.8 ± 0.7), slicing (8.6 ± 0.9), puncture (5.8 ± 1.0) and aspiration (3.3 ± 1.1), were significantly higher when compared to the study in which the follicular oocytes were recovered from goat ovaries using three different methods (aspiration, puncture and slicing) (13). In a study conducted by Shirazi (14) the percentage of recovered oocytes was highest when using the slicing technique, compared to aspiration (52% vs. 22%). A study conducted by Morton et al. (10). showed that the recovery of oocytes per ovary considered to be viable for the IVM procedure were 3.3 ± 0.5, 3.1 ± 0.3, 3.3 ± 03 and 3.0 ± 0.4 respectively, for the syringe and aspiration technique set at the flow rates of 25, 50 and 100 mmHg. The oocyte recovery rates were 9.5 ± 0.4, 9.5 ± 0.4 and 6.8 ± 0.3 respectively, for three different oocyte harvesting methods (puncturing, slicing and aspiration in sheep) (7). Based on the results of Dadashpour Davachi et al. (8), the proportion of oocyte recovery rate per ovary were 6.8 ± 0.3, 2.3 ± 0.4, 2.3 ± 0.2 and 3.8 ± 0.1 respectively, for ORC and aspiration (set a flow rates of 10, 15 and 20mL / min, needle). The results of the present study recorded no significant differences in the percentage of those oocytes reaching metaphase II stage (76.7% ≤ MII ≤ 79.3%). Arav (15) had previously established a new oocytes recovery method in cattle called Transillumination- Aspiration Ovary (TAO), the results showed that by using TAO the oocyte recovery rates were significantly improved (7.3 oocytes/ovary), while there

were no decreases reported in the rate of IVM (81%). The results of a study conducted by Kochhar et al. (16) reported an 85.7% maturation rate for ovine oocytes with ≥ 3 layers of cumulus cells. Previously, Dadashpour Davachi et al. (5), also reported 77.0 ± 2.7, 77.2 ± 1.9, 53.0 ± 2.1 and 2.2 ± 1.1 maturation rates in ovine oocytes respectively for COC's with ≥ 5, 3 - 4, 1 - 2 and no cumulus cell layers. In the present study, the recovery of class I oocytes using the ORC, slicing and puncture were 82%, 80% and 80% respectively. Based on the results of Morton et al. (10). The recovery of oocytes class I using aspiration technique was 86%. However, Shirazi et al. (14), reported a low recovery rate of 52% and 22% respectively using slicing and aspiration methods. Rodriguez et al. (17), recorded a low recovery rate of approximately 60%, for post mortem ovine oocyte retrieval. In a study conducted by Pawashe et al. (13), aspiration, puncturing and slicing were evaluated in goats for oocyte recovery. The results recorded from those mentioned techniques indicated significantly ($P < 0.05$) the higher number of good quality and usable oocytes enclosed with compact cumulus cells were obtained per ovary following the slicing, compared to aspiration or puncturing. Dadashpour Davachi et al. (8) reported a 77% recovery rate for oocytes class I + II + III using ORC and 90% when using aspiration technique (set at flow rate of 10 mL / min H2O). In this study, results showed that oocytes collected via ORC were more competent for IVF. Moreover, the results of the present study indicated that oocytes obtained via ORC showed higher percentage of monospermic fertilization. In a study conducted by Shabankareh and Zandi et al (18), results showed that cleavage and blastocysts rates were 73% and 20% respectively. The results of the present study, when using ORC for oocyte harvesting was significantly higher compared to blastocysts rate reported by Shabankareh (18). Various earlier studies have shown that the rate of blastocysts development was between 22-27% (19-22). The results of a study conducted by Arav (15) revealed that using TAO lead to 52% cleavage and 26% blastocysts stage. Results of a study performed by Morton et al. (10) using two different oocytes harvesting method, aspiration technique (set at 25, 50 and 100mm flow rates) and syringe, indicated that the proportion of cleaved oocytes were 31%, 50%, 47% and 44% respectively and the percentage of blastocysts formation (%cleaved oocytes) were 19.5%, 54%, 44% and 58% respectively. Oocyte competence in ewes was affected by oocyte harvesting technique and it appeared that the harvesting technique would improve developmental competence. The possible explanation for these findings may be related to the critical role of cumulus cells in oocytes competence. Previously researches have indicated that there are several metabolites and growth factors which produced in cumulus cells. These micro and macro molecules have great impact on the oocytes competence and subsequent embryonic developmental potential. Oocyte harvesting technique would be considered as a first external stressor which oocytes are faced with. Aspiration technique may influence the gap-junctional communication between oocytes and their cumulus investments. Therefore, many useful metabolites for the oocyte and embryo competence which only provided by the cumulus investments cannot reach the oocytes. This may lead to some deterioration in the fertility rate or even in the subsequent embryonic development. While we can proposed that other techniques especially ORC have not influenced the gap-junctions in COC's. We suggest more studies to investigate the integrity of these gap-junctions by applying Lucifer yellow which can pass through these junctions. It may provide a great indication regarding the gap-junctions integrity.

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Authors' Contribution

All authors have contributed to this work equally.

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