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In vitro embryo production from ewes at different physiological stages

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ABSTRACT

Background: The collection of ovaries from slaughterhouses is an important source of oocytes for *in vitro* embryo production. On the other hand, the physiological stage of slaughtered females varies and influences embryo production.
Objectives: The study examined the *in vitro* efficiency of embryos and demi-embryos from young, non-pregnant adult, and pregnant adult ewes from a local slaughterhouse.
Methods: One thousand three hundred ovaries were collected from August to October 2020. The recovered oocytes were matured, fertilized, and cultured at 5% CO_2, 38.5°C, and 100% humidity. Embryo bisection was performed in 96 blastocysts (n = 32 per treatment). The demi-embryo pairs were incubated for their reconstitution for 12 h. SAS was used for data analysis.
Results: The number of oocytes collected from the experimental group of non-pregnant adult ewes was higher ($p \leq 0.007$) than those collected from the group of pregnant adult ewes (2.67 ± 0.19 vs. 2.18 ± 0.15 oocytes/group, respectively). The blastocyst rate was higher ($p \leq 0.0001$) in the non-pregnant adult group (36.39%) than in the young (17.96%). The ratio of demi-embryos that recovered the blastocoelic cavity was higher ($p < 0.05$) in the young group (81.25%) than in the pregnant adult group (59.38%). The diameter of the demi-embryos was higher ($p < 0.05$) in the non-pregnant adult group (186.54 ± 8.70 μm) than those in the young and pregnant adult groups.
Conclusions: In conclusion, the *in vitro* embryo production efficiency was highest when using oocytes from non-pregnant adult ewes under the conditions of this study.

Keywords: Sheep; *in vitro* fertilization; embryonic development

INTRODUCTION

In vitro embryo production (IVEP) has been implemented in genetic improvement programs and basic research on animal reproduction. Blastocysts can be split to increase the number of viable demi-embryos for transfer to recipient females and increase the number of offspring [1]. On the other hand, the efficiency of embryo bisection is affected by the quality and origin of the whole embryo [2]. Cumulus-oocyte complexes (COCs) are necessary for IVEP and conducting reproductive biotechnology studies [3]. The collection of ovaries from slaughterhouses is a common and inexpensive practice for producing embryos [4], but the physiological stage of the female oocyte donor affects IVEP [5,6]. This heterogeneity of

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Conflict of Interest

The authors declare no conflicts of interest.

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slaughtered females is usually considerable and influences *in vitro* blastocyst rate (20–50%) [7,8], possibly due to structural differences related to oocyte competence [5,6,9] and the susceptibility to *in vitro* manipulation [10]. Oocyte quality is one of the crucial factors affecting IVEP programs. Several studies have reported that the low quality of prepubertal oocytes (28–42 days old) compared to adult oocytes (3–6 years old) could be due to a deficiency in cytoplasmic and nuclear maturation, which influences embryo development [5,11,12]. On the other hand, mitochondrial activity decreases with age, and some important processes are affected, such as calcium signaling, reactive oxygen species homeostasis [13], ATP production, and organelle development [5], as well as the follicular environment in which oocytes are found [14,15]. In addition, oocyte recovery from pregnant females is another alternative of interest to produce *in vitro* bovine embryos of economic importance [16]. In some countries, the diagnosis of pregnancy in bovines is not a common practice. Consequently, some pregnant females are slaughtered at the first weeks of gestation [17]; something similar could happen in ewes. This opens the opportunity to evaluate the viability of oocytes obtained from pregnant ewes. To the best of the authors' knowledge, the information on oocyte recovery from pregnant and young ewes (4–8 months old) is scarce. Therefore, this study evaluated the *in vitro* efficiency of embryos and demi-embryos from young females and non-pregnant adult and pregnant adult ewes obtained from a slaughterhouse.

MATERIALS AND METHODS

Ethics statement

The ovaries were collected from a slaughterhouse based on the Official Mexican Standard NOM-033-ZOO-1995, Humanitarian Slaughter of Domestic and Wild Animals [18], from August to October 2020 during the sheep reproductive season. The entire experiment was conducted according to the guidelines of the Ethical Committee (No. CECBS1913) of Universidad Autónoma Metropolitana, Mexico.

Ovary collection

Ovaries were collected from young females (4–8 months old), non-pregnant adults (>2 years old), and pregnant adult ewes (> 2 years old and < 3 months of gestation) of different genotypes from a local slaughterhouse. The slaughterhouse operator provided the individual histories of the ewes. The information of age and pregnancy status was confirmed by the morphological mandibular teeth and the presence and size of the fetus. The ovaries were transported to the laboratory in a physiological saline solution (0.9% NaCl) supplemented with 50 μg gentamicin sulfate at 30–35ºC within three hours of collection.

COCs recovery and *in vitro* maturation

The COCs recovered per ovary were determined after follicular puncture and split into eight replicates. The COCs were retrieved by aspiration of follicles with diameters of 2–6 mm, using an 18-gauge needle (18 × 38 mm) and a hypodermic syringe with TCM-199 medium containing HEPES, 50 μg mL^{-1} gentamicin sulfate, and 50 IU heparin mL^{-1} [19]. The COCs were evaluated with a stereoscopic microscope (Leica S8APO; Leica Microsystems, Germany). Only COCs surrounded by at least five granulosa cell layers and homogeneous cytoplasm were selected for maturation and subsequent development [20,21]. The COCs were washed and cultured for 24 h in groups of 30–40 per well (Nunc, Roskilde, Denmark) with 500 μL TCM-199 medium, supplemented with 10% (v/v) SFB (Biowest; Mayimex, Mexico), 5 μg mL^{-1} FSH (Folltropin-V; Bioniche, Canada), 5 IU mL^{-1} hCG (Chorulon; Merck Shar, USA), and 1 μg mL^{-1} 17-β estradiol

(Sigma Aldrich, Mexico) [22] plus 200 µL mineral oil (Sigma Aldrich). The culture conditions for the entire experiment were 38.5°C, 5% CO_2, and humidity at saturation [22].

Sperm preparation and *in vitro* fertilization

Fertilization was conducted using the fresh semen from a Rideau Arcott ram of known fertility with an artificial vagina. The semen was maintained at 22°C for 90 min [23], then washed in a commercial fertilization medium (Modified Tyrode; In vitro SA, Mexico) and centrifuged at 225 ×g for 3 min. Capacitation and spermatozoa selection were performed using the Swim-up technique [24]. Briefly, 100 µL of centrifuged semen were layered gently under 1 mL of fertilization media. The tube was slanted and incubated for 5 min at 38.5ºC and 5% CO_2. The swim-up sperm fraction was used for *in vitro* fertilization. After maturation, the COCs were washed and placed in wells containing 500 µL of fertilization medium plus 200 µL of mineral oil. The COCs were fertilized with 1×10^6 spermatozoa mL^{-1} and cultured for 22 h [19].

In vitro embryo culture

The presumptive zygotes were washed and denuded using a narrow bore pipette. Groups of 30–40 zygotes were transferred to wells with 500 µL of Cleavage medium (Cook IVF, Australia) plus 200 µL of mineral oil and left for 72 h. Subsequently, the number of embryos at the morula stage was determined. The morulae were washed and transferred to wells with 500 µL of Blastocyst medium (Cook IVF) plus 200 µL of mineral oil [7] and stored for 96 h more to reach the blastocyst stage (168 h total). The number of blastocysts was determined at 168 h, considering the start of culture in the cleavage medium as time zero. The embryos were evaluated using an inverted microscope (Nikon Eclipse TS100; Nikon, Japan) according to the morphological criteria established by Ushijima et al. [25].

Bisection of blastocysts and demi-embryo survival

Embryo bisection was performed in 96 (n = 32 per treatment) excellent quality expanded blastocysts selected at random. Only blastocysts with clear visual differentiation between the inner cell mass (ICM) and trophectoderm, dark and compact ICM, without visible defects in the trophectoderm, and intact zona pellucida were selected for the bisection process [25]. The bisection procedure was performed as indicated by Hashiyada [2]. Briefly, each embryo was placed in a 50 µL drop of biopsy medium (IVF Bioscience, England) and oriented symmetrically. Vertical pressure with a microblade was applied to the zona pellucida until a proportional cut was made. Immediately, pairs of demi-embryos were placed in 50 µL drops of Blastocyst medium (Cook IVF) and cultured for 12 h. The demi-embryo survival was evaluated based on the morphological criteria [25].

Blastocyst diameter was measured before embryo bisection, and after *in vitro* culture, the average diameter of the demi-embryo surviving was determined. The measurements were carried out with a digital camera (AmScope, United States) adapted to the inverted microscope (×200). The mean diameter (µm) of two perpendicular lines through the embryo was recorded, and in the case of whole blastocysts, the zona pellucida was included [26].

Statistical analysis

The treatments were the different physiological stages of the ewe from the slaughterhouse, T_1 = young, T_2 = non-pregnant adult, and T_3 = pregnant adult. The data were analyzed according to Gbur et al. [27] with the SAS software [28]. The number of oocytes obtained per ovary was analyzed with the GLIMMIX procedure, assuming a Poisson distribution and the Logit link function. The number of morulae and blastocysts was analyzed with the GLIMMIX

procedure, assuming a Binomial distribution and the Logit link function. The number of reconstituted embryos was analyzed using the GENMOD procedure. A Binomial distribution and the Logit link function were assumed. Finally, the diameter of the demi-embryos was modeled, including the original embryo diameter as a covariate, with the MIXED procedure considering the embryo as an experimental unit. The least-squares means ± standard error (LSM ± SE) are reported throughout the document. The statistical model used is as follows:

$$y_{ij} = \mu + T_i + \beta_1 (x_{ij} - \bar{X}) + \varepsilon_{ij}$$

where:

y_{ij} = diameter of the demi-embryo reconstituted in the jth replicate of the ith physiological stage of the ewe; μ = general mean; T_i = fixed effect of the ith physiological stage of the slaughtered females; i= young, non-pregnant adult, and pregnant adult ewes; β_1 = regression coefficient for the covariate diameter of the original embryo before bisection; ε_{ij} = random error associated with the measurement of the jth diameter of the demi-embryo in the ith physiological stage of the ewe, which $\varepsilon_{ij} \sim NIID(0, \sigma^2_e)$ was assumed. The ESTIMATE option was used for all variables to test for treatment differences. Data with $p < 0.05$ were considered significant.

RESULTS

COCs recovery

Table 1 lists the average COCs recovered from ewe ovaries from the slaughterhouse. In general, the number of COCs ranged from 2.18 to 2.41 per ovary. The COCs recovered were similar in young ewes and non-pregnant adults ($p < 0.05$). In the group of pregnant adult ewes, however, the COCs recovery rate was higher ($p \leq 0.007$) than that of the group of non-pregnant adult ewes.

IVEP

In total, 3,185 COCs were matured, of which 76.5% reached the morula stage, and 21.3% developed into the blastocyst stage. No significant differences ($p > 0.05$) were observed between the groups for morula rate (**Table 2**). However, the blastocyst rate was 17% higher ($p \leq 0.0001$) in the group of non-pregnant adult ewes than in the other two groups.

Table 1. Recovery of COCs from ovaries of slaughtered ewes at different physiological stages

Physiological stage of ewe	No. of ovaries	COCs recovered	COCs per ovary (LSM ± SE)[a]
Young	870	2,009	2.41 ± 0.13[b,c]
Non-pregnant adult	163	421	2.67 ± 0.19[b]
Pregnant adult	267	586	2.18 ± 0.15[c]

COC, cumulus-oocyte complex; LSM, least-squares means.
[a]LSM ± SE in the same column with different letters are statistically different ($p < 0.05$).

Table 2. Production of morula and blastocysts *in vitro* from oocytes recovered from ovaries at different physiological stages

Physiological stage of ewe	COCs recovered	Morula rate (LSM ± SE, %)[a]	Blastocyst rate (LSM ± SE, %)[a]
Young	2,009	1,474 (74.36 ± 9.1)	354 (17.96 ± 1.3)[c]
Non-pregnant adult	590	455 (78.25 ± 4.4)	219 (36.39 ± 3.5)[b]
Pregnant adult	586	421 (77.04 ± 4.8)	105 (18.35 ± 2.5)[c]

COC, cumulus-oocyte complex; LSM, least-squares means.
[a]LSM ± SE, % in the same column with different letters are statistically different ($p < 0.05$).

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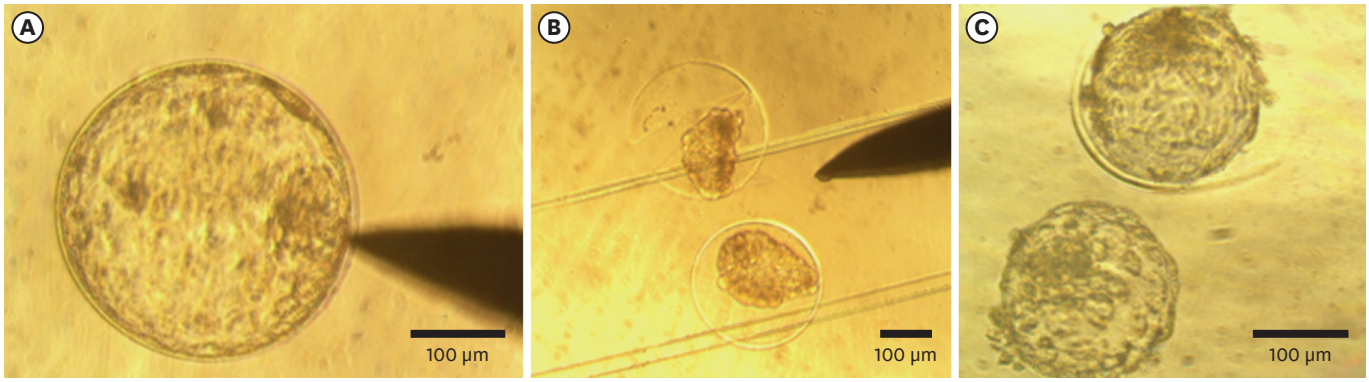

Fig. 1. Embryo bisection process and demi-embryo survival after 12 h of *in vitro* culture. (A) Expanded blastocyst, (B) pair of demi-embryos, and (C) reconstituted demi-embryos (×200).

Table 3. Survival of demi-embryos obtained from blastocysts produced *in vitro* from oocytes recovered from slaughtered ewes at different physiological stages

Physiological stage of ewe	Original blastocyst	Demi-embryos	Reconstituted demi-embryos	Demi-embryo rate (LSM ± SE, %)[a]
Young	32	64	52	81.25 ± 4.87[b]
Non-pregnant adult	32	64	44	68.75 ± 5.79[b,c]
Pregnant adult	32	64	38	59.38 ± 6.13[c]

LSM, least-squares means.
[a]LSM ± SE, % in the same column with different letters are statistically different ($p < 0.05$).

Demi-embryo survival

Demi-embryo survival after 12 h of culture (**Fig. 1**) in all groups was higher ($p < 0.05$) than the original number of bisected blastocysts (139.5%, 134/96). The percentage of demi-embryos produced immediately after bisection (64 halves) ranged from 59.4 to 81.3%. No significant difference ($p > 0.05$) was observed between young and non-pregnant adult ewes (**Table 3**). On the other hand, the demi-embryo rate was 22% higher ($p <0.05$) in young ewes than in pregnant adult ewes.

Diameter of demi-embryos

The diameter of the bisected embryos was considered as a covariate for each physiological stage, young (226.25 ± 6.51), non-pregnant adult (235.00 ± 6.51), and pregnant adult ewes (212.85 ± 6.51), but it was not statistically significant ($p \geq 0.20$). The average diameter of the reconstituted demi-embryos after 12 h of culture in non-pregnant adults was higher ($p < 0.05$) than in young and pregnant adult ewes (186.54 ± 8.70 vs. 165.50 ± 8.03 and 158.98 ± 9.39 μm, respectively) (**Fig. 2**). On the other hand, the difference between young and pregnant adult ewes (165.50 ± 8.03 vs. 158.98 ± 9.39 μm) was non-significant ($p > 0.05$). In general, the average diameter for all groups was 75.5% (170/225 μm) of the average diameter of the original blastocyst.

DISCUSSION

The collection of ovaries from slaughterhouses is one of the main sources of COCs for IVEP [29]. To the best of the authors' knowledge, the information about the efficiency of oocytes from pregnant (< 3 months of gestation) and young ewes (4–8 months old) on *in vitro* embryo and demi-embryo production is limited. One thousand three hundred ovaries were collected from ewes at different physiological stages to evaluate their competence in IVEP. In total, 3,016 COCs were recovered, with a general average of 2.3 COCs per ovary, which was higher

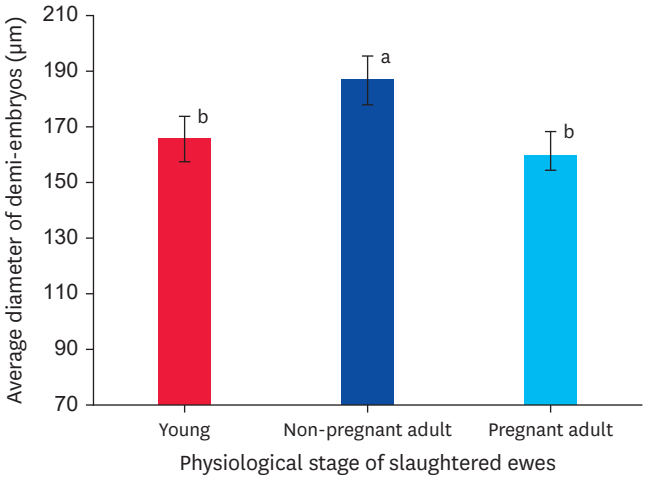

Fig. 2. Mean diameter of demi-embryos (after 12 h of culture) produced from slaughtered ewes at different physiological stages. Each bar represents the least-squares mean ± standard error. Different superscript letters (a and b) between columns represent the significant differences ($p < 0.05$).

than the 3.7 COCs per ewe (two ovaries) reported by Davachi et al. [29]. In the present study, a higher number of COCs per ovary was collected in non-pregnant ewes compared to pregnant ones. Furthermore, the number of COCs was similar in young and non-pregnant adult ewes. Similar results were reported in multiparous ewes by ovarian puncture (2.3–3.8 oocytes per ovary) [29]. On the other hand, some authors reported a higher number of oocytes per ovary (5.8 ± 1.0) in 1.5- to 3.5-year-old ewes during the reproductive season [3] and 3.4 oocytes per ovary with less than four granulosa cell layers [29]. The differences in the recovery of COCs could be because, in the present study, the selection of oocytes was stricter based on the number of granulosa cell layers (> 5) [20]. Unfortunately, the number of COCs with less than five granulosa cell layers was not recorded, avoiding further comparisons. Moreover, pregnant adult ewes showed a tendency ($p = 0.07$) to produce a lower number of COCs, possibly due to the presence of atretic follicles reported in pregnant ewes [30]. Nevertheless, the results obtained in the present study confirm the presence of morphologically viable COCs in the ovaries of pregnant females.

In sheep, blastocyst efficiency in IVEP ranges from 30 to 40% [31]. In this study, the morula rate ranged from 74.4 to 78.3%, and non-significant differences were found between the physiological stages tested. On the other hand, the blastocyst rate was higher in non-pregnant adults than in young and pregnant adult ewes. Similar results were reported by Leoni et al. [31], who found differences in the blastocyst rate in adults compared to prepubertal ewes (30–40 days old) (34.5 vs. 12.1%, $p < 0.01$). Similarly, Leoni et al. [5] obtained a higher blastocyst rate in adults than in prepubertal ewes (34.43 vs. 11.94%, $p < 0.01$).

The difference between young ewes and pregnant adult ewes was non-significant, and the percentage in both groups was half the blastocyst rate of non-pregnant adult ewes (> 17%). This is important because although the efficiency is low, the results confirm that it is possible to produce viable blastocysts from young and pregnant ewes.

In a study on prepubertal ewes, a low blastocyst production rate was also reported (6–11%) [6]. The variation in the IVEP rate could be because, in the oocytes from young and prepubertal ewes, there are deficiencies in the storage of mRNAs in germinal vesicles

involved in the compaction and blastulation processes [32]. Similarly, it has been suggested that a possible relationship exists between reduced developmental competence and the distribution of E-cadherin, which is important in genome activation [33]. In addition, delayed nuclear maturation and variations in cytoplasmic maturation have been reported [34], possibly due to the lower ATP content [5]. On the other hand, complete development of organelles, such as the smooth endoplasmic reticulum, the Golgi apparatus membrane, and a greater number of mitochondria, have been observed in adult oocytes [5]. This could influence their competence because the redistribution of organelles and morphological adaptations could produce and store energy essential for embryonic development [9]. Therefore, these results highlight the need for additional studies to evaluate the molecular differences in oocytes from ewes at different physiological stages and their impact on nuclear and cytoplasmic maturation, which are vital processes influencing the final efficiency of IVEP.

The low competence of oocytes from pregnant adult ewes is also reported elsewhere [30]. Ewes in early gestation showed lower follicular growth (< 3 mm) and a high proportion of atretic follicles (90%) [30]. Furthermore, in sheep, the number of follicles < 2 mm and antral follicles significantly decrease during the last two-thirds of gestation [35]. In cattle, the cleavage and blastocyst rates were higher in the presence of the corpus luteum than in its absence (84 vs. 69% and 43 vs. 23%, respectively), negatively affecting the oocyte competence [8]. Oocyte degeneration could be due to prolonged exposure to high progesterone and low FSH concentrations [15]. In addition, this hormonal change favors the establishment of pregnancy due to a decrease in estradiol synthesis, which is necessary for the luteolysis process [36]. In general, in the present study, the competence of oocytes from pregnant adult ewes was lower than in non-pregnant adult ewes but similar to the competence of oocytes recovered from young ewes. In this sense, the results obtained in the present study confirm the competence of COCs from pregnant ewes on *in vitro* production of viable embryos. Therefore, they could be a suitable alternative to producing embryos from high genetic merit sheep. In the present study, however, ovaries from pregnant ewes slaughtered in a slaughterhouse were considered. Therefore, the feasibility of using the "Ovum pick-up" method in live pregnant ewes, as has been carried out in cows, needs to be evaluated [16].

On the other hand, the age of the oocyte influences the tolerance of embryos to *in vitro* manipulation [10]. Bisection and *in vitro* culture allow the identification of viable demi-embryos to increase the number of available embryos [1]. In the present study, the total rate of viable reconstituted demi-embryos was 139.5% of the manipulated blastocysts, which agrees with 152% reported by Hashiyada [2] in cattle. The groups of young and non-pregnant adult ewes had a similar reconstitution rate. Therefore, the quality possibly has an important influence on embryo reconstitution. In swine, after 48 h of culture, the diameter of excellent, good, and low-quality demi-embryos was measured; the differences between these categories (211, 162, and 122 μm, respectively) were significant ($p < 0.01$). In addition, a strong correlation ($r = 0.85$) was observed between the embryonic diameter and the number of cells [37]. Therefore, diameter is an important indicator of embryo quality. In the present study, the diameter was larger in demi-embryos obtained from non-pregnant adult ewe oocytes (> 185 μm) than those obtained from young and pregnant adult ewes (≤ 165 μm for both groups). Thus, the mean diameter for all groups was 75.5% (170/225 μm) of the average diameter of the original embryo. Although the percentage appears low, the diameter of the demi-embryos produced is greater than that reported in other studies, even when compared to the diameter of intact embryos. In bovines, the diameter of blastocysts produced by blastomere isolate (from a four-cell embryo) was similar ($p > 0.05$) to that of intact embryos

grown to the same stage, 143 vs. 150 μm, respectively [38]. The average diameter of blastocysts produced from intact embryos in human was 121 μm [39]. The average diameter of demi-embryos from all groups (170 μm) indicates that embryo size is a good quality indicator because it is important in the secretion of interferon-T, an important protein for maternal recognition [40].

In conclusion, the IVEP efficiency was higher when oocytes from non-pregnant adult ewes were used than when they were from young or pregnant adult ewes from a slaughterhouse. Although embryo production efficiency was low in young and pregnant adult ewes, using animals in these stages is an alternative when the availability of ewe oocyte donors is limited.

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