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ESTRATEGIAS PARA AUMENTAR LA EFICIENCIA EN LA PRODUCCIÓN *IN VITRO* DE EMBRIONES OVINOS

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ABREVIATURAS

IVEP	*In vitro* embryo production
COCs	Cumulus-oocyte complexes
CCs	Cumulus cells
ICM	Inner cell mass
TE	Trophectoderm cells
GDF9	Growth differentiation factor 9
BMP15	Bone morphogenetic protein 15
FGF8	Fibroblast growth factor 8
DOs	Denuded oocytes
SE	Standard error
LSM	Least-squares means
ATP	Adenosine triphosphate
CI	Confidence interval
OR	Odds ratio
τ^2	Variability
I^2	Heterogeneity
FBS	Fetal Bovine Serum
hCG	Human Chorionic Gonadotropin

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RESUMEN GENERAL

ESTRATEGIAS PARA AUMENTAR LA EFICIENCIA EN LA PRODUCCIÓN *IN VITRO* DE EMBRIONES OVINOS[1]

La eficiencia de la producción *in vitro* de embriones ovinos (IVEP) es baja, debido a varios factores intrínsecos y extrínsecos que limitan el desarrollo embrionario. El objetivo de la presente tesis fue implementar estrategias para mejorar la eficiencia en la IVEP en ovinos. Primero, se realizó una búsqueda de literatura sistemática de la importancia de la bipartición embrionaria y reconstitución de semi-embriones, como alternativa para aumentar la disponibilidad de IVEP. El artículo "Demi-embryo reconstitution, a factor to consider for the success of embryo bisection. Review" enfatiza la importancia de la calidad del embrión bisectado para el éxito de la técnica. Después, el trabajo "*In vitro* embryo production from ewes at different physiological stages" resalta las diferencias (p<0.01) en la eficiencia de la IVEP cuando se utilizan ovocitos de hembras jóvenes, adultas no gestantes y adultas gestantes (18, 36 y 18%, respectivamente). Además, la reconstitución de semi-embriones fue mayor en el grupo de ovejas jóvenes y adultas no gestantes (>68%). En el tercer artículo, "Effect of the developmental stage of bisected embryos on ruminant reproductive efficiency: Meta-analysis", los resultados indicaron heterogeneidad entre estudios y falta de evidencia para afirmar sesgo de publicación; sin diferencias (p>0.01) entre la bisección de mórulas y blastocistos en el porcentaje de gestación, sobrevivencia de semi-embriones y pares de semi-embriones. Por último, en el artículo "Co-culture of low-quality ovine cumulus-oocyte complexes with denuded oocytes during *in vitro* maturation" se encontró que el co-cultivo de complejos cúmulo-ovocito de baja calidad (<4 capas de células del cúmulo) con ovocitos desnudos durante la maduración *in vitro*, aumentó (p<0.05) 10% el porcentaje de mórulas y blastocistos, así como también 22 µm más el diámetro de embriones que el grupo sin co-cultivo. En conclusión, la eficiencia de la IVEP mejoró al utilizar la bipartición embrionaria, co-cultivo y cuando se seleccionan los complejos cúmulo-ovocito de acuerdo con su origen.

Palabras clave: Ovocito, fertilización *in vitro*, embrión.

[1]Tesis de Doctorado en Ciencias en Innovación Ganadera, Posgrado en Producción Animal, Universidad Autónoma Chapingo
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GENERAL ABSTRACT

STRATEGIES TO INCREASE EFFICIENCY OF *IN VITRO* PRODUCTION OF SHEEP EMBRYOS[1]

The efficiency of *in vitro* sheep embryo production (IVEP) is low, due to several intrinsic and extrinsic factors that limit embryo development. The objective of this thesis was to implement strategies to improve the efficiency of IVEP. First, a systematic literature search of the importance of embryo bisection and semi-embryo reconstitution, as an alternative to increase IVEP availability, was performed. The article "Demi-embryo reconstitution, a factor to consider for the success of embryo bisection. Review" highlights the importance of the quality of the bisected embryo for the success of the technique. Later, the article "*In vitro* embryo production from ewes at different physiological stages" highlights the differences (p<0.01) in the efficiency of IVEP when using COCs from young females, non-pregnant adults, and pregnant adult ewes (18, 36, and 18%, respectively). In addition, semi-embryos reconstitution was greater in young and non-pregnant adult ewes (>68%). In the paper "Effect of the developmental stage of bisected embryos on ruminant reproductive efficiency: Meta-analysis", the results indicated heterogeneity between studies and lack of evidence to elucidate publication bias; no differences (p>0.01) between the bisection of morulae and blastocysts in pregnancy rate, survival of semi-embryos and pairs of semi-embryos. Finally, the article "Co-culture of low-quality ovine cumulus-oocyte complexes with denuded oocytes during *in vitro* maturation" showed that co-culture of low-quality COCs (<4 layers of cumulus cells) with ODs during *in vitro* maturation increased (p<0.05) 10% the morulae and blastocysts rate, as well as 22 µm greater diameter of embryos than the group without co-culture. In conclusion, the efficiency of IVEP improved when embryo bisection and co-culture were used, and when cumulus-oocyte complexes were selected according to their origin.

Keywords: Oocyte, *in vitro* fertilization, embryo.

[1]Doctoral Thesis in Livestock Innovation, Graduate Program in Animal Production, Universidad Autónoma Chapingo

Author: Alfredo Lorenzo Torres
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1. INTRODUCCIÓN GENERAL

La producción *in vitro* de embriones (IVEP) es una técnica que permite el desarrollo embrionario en diferentes etapas como mórula, blastocisto y blastocisto eclosionado, para utilizarse en investigación o para su aplicación comercial. La técnica consiste en procesos fisiológicos complejos que se realizan en condiciones de laboratorio, simulando las condiciones naturales que la hembra proporciona (Zhu et al*.,* 2018), tales como maduración del ovocito, capacitación espermática, fertilización y desarrollo embrionario. Así, los embriones producidos pueden transferirse directamente a una hembra receptora o crioconservarse para su posterior uso (Paramio & Izquierdo, 2016). La IVEP permite generar progenie de hembras prepúberes, reduciendo el intervalo generacional, aumentando el progreso genético anual; además, producir crías de hembras infértiles, preñadas, lactantes o sacrificadas en rastros. Sin embargo, la técnica es impredecible y variable, lo que limita su aplicación comercial en gran escala (Paramio & Izquierdo, 2016). La eficiencia de la IVEP de embriones ovinos es de 20 a 50 % de blastocistos expandidos, por lo que es necesario desarrollar estrategias para mejorar dicha eficiencia y la calidad embrionaria (Zhu et al., 2018).

La bisección embrionaria es una biotecnología que permite obtener semi-embriones para investigación o la industria ganadera (Godke et al., 2014; Praharani, 2019). La bisección se ha realizado en varias especies ganaderas con el objetivo de aumentar la disponibilidad de embriones y el número de crías (Hashiyada, 2017; Chesné et al., 1987). Esta biotecnología podría aumentar la disponibilidad de embriones *in vitro*. No obstante, deben considerarse varios factores, entre ellos la etapa de desarrollo del embrión bisectado (Illmensee & Levanduski, 2010). Por otra parte, la colecta de COCs (complejos cúmulo-ovocito) en rastros es una práctica común y barata para producir embriones (Wani et al., 2000). Sin embargo, existe heterogeneidad en la etapa fisiológica de las hembras sacrificadas y, por consiguiente, diferencias moleculares en los COCs que se colectan (Leoni et al., 2015). Por lo que es importante evaluar la eficiencia de IVEP utilizando COCs provenientes de hembras en diferentes etapas fisiológicas. La selección de COCs

es una práctica común que a menudo se basa en criterios morfológicos y que influye en la producción de blastocistos (De Bem et al., 2014). El co-cultivo de ovocitos desnudos (ODs) y COCs viables incrementa el porcentaje y la calidad de blastocistos, debido a la sinergia acumulativa en la maduración nuclear y citoplasmática, la fertilización y la regulación del estrés oxidativo en los ovocitos (Dey et al., 2012). Lo anterior debido a que el ovocito secreta proteínas como el factor de diferenciación del crecimiento 9 (GDF9), la proteína morfogenética ósea 15 (BMP15) y el factor de crecimiento de fibroblastos 8 (FGF8) que promueven la proliferación y diferenciación de las células del cúmulo (CCs) (Alam & Miyano, 2020). Por lo que el co-cultivo con ODs podría tener mayor influencia en la competencia de COCs de baja calidad. El objetivo de esta tesis fue desarrollar estrategias que permitan mejorar la eficiencia de embriones ovinos producidos *in vitro.*

El Capítulo 2 hace referencia a la revisión de literatura, de la cual se generó un artículo de revisión titulado “Demi-embryo reconstitution, a factor to consider for the success of embryo bisection. Review”. En este escrito se enfatiza la importancia de la reconstitución embrionaria después del proceso de bipartición.

El Capítulo 3 es un artículo derivado del trabajo de tesis titulado “*In vitro* embryo and production from ewes at different physiological stages”. Este trabajo resume la eficiencia productiva de embriones provenientes de ovejas con diferentes etapas fisiológicas.

El Capítulo 4 incluye el artículo “Effect of the developmental stage of bisected embryos on ruminant reproductive efficiency: Meta-analysis”, que resalta la importancia de la etapa de desarrollo en la selección del embrión bisectado.

Finalmente, el capítulo 5 hace referencia al artículo “Co-culture of low-quality ovine cumulus-oocyte complexes with denuded oocytes during *in vitro* maturation”. En este trabajo se plantea una estrategia para mejorar la competencia de ovocitos.

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2. Demi-embryo reconstitution, a factor to consider for the success of embryo bisection. Review

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Abstract:

For many years it has been sought to increase the reproductive efficiency of livestock using biotechnologies such as embryo bisection. However, despite its potential in livestock, its level of adoption is limited. The present work reviews the importance of demi-embryo reconstitution, after bisection, and the main factors that limit its success in livestock. It is possible to increase its level of adoption if it is possible to increase the efficiency currently obtained with this technique, this can be achieved by making a more precise selection of the embryos subjected to bisection. Embryo quality is one of the most important factors related to the potential to reconstitute into viable demi-embryos after bisection, which can be used with greater reliability in embryo transfer programs.

Key words: Embryo bisection, Demi-embryo reconstitution, Embryo development**.**

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Introduction

Embryo bisection is a reproductive biotechnology that allows obtaining identical demiembryos, to be used in research[1] or the livestock industry[2]. The aim of embryo bisection is to increase the number of semi-embryos available for transfer and therefore, the offspring of genetically superior animals[1,3,4]. This technique can be applied to embryos developed in the morula or blastocyst stage and consists of obtaining two similar halves by mechanical bisection[5,6,7]. Morula bisection can be carried out in any position of the embryo, due to its symmetric morphology[8]. In the case of blastocysts is important the symmetrical orientation of the embryo to obtain a proportional distribution of the inner cell mass (ICM) and trophectoderm (TE) in the resulting demi-embryos[9].

Embryo bisection is performed in embryos of several species[10,11,12], in order to increase embryo availability[12], pregnancy rate[13], and the number of offspring[5,14,15]. However, there are studies where the survival of demi-embryos was low[16,17], even lower compared to the use of whole embryos[18]. This could be associated with the fact that embryo bisection is an invasive technique[6] and the procedure causes cellular damage[19]. Therefore, the success of the technique could be influenced by factors associated with the original embryo[20,21,22] and its ability to reconstitute itself in the resulting demi-embryos[23]. The objective of this review is to highlight the importance of demi-embryo reconstitution in embryo bisection programs, as well as to discuss the main factors that influence its success.

Importance of embryo bisection in livestock

Embryo bisection has been carried out in different livestock species of interest, such as rabbits[19], sheep[24], bovines[12], goats[10], equines[25], swine[26], and even in humans[22]. Despite it is an invasive technique, it is practical and it does not require cell reprogramming like cloning[6]. Embryo bisection in livestock allows to produce identical twins for experimental use[14], reducing the number of animals needed per treatment for comparison tests[27] or to increase the availability of transferred embryos[28]. In addition, obtaining identical twins facilitates the evaluation of sires or maternal trait tests[29] and, lastly, to maintain desirable characteristics in cattle[3].

Embryo bisection has allowed to increase pregnancy rate[29] and the number of offspring[13], compared to the transfer of whole embryos[13,24]. Most authors have reported a higher number of semi-embryos available for transfer in relation to the number of bisected embryos, thus increasing the efficiency in the number of offspring (Table 1). However, there is a great variation (75 to 118 %, efficiency), which could be mainly associated with factors related to the original embryo. In ewes, the pregnancy rate was 64 % when they received airs of demiembryos, obtaining 118 % efficiency in offspring[14]. Likewise, there was a higher percentage of embryo survival when transferring two demi-embryos per recipient ewe (101 %, 710/705), compared to the transfer of two whole embryos (62 %, 771/1252) considering the number of original embryos[24]. Further, 30 % more lambs were born after the transfer of pairs of demiembryos, compared with whole embryos (85 *vs* 55 %, $P<0.05$)[30].

Table 1: Efficiency of embryo bisection in the production of offspring in relation to the number of bisected embryos

Species	Bisected embryos	Number of offspring born	Efficiency (%)	Reference
Bovine	36	27	75	13
Bovine	50	61*	105	15
Bovine	11	12*	109	5
Sheep	40	34	85	30
Sheep	24	21**	88	16
Sheep	705	710*	101	24
Sheep	16	17	106	31
Sheep	39	46	118	14

* Number of fetuses diagnosed by ultrasound between 30 and 80 d of gestation or ** by sacrifice surgery after slaughter. Efficiency (%)= Number of offspring born / embryos bisected.

On the other hand, in some studies, low efficiency was obtained with embryo bisection [16,32,33]. It has been reported that a lower percentage of lambs were born after the transfer of bisected sheep embryos, compared to whole embryos (27 *vs* 52 %, $P<0.05$)[34]. However, in beef cattle, despite the problems being associated with twin gestation[4], implementing embryo bisection represents an economic benefit once the number of offspring is increased[2,17,35]. Therefore, embryo bisection can be implemented in embryo transfer programs[2,24].

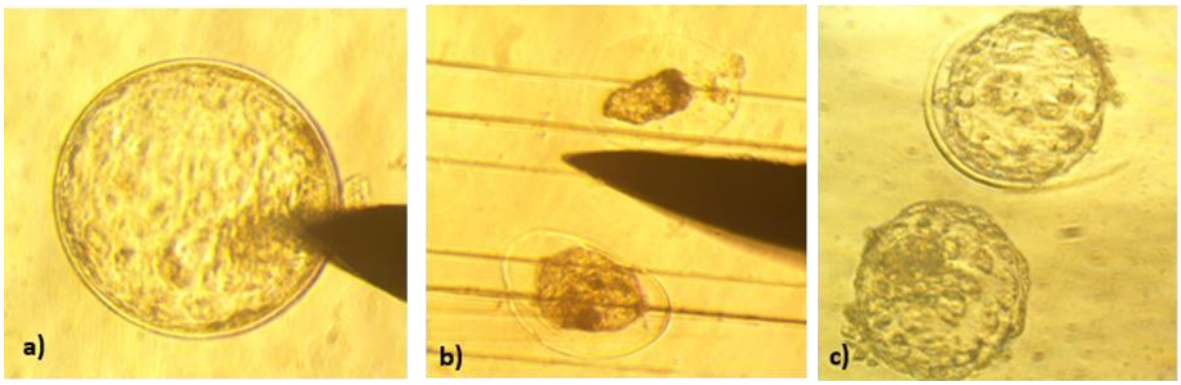
Embryonic reconstitution after bisection

Embryo reconstitution is an indicator of the ability of demi-embryos to become offspring after their transfer to a recipient female[18,29]. In adult tissues, stem cells are responsible for repairing injuries and regenerating tissues[23,36] due to aging or diseases[37]. In the case of embryos, something similar happens, through the replacement of specialized cells that have been lost due to some alteration[23]. Embryos are capable of repairing their injuries, adapting to environmental conditions to survive after their reconstitution[23]. Embryonic cells in early stages can adapt both in mitotic rate and differentiation process[38], due to the plasticity they present[39,40]. In addition, it has been proven that embryonic cell conglomerates in the early stages of development can become living organisms through cell reorganization, such as the case of blastocyst splitting[41].

Thus, a group of cells has properties that exceed the potency of any of the individual cells within the group for cell reconstitution, which could be a joint effect[23]. The extruded cells or even cell debris observed after embryo bisection could contain enough viable cells to proliferate and reorganize, originating another functional embryo[8]. At the time of embryo bisection, the resulting halves can be cultured *in vitro* for 2 to 48 h[5,26]. In each demi-embryo, the ICM is reorganized and

the reconstitution of the blastocele begins immediately[5,29,42]. The union of the edges of trophoblastic cells during bisection is responsible for the trophoblast's ability to reconstitute itself[5] since this group of cells secretes fluid into the blastocele, a process regulated by genes[43,44]. This allows the spherical formation of demiembryos[42], being the trophoblastic cells important for embryo implantation[45]. Likewise, an embryo, even if it has lost half of its cells, it is still an organism and a characteristic of organisms is to repair their lesions, regenerating them to continue their development[23]. In the laboratory, it was bisected sheep expanded blastocysts produced *in vitro* with a microblade (Figure 1a), using the procedure called scratched bottom dish[46] (Figure 1b) and it was observed a completely spherical reconstitution (70 % the size of the original embryo) 12 h after *in vitro* culture (Figure 1c).

Figure 1: Embryo bisection process and reconstitution of the demi-embryos after 12 h of *in vitro* culture



a) Expanded blastocyst oriented symmetrically with respect to the bisecting microblade, b) resulting demi-embryos, and c) reconstituted demi-embryos. ×200.

Several authors have reported a reconstitution percentage ranging from 90 to 178 % (Table 2).

Table 2: Reconstitution efficiency of bovine demi-embryos after *in vitro* culture for 2-48 h post-bisection

Bisected embryos	Number of demi-embryos	Reconstitution* (%)	Reference
21	19	90	29
11	16	145	5
176	268	152	12
19	30	158	47
230	408	178	28

*Reconstitution (%) = Number of demi-embryos / bisected embryos.

The evaluation of embryo reconstitution could allow the selection of viable demi-embryos and it can be a useful tool in embryo transfer programs[18].

Factors that affect demi-embryo reconstitution

Embryo bisection technique

Embryo bisection is a technique that allows the production of identical twins in embryo transfer programs[5]. In the 80s, the technique required up to six embryo manipulation and bisection instruments[5]. However, over time, different methodologies have been developed to simplify the technique[1], since the procedure required up to 15 min to bisect an embryo[48]. In addition, the use of cutting instruments, such as microblade[5,25,49] or glass needle[50,51,52], have been extensively studied, with the objective of minimizing cellular damage at the time of cutting[53]. Thus, the success of the technique depends on the minimum damage produced to the embryos[19], since the procedure generates 10 to 13% of cell loss[47,54].

On this regard, the microblade embryo bisection method has proven to be practical and with an application under field conditions[1]. The implementation of this technique has been simplified by means of vertical pressure at the time of embryo bisection, using a microblade adapted to a single micromanipulator, without the use of an embryo holding micropipette[12,15,34]. In the laboratory, has been observed that the use of the scratched bottom dish technique[46] with 50 µL of a commercial bisecting medium, facilitates embryonic fixation and prevents cell adhesion to bisecting and culture materials. This makes it possible to bisect groups of five embryos in approximately 3 min, making the application of this biotechnology more practical and without subjecting the embryos to prolonged stress.

On the other hand, there is evidence showing that the technician who performs the embryo bisection influences the productive response. In sheep, it has been evaluated the effect of the technician at the time of bisection, finding a significant difference between the two technicians in pregnancy rate (66 *vs* 75 %, P<0.05) and demi-embryo survival (51 *vs* 44 %, P<0.01)[55]. Thus, proper training of the technician should be considered before implementing embryonic bisection.

Developmental stage

The stage of embryonic development, morula, early blastocyst, or expanded blastocyst at the time of bisection, is one of the most important factors that affect the pregnancy rate of transferred embryos[8]. After the bisection of mouse embryos, it was found that, in the morula stage, the demi-embryos are reconstituted in a lower percentage than in the blastocyst stage (74 *vs* 90 %, P=0.001) after *in vitro* culture for 24 h[45]. In cattle, no significant differences were reported when transferring demi-embryos from morulae, early blastocysts, and expanded blastocysts, on pregnancy rate (51-65 %, P>0.1)[53]. In another study, there was a lower pregnancy rate using bisected morulae (7/44, 16 %) compared to early blastocysts (58/96, 60 %), P <0.01[8]. Likewise, it has been reported a higher percentage of viable fetuses after blastocyst bisection compared to morulae, 91 (10/11) *vs* 30 % (3/10), P<0.05, at d 70 of gestation[47]. Finally, in sheep, six pairs of identical twins were obtained from blastocyst bisection, without success in morula bisection (P<0.05)[16].
In a practical way, it appears that bisecting morulae is easier due to the morphological symmetry

they present, however, in the laboratory, it was observed that bisecting expanded blastocysts and even hatched blastocysts was easier once the ICM and TE were clearly identified. Furthermore, some authors have reported a higher pregnancy rate using blastocysts compared to morulae (Table 3), perhaps because they are more tolerant to manipulation and are less affected by the loss of the zona pellucida[8,54]. This could be due to the fact that embryogenesis is strictly regulated in time[22] and the more developed the embryos are, the more tolerant they are.

Table 3: Effect of the developmental stage of the whole embryo on the pregnancy rate of transferred semi-embryos

Species	Developmental stage , % (n)			Reference
	Morula	Blastocyst	Expanded blastocyst	
Caprine	0 (5)	33 (9)	55 (11)*	10
Ovine	60 (20)	88 (24)	-	16
Bovine	48 (162)	60 (96)	54 (28)	8
Bovine	51 (71)	64 (61)	58 (12)	53
Bovine	39 (139)	36 (33)	30 (10)	12

%= Pregnancy rate; n= Number of recipient females; *Hatched blastocyst.

Embryo quality

There is wide evidence for the use of excellent quality embryos for bisection purposes[12,40,56,57]. The embryos selected for bisection must meet certain morphological criteria, from which the success of demi-embryo reconstitution will depend[28] and, consequently, the pregnancy rate[12,51]. The quality of the embryos must be excellent or good, depending on the morphological criteria[58] because when they are of low quality (fair and bad) are more vulnerable to the bisection process[12,47].

In bovines, when bisecting morulae, a higher percentage of survival was obtained in the group of excellent and good quality, compared to morulae of regular and poor quality, 167 (20/12) *vs* 75% (9/12), $P<0.001$[47]. On the other hand, 42 % pregnancy was found in cows after thawing and transferring excellent quality demi-embryos, while when transferring demi-embryos from low-quality embryos, no female became pregnant[51]. Likewise, it has been reported a higher percentage of development in pairs of demi-embryos, when bisecting bovine embryos of excellent quality, compared to those of good quality (76 *vs* 40 %, $P<0.05$)[12]. Therefore, evaluation of embryo quality subjected to bisection should be considered to obtain positive results. However, the morphological evaluation of embryos subjected to bisection is a subjective aspect, based on the experience of the researcher.

In the experience in manipulating sheep embryos of this research team, was found a higher percentage of demi-embryo reconstitution (145 %, approximately), when bisecting embryos of excellent quality and with a diameter greater than 230 µm. In the resulting demi-embryos, after 12

h of *in vitro* culture, was found that when they were reconstituted, they had an average diameter of 176 ± 10.03 µm (Figure 2), similar to that reported in quality two porcine demi-embryos (161.6 ± 25.7 µm)[26], but higher than that reported in human demi-embryos (121 µm)[22]. Therefore, the embryonic diameter has been proposed as an indicator of quality[25,59] since the size of the embryo is important in maternal recognition[60].

On the other hand, the size of the embryo is also associated with the number of cells[61] and, consequently, with the embryo quality. In poor quality embryos, there is a deficiency in the rate of cell division, mitosis, and consequently in the number of cells[47]. Thus, the size of the embryo is proportional to the number of cells used for its reconstitution[22]. There is a 50 % recovery of cells from the original embryos in the resulting demi-embryos depending on the quality and uniformity of the bisection process[26,28]. This suggests that bisecting bigger size embryos will produce semi-embryos with more ICM and TE cells, thus increasing their survival. In the laboratory, was obtained an average of 68 ± 11.3 cells in reconstituted demiembryos, after 12 h of *in vitro* culture (Figure 3), from embryos with 122 ± 6.6 cells. In this sense, active cell proliferation can be a criterion of embryonic quality[62]. Based on the above, the diameter of the embryo could be an objective criterion to select embryos for bisection, in order to achieve the greatest possible success in demi-embryos reconstitution.

Figure 2. *In vitro* reconstitution of demi-embryos after 12 h of culture. ×200

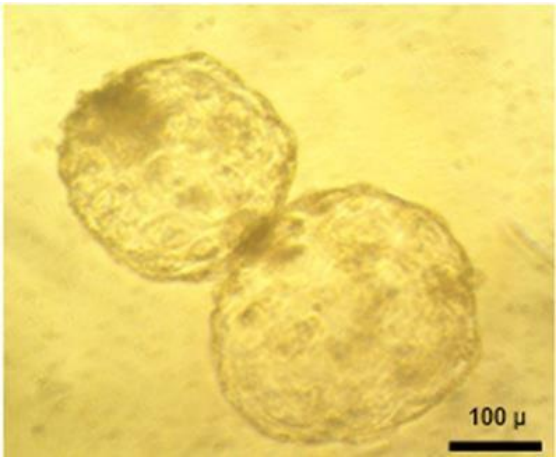
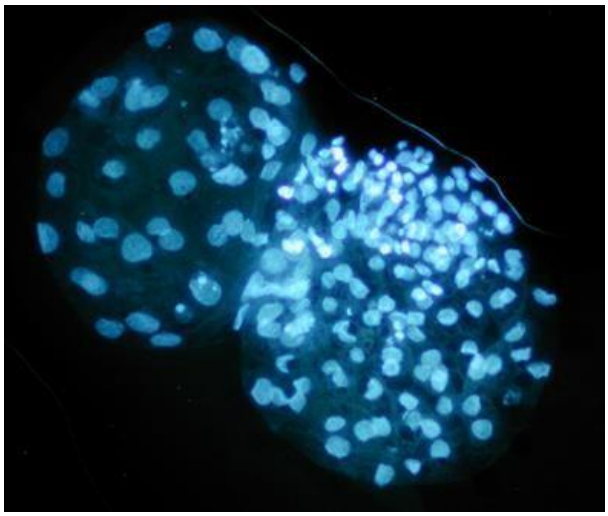


Figure 3. Cell staining (Hoechst) of *in vitro* demi-embryos after 12 h of culture. ×200



In vitro or *in vivo* produced embryos

There are differences between embryos produced *in vitro* or *in vivo*, both in morphology and in molecular components[63], where *in vivo* produced embryos are of better quality. Nevertheless, a high percentage of *in vitro* survival of ovine demi-embryos has been reported after bisection (80-85 %), and 33 % pregnancy after transferring pairs of demi-embryos to recipient ewes (5/15)[34]. On the other hand, for embryos produced *in vivo*, some authors reported greater pregnancy rates. In bisected bovine embryos produced *in vivo* and cultured the demi-embryos *in vitro*, a high percentage of reconstitution was reported (47/60 , 78.3 %)[15]. In bovines, there was 53 % (18/34) pregnancy when transferring embryos produced *in vivo*, bisected, and cultured *in vitro*[18]. Thus, the efficiency of embryo bisection in relation to the origin of the embryo seems to be lower in embryos from *in vitro* conditions. This could be due to the low quality and efficiency of *in vitro* production[63]. Therefore, it is necessary to improve efficiency in both embryo production procedures, once the two of them are focused on improving productivity in livestock production[64].

Effect of breed and age of embryo donors for bisection

There are other little-studied factors that could affect the embryo's fate under the bisection process. In sheep, the effect of the breed was evaluated, without finding significant differences in pregnancy rate and survival of demi-embryos between embryos from Gotland and Finnish Texel (69 *vs* 50 % and 42 *vs* 26 %, respectively, $P>0.05$) and between Danish Texel and Finnish Texel breeds (74 *vs* 74 % and 50 *vs* 50 %, respectively, $P>0.05$)[55]. In addition, the effect of the donor age has been reported, without finding differences in pregnancy rate between embryos generated from adult females (24 months of age) and young (approximately 10 mo of age) (74 *vs* 74 %, $P>0.05$), however, demi-embryo survival (51 *vs* 47, $P<0.05$) and percentage of identical twins was higher in adult ewes than in young (38 *vs* 27, $P<0.01$)[55]. This could be related to a lower survival capacity of whole embryos from young ewes[65,66,67], which is confirmed after transferring demi-embryos[55].

Conclusions

Demi-embryos reconstitution is a key factor for the success of embryo bisection and the highest efficiency is obtained by selecting excellent quality embryos regardless of their developmental stage. The results of the literature show the potential of the bisection technique; therefore, its application should be considered to improve the efficiency of embryo transfer programs.

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3. In vitro embryo production from ewes at different physiological stages

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ABSTRACT

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 variesand influences embryo production. 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. 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. The number of oocytes collected from the experimental group of non-pregnantadult 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. 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 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

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Conflict of Interest
The authors declare no conflicts of interest.

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susceptibility to *in vitro* manipulation [10]. Oocyte quality is one of the crucial factors affectingIVEP 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 recoveryfrom 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, usingan 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 for24 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. Groupsof 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) accordingto 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 embryowas 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:

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

where:

y_{ij} = diameter of the demi-embryo reconstituted in the jth replicate of the ith physiological stageof 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 forthe covariate diameter of the original embryo before bisection; $\mathcal{E}_{ij}$ = random error associated with the measurement of the jth diameter of the demi-embryo in the ith physiological stage of the ewe, which $\mathcal{E}_{ij}$~NIID(0, σ^2) was assumed. The ESTIMATE option was used for all variables to test for treatment differences. Data with $p < 0.05$ were considered significant.[e]

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 2.67 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[bc]
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 ($p < 0.05$).

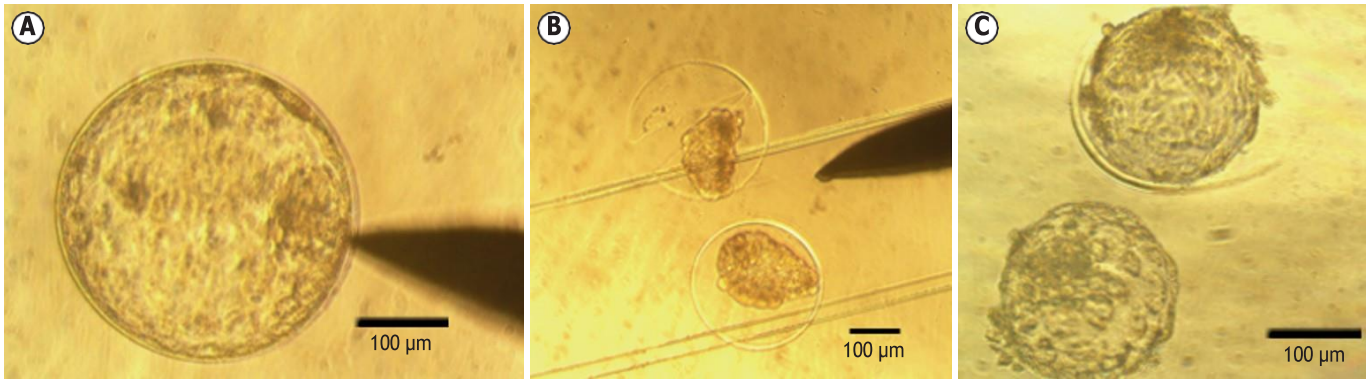

Fig. 1. Embryo bisection process and demi-embryo survival after 12 h of *in vitro* culture. (A) Expanded blastocyst, (B) pair of demi-embryos, and (C) reconstituted demi-embryos (×200).

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[bc]
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

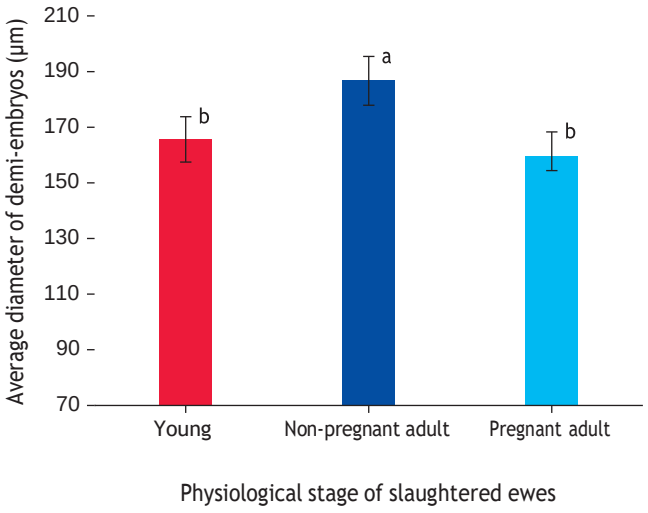

Fig. 2. Mean diameter of demi-embryos (after 12 h of culture) produced from slaughtered ewes at different physiological stages. Each bar represents the least-squares mean ± standard error. Different superscript letters (a and b) between columns represent the significant differences ($p < 0.05$).

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 viableembryos. Therefore, they could be a suitable alternative to producing embryos from high genetic merit sheep. In the present study, however, ovaries from pregnant ewes slaughteredin 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 animportant influence on embryo reconstitution. In swine, after 48 h of culture, the diameterof 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, astrong 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 diameterof 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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Effect of the developmental stage of bisected embryos on sheep and bovine reproductive efficiency: Meta-analysis

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Search Terms:	Bovine, Ovine, Pregnancy, Reproduction
Abstract:	The developmental stage of the embryo at the time of bisection is one of the most important factors influencing the reproductive parameters of demi-embryo recipients. Therefore, it is required to determine the optimal stage for bisection. The objective of this study was to evaluate the effect of the developmental stage of the embryo at the time of bisection on the reproductive efficiency of ovine and bovine demi-embryos recipients using meta-analytical procedures. The reviewed literature included the analysis of demi-embryos obtained from 1,174 morulae and 1,368 bisected blastocysts from ten studies. The variables studied were pregnancy rate, survival of demi-embryos, and survival of pairs of demi-embryos from 8, 7, and 4 studies, respectively. The METABIN, FOREST META, and FUNNEL procedures of the R program (version 4.1.2) were used to analyze data with classical meta-analysis and the GLIMMIX procedure of SAS 9.4. using the logistic binomial model. There was no difference ($p>0.05$) between the bisection of morulae or blastocysts for pregnancy rate (0.52±0.04 vs. 0.53±0.04), survival of demi-embryos (0.42±0.03 vs. 0.42±0.03) and survival of pairs of demi-embryos (0.17±0.02 vs. 0.23±0.03). The results indicated heterogeneity and lack of evidence ($p<0.01$) to affirm publication bias for the variables studied. The results of this meta-analysis indicate the feasibility of splitting morulae and blastocysts from sheep and cattle, without compromising the surviving capacity of the demi-embryos transferred to recipient females.

4. Effect of the developmental stage of bisected embryos on sheep and bovine reproductive efficiency: Meta-analysis

ABSTRACT

The developmental stage of the embryo at the time of bisection is one of the most important factors influencing the reproductive parameters of demi-embryo recipients. Therefore, it is required to determine the optimal stage for bisection. The objective of this study was to evaluate the effect of the developmental stage of the embryo at the time of bisection on the reproductive efficiency of ovine and bovine demi-embryos recipients using meta-analytical procedures. The reviewed literature included the analysis of demi-embryos obtained from 1,174 morulae and 1,368 bisected blastocysts from ten studies. The variables studied were pregnancy rate, survival of demi-embryos, and survival of pairs of demi-embryos from 8, 7, and 4 studies, respectively. The METABIN, FOREST META, and FUNNEL procedures of the R program (version 4.1.2) were used to analyze data with classical meta-analysis and the GLIMMIX procedure of SAS 9.4. using the logistic binomial model. There was no difference (p>0.05) between the bisection of morulae or blastocysts for pregnancy rate (0.52±0.04 *vs*. 0.53±0.04), survival of demi-embryos (0.42±0.03 *vs.* 0.42±0.03) and survival of pairs of demi-embryos (0.17±0.02 *vs*. 0.23±0.03). The results indicated heterogeneity and lack of evidence (p<0.01) to affirm publication bias for the variables studied. The results of this meta-analysis indicate the feasibility of splitting morulae and blastocysts from sheep and cattle, without compromising the surviving capacity of the demi-embryos transferred to recipient females.

Keywords: developmental stage, embryo bisection, demi-embryo survival.

INTRODUCTION

Embryo bisection is an assisted reproductive biotechnology that increases the availability of transferable embryos and the number of offspring in livestock (Lambeth et al., 1983; Yang et al., 2007; Wakchaure & Ganguly, 2016). Bipartition has been carried out in embryos of rabbits (Skrzyszowska et al., 1997), sheep (Vivanco et al., 1991), cattle (Hashiyada, 2017), goats (Tsunoda et al., 1985), horses (McKinnon et al., 1989), pigs (Reichelt & Niemann, 1994), and humans (Noli et al., 2017). The procedure is performed by symmetrically bisecting the embryo to obtain two halves capable of reconstitution and generating two independent individuals (Lorenzo-Torres et al., 2022). After bisection, the inner cell mass (ICM) of each half reorganizes and blastocoel formation begins (Daniel, 1963). This is because the embryos can repair their injuries to adapt and survive after the cell damage caused by the procedure (Condic, 2014).

There is a large number of factors that negatively affect the efficiency of the technique (Arave et al., 1987; Rahbaran et al., 2021; Lorenzo-Torres et al., 2022), i. e. type of splitting medium (Shelton, 1992), type of transfer (Hashiyada, 2017), bipartition technique (Godke et al., 2014), embryo quality (McEvoy & Sreenan, 1990), and embryonic stage of development (Illmensee & Levanduski, 2010), the latter frequently referred as one of the most important. Bisection can be performed on embryos at different stages of development, early morula (Williams et al., 1984), compact morula (Gray et al., 1991), early blastocyst (Kippax et al., 1991), expanded blastocyst (Illmensee & Levanduski, 2010), and hatched blastocyst (Tsunoda et al., 1985). Since the 1980s, studies have been conducted to determine the most appropriate stage of embryonic development for bisection, however, the results are not consistent. Some studies have found a higher pregnancy rate, fetal survival, and number of identical twins in early blastocysts than in early morulae after bisection (Williams et al., 1984; McEvoy & Sreenan, 1990; Hashiyada, 2017). It has been argued that blastocysts are more tolerant to bisection and less affected by the absence of zona pellucida (Williams et al., 1984). Nevertheless, Arave et al. (1987) reported nearly a 2-fold efficiency for the same variables using early morulae instead of blastocysts. In other studies, no

differences were reported in bisected morulae or blastocysts (Kippax et al., 1991; Shelton, 1992); or bisected expanded blastocysts compared to hatched blastocysts (Rangel, 1991; Gray et al., 1991; Hashiyada, 2017). This inconsistency could be because, in some cases, the studies included a low number of experimental units or data came from studies carried out under different conditions, at a commercial or experimental level, in which there was a great variability of the results. One alternative to integrate the results of individual experiments available in the scientific literature is to use meta-analytic methods (Lean et al., 2009). To our knowledge, there is no meta-analysis considering the effect of the developmental stage on embryonic bisection. The hypothesis is that bisection can be performed in embryos at the morula and blastocyst stage, without negatively influencing the viability of the resulting demi-embryos. The objective of this meta-analysis was to evaluate the effect of the developmental stage of sheep and bovine embryos at the time of bisection on the reproductive efficiency of demi-embryo recipients, through meta-analytical methods, using published data in the literature.

MATERIALS AND METHODS

Search of the published literature

A systematic search of the published literature from 1980 to 2021 was performed to identify experiments that evaluated the effect of developmental stage on the bisection of ovine and bovine embryos. The search of the literature was performed with the search engines: Web of Science, Scopus, and PubMed; and conducted from September to December 2021 using the keywords: embryo bisection, embryo transfer, demi-embryos, and ruminants.

Inclusion and exclusion criteria

All published literature was screened using standardized criteria based on the hypothesis and the objective of the meta-analysis, including experiments that evaluated only the effect of embryo developmental stage on the bisection of sheep and bovine embryos. Studies that considered factorial designs and evaluated the interaction of other factors with developmental stage were

discarded. The experiments studied were those that reported the number of successes or failures for pregnancy rate, survival of demi-embryos, and demi-embryo pairs.

Experiments included or excluded

The selection of the experiments included in the meta-analysis was based on the prism diagram of Moher et al. (2009) (Figure 1).

Figure 1. PRISMA information flow diagram through the different phases of a systematic review from the initial search to the selection of the studies included in the meta-analysis. The information was collected with different search engines and the results were screened to remove duplicated studies. The studies selected were those that reported the proportions of success and failure for each response variable.

Data extraction

Success or failure data on the total number of events were extracted from tables or figures in each study. Data reported as a percentage were used to obtain the number of successes or failures per treatment. The information collected from each article was the author(s), year of publication, species, breed of the bisected embryo, method of obtaining embryos, quality of the bisected embryo, holding method, use of surrogate zona pellucida, post bipartition culture, incubation of demi-embryos, type of bisection medium, time of transfer of demi-embryos, type of transfer, developmental stage of the bisected embryo, number of bisected embryos, number of halves, number of halves transferred per recipient, number of recipients, and pregnancy diagnosis method. In the case of the treatments (developmental stage of the bisected embryo), five developmental stages were originally identified: early morula, compact morula, early blastocyst, blastocyst, expanded blastocyst, and hatched blastocyst. However, some studies did not report a specific description of the developmental stage and only mentioned two stages: morula and blastocyst. Therefore, we decided to standardize the developmental stage in morula (early and compact), blastocyst (early and expanded), and hatched blastocyst (the latter was not considered in the analysis due to the lack of comparable groups) (Table 1). The response variables were the pregnancy rate of females receiving demi-embryos, survival of demi-embryos, and survival of pairs of demi-embryos. The results of each study were

standardized in the number of successes or failures for the two developmental stages according to the number of demi-embryos available.

Table 1. Summary of the experimental comparison used in the meta-analysis, including the general description and the developmental stages used as treatments.

Statistical analysis

Data from different studies were used for the variables pregnancy rate (n=8), survival of demi-embryos (n=7), and survival of pairs of demi-embryos (n=4). The three variables were analyzed with the classic methods of meta-analysis for variables with a binomial distribution. The procedures used were METABIN and FOREST META of the R program (Version 4.1.2) to generate tree graphs for each response variable. In addition, the FUNNEL procedure was used to generate the funnel plots. Additionally, a sensitivity analysis was performed for each variable with the FOREST META procedure. The heterogeneity of results between comparisons was quantified using the I^2 statistic (Higgins & Thompson, 2002). Finally, a logistic model was used for each variable using the GLIMMIX procedure of SAS 9.3, assuming a binomial distribution, the logit link function, and the study as a random effect. For all variables, the ESTIMATE and LSMEANS options were used to obtain and test differences between adjusted probability means for each developmental stage. Results with $p<0.05$ were considered statistically significant.

RESULTS

Pregnancy rate of females receiving demi-embryos

Eight studies using 2,021 morula recipients and 1,038 blastocyst recipients were included in the pregnancy rate assessment. The evaluation of the effect of the bisected embryos in the morula and blastocyst stage with the classic meta-analysis is similar in pregnancy rate (Figure 2) with a joint estimation of odds ratio (OR)=0.7; 95% confidence interval (CI)= 0.43 to 1.13. Furthermore, the results indicated heterogeneity (I^2=77%, $p<0.01$) and variability between studies (τ^2= 0.3177, $p<0.01$). However, it can be noted that in most of the studies there is consistency in the result of no difference between the 2 groups, and in 2 of the 3 studies in which there are discrepancies the sample size was small. In the simple

scatter plot in Figure 2, a slight asymmetry can be seen. However, there is not enough evidence to affirm that there is a publication bias effect. In addition, there is no reason to think that there are preferences to find differences between the two groups.

Figure 2. Forest and funnel plot representation of the estimated odds ratio for the pregnancy rate of females receiving bisected embryos at different developmental stages.

In the sensitivity analysis, one at a time of the studies was excluded to verify its influence on the results of the meta-analysis (Figure 3), as these were similar, both in direction and magnitude of the effect, it can be assumed that the analysis is robust, since the absence of any of the studies did not substantially modify the results when eliminated from the meta-analysis.

Figure 3. Graphical representation of sensitivity analysis excluding one study at a time in the joint estimation of the meta-analysis for the pregnancy rate of females receiving bisected embryos at different developmental stages.

Survival of demi-embryos

The survival of demi-embryos was evaluated considering seven studies, where 1,782 demi-embryos of 891 bisected morulae and 1,116 demi-embryos of 558 bisected blastocysts were transferred. The meta-analysis evaluated the effect of bisected embryos at different developmental stages without finding differences ($p>0.05$) in the survival of demi-embryos (Figure 4). The joint estimate of the variable was OR=0.92; 95% CI= 0.52 to 1.16 with evidence of heterogeneity between studies (I^2=71%; τ^2=0.4248, p<0.01). Most of the studies agreed with no difference between the groups. However, in other studies there was no agreement favoring one or the other, so this result should be taken carefully. A slight asymmetry is observed in the funnel plot. Nevertheless, there is not enough evidence to assume publication bias, although this could be due to the low number of studies.

Figure 4. Forest and funnel plot representation of the estimated odds ratio for the survival of demi-embryos produced by bisected embryos at different developmental stages.

In the graph of the sensitivity analysis (Figure 5), none of the studies seems to modify the results when one is eliminated from the meta-analysis, so there was

no effect of the developmental stage at the time of embryo bisection on the survival of demi-embryos.

Figure 5. Graphical representation of sensitivity analysis excluding one study at the time in the joint estimation of the meta-analysis for the survival of demi-embryos from bisected embryos at different developmental stages.

Survival of pairs of demi-embryos

Four studies were considered in the analysis of this variable. Data from 427 pairs of demi-embryos from morulae and 202 pairs of bisected blastocysts were used. The survival of pairs of demi-embryos with the classical meta-analysis showed a joint estimate of OR= 0.72; 95% CI=0.26 to 1.97 with heterogeneity (I^2=70%, p<0.01) in the variability between studies (τ^2=0.6612, p=0.02) (Figure 6). In this case, three studies showed no differences between the groups, and only one differed with these results. It is noteworthy that this study is the one carried out by Williams et al. (1984), which disagrees with the results obtained by most studies in all the studied variables. It is expected that there must be confounding factors that explains this discrepancy; however, the meta-analysis on this variable was performed with a small sample, therefore, the results should be taken carefully. In the case of the funnel plot, the studies are not clearly visualized to assume symmetry or not, probably due to the small number of studies.

Figure 6. Forest and funnel plot representation of the estimated odds ratios for the survival of pairs of demi-embryos produced by bisected embryos at different developmental stages.

In the graph of the sensitivity analysis excluding one study at each step (Figure 7), the exclusion of one particular study did not modify the results of the meta-analysis as a whole. Therefore, there is not enough evidence showing an effect of the developmental stage of the bisected embryo on the survival of pairs of demi-embryos.

Figure 7. Graphical representation of the sensitivity analysis excluding one study at a time in the joint estimation of the meta-analysis for the survival of pairs of demi-embryos from bisected embryos at different developmental stages.

DISCUSSION

Embryo bisection has been used to increase the availability of transferable embryos (Rho et al., 1998), the number of offspring in cattle (Vintila et al., 1995;

Wakchaure & Ganguly, 2016), and as a source of identical demi-embryos for gene expression analysis (Velásquez et al., 2017). The developmental stage of the bisected embryo is one of the most important factors determining the success of the bisection process (Illmensee & Levanduski, 2010). Since the 1980s, several studies have been carried out to determine the ideal developmental stage to perform the bisection and to evaluate its effect on reproductive variables of females receiving demi-embryos (Ozil, 1983; Williams et al., 1984; Arave et al., 1987; Hashiyada, 2017). However, the results have been inconsistent and, because in some studies a low number of experimental units were used (bisected embryos), they are not robust enough to provide sufficient statistical evidence of the ideal developmental stage for the bisection process.

To our knowledge, there is no meta-analysis evaluating the effect of the stage of the bisected embryo on its subsequent development. In the present meta-analysis, the effect of the developmental stage of sheep and bovine embryos at the time of bisection on the reproductive efficiency of demi-embryo recipients was evaluated, using meta-analytical methods. Demi-embryos from 1,174 morulae and 1,368 bisected blastocysts from ten studies were considered in the meta-analysis. This data set provides sufficient statistical evidence and important consideration of the need for meta-analytic studies to determine the ideal developmental stage of the bisected embryo and the opportunity to test the initial hypothesis, highlighting the necessity of the present study. This research hypothesized that embryo bisection can be carried out in embryos at the morula and blastocyst stage, without influencing the reproductive response of females receiving demi-embryos. Supporting this hypothesis, the results of the meta-analysis indicate that bisection can be performed at both stages without affecting the reproductive response of recipient females of ovine and bovine demi-embryos. The least-squares means for pregnancy rate, survival of embryos, and survival of pairs of demi-embryos were similar (p>0.05) in morulae and blastocysts (Table 2). Despite the structural differences, it appears feasible to bisect embryos from these developmental stages.

Table 2. Effect of the developmental stage of the bisected embryo on the reproductive efficiency of ovine and bovine demi-embryo recipients. †

Embryos are able of repairing injuries caused by external interventions to adapt and survive (Condic, 2014). After the bisection process, the cells of the demi-embryos tend to organize and reconstitute themselves (Daniel, 1963), secreting fluid into the blastocoel from the trophoblast cells (Watson et al., 2004), thus allowing the spherical demi-embryo reconstitution (Wang et al., 1990). Morula-stage embryos are easier to bisect because of their symmetrical morphology. However, approximately 80% of morulae develop up to blastocysts under *in vitro* conditions (Rho et al., 1998), indicating that they have yet to overcome several cellular processes. For example, minimizing cytogenetic aberrations (Lagalla et al., 2020), finishing the sealing of the embryo by binding proteins such as e-cadherin, alpha-catenin, and beta-catenin, the pumping of fluid into the embryo, the blastocoel formation, and the differentiation of cell lineages (Lim & Plachta, 2021).

It is feasible to think that the normal development of the embryo could be interrupted in the bisected morulae. However, the results of the present meta-analysis showed the feasibility of bisecting morulae. In the case of blastocysts, lineage differentiation has already occurred, allowing visualization of the ICM and trophoblast cells. In this case, the bisection must ensure the same cell proportion of both lineages in each demi-embryo (Escribá et al., 2002). In blastocysts, the presence of aquaporins and Na^+K^+ ATPase activity is essential to maintain an iso-osmolar gradient (Huang et al., 2006) and water flow towards the blastocoel (Huang et al., 2006; Lim & Plachta, 2021), a process that possibly supports the rapid demi-embryos reconstitution, since a relationship between the blastocoel formation and the ability of the demi-embryos to survive has been proposed (McEvoy & Sreenan, 1990). In general, bisection is an invasive procedure that causes 10 to 13% cell loss (McEvoy & Sreenan, 1990) and probably alters the development of demi-embryos influencing their gene expression (Velásquez et al., 2017). Therefore, it is important to select the bisected embryo, which must be of the highest quality (McEvoy & Sreenan, 1990), according to several morphological criteria (Stringfellow & Seidel, 1990).

One of the limitations of this meta-analysis was probably the low number of studies used for each response variable, this caused a wide variance between studies (0.31 to 0.66) and great heterogeneity (70 to 77%). The results show statistical heterogeneity when the true effects that are evaluated differ between studies and may be detectable if the variation between the results of the studies is above the expected (Higgins & Thompson, 2002). This heterogeneity could be due to the conditions in which the included studies were carried out and to the variables described in Table 1. In addition, in the case of the funnel plots, the studies could not be visualized clearly to assume symmetry or not, probably due to the reduced number of studies included. In the sensitivity analyses, when one study was excluded at a time, the excluded study did not change the results of the meta-analyses as a whole. Therefore, there is not sufficient evidence to elucidate an effect of the developmental stage (morula and blastocyst) at the time of the bisection process on the analyzed variables.

Embryo development is sequential and is strictly regulated by genes (Noli et al., 2017). This has been classified into several categories (early morula, compact morula, early blastocyst, blastocyst, expanded blastocyst, and hatched blastocyst) (Bó & Mapletoft, 2018). In the case of this meta-analysis, the morula (early and compact) and blastocyst (early, blastocyst, and expanded) categories were clustered, without considering hatched blastocysts. This clustering was carried out because, in some of the studies included in the meta-analysis, the precise embryo classification in terms of their developmental progress was not specified. Hence, it is necessary to provide more information about the characteristics of the embryos used in future studies to obtain objective results. In addition, the hatched blastocysts are more robust and have overcome the hatching process, then, they should be considered for future bisection studies, since they could have better survival (Velásquez et al., 2016). Finally, there was interest in knowing how some variables described in Table 1 had a statistical influence, including the species and breed of the bisected embryo, which have been evaluated in individual studies (Rangel-Santos, 1991; Vivanco et al., 1991; Hashiyada, 2017). However, the data structure did not allow for to inclusion of

any of them in the meta-analysis. Similarly, sorting the embryos before the bisection process is essential for the survival of the demi-embryos (McEvoy & Sreenan, 1990). Therefore, the bisection of morulae and blastocysts is recommended if they are of excellent quality.

Conclusions

The results of this meta-analysis indicate the feasibility of bisecting ovine and bovine embryos at morula and blastocyst stages, without negatively influencing the reproductive response of demi-embryo recipients. The pregnancy rate, survival of demi-embryos, and survival of pairs of demi-embryos were not compromised by the bisection process of embryos at different developmental stages. In addition, it was observed that a reduced number of experimental units in individual studies may influence the evaluation of the effect of the bisected embryo stage. Finally, as the productive response is acceptable, studies that apply and improve embryo bisection as a routine practice in livestock are justified.

CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

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TABLES

Table 1. Summary of the experimental comparison used in the meta-analysis, including the general description and the developmental stages used as treatments.

Study	Species	Breed	Developmental stage of bisected embryos (n)			Embryo quality[†]	Holding pipets	Surrogate zona pellucida
			Morula[‡]	Blastocyst[§]	Hatched blastocyst[¶]			
Williams et al., 1984	Bovine	-	103	62	-	1	Yes	Yes
Arave et al., 1987	Bovine	Holstein	64	27	-	1	Yes	Yes
McEvoy & Sreenan, 1990	Bovine	-	10	11	-	1	Yes	No
Gray et al., 1991	Bovine	-	660	333	-	1	Yes	Yes
Kippax et al., 1991	Bovine	Holstein	71	73	-	1	Yes	No
Rangel-Santos, 1991a	Sheep	Romney Marsh	-	8	19	1 and 2	No	No
Rangel-Santos, 1991b	Sheep	Drysdale	-	766	145	1 and 2	No	No
Rangel-Santos, 1991c	Sheep	Texel	13	21	-	1	No	No
Shelton, 1992	Sheep	Merino	20	24	-	-	No	No
Hashiyada, 2017	Bovine	-	233	43	-	-	No	No
Total (n)			1,174	1,368	164			

[†]Quality of bisected embryo= 1: excellent, 1 and 2: good and regular.
[‡]Morula: early and compact.
[§]Blastocyst: early, blastocyst, and expanded.
[¶]Stage was not considered in the meta-analysis due to the lack of comparable groups.

Table 2. Effect of the developmental stage of the bisected embryo on the reproductive efficiency of ovine and bovine demi-embryo recipients.[†]

Response variable	Developmental stage of bisected embryo (LSM ± SE)[‡]		*P*-value
	Morula	Blastocyst	
Pregnancy rate	0.52 ± 0.04	0.53 ± 0.04	0.12
Survival of demi-embryos	0.42 ± 0.03	0.42 ± 0.03	0.14
Survival of pairs of demi-embryos	0.17 ± 0.02	0.23 ± 0.03	0.14

[†]Logistic model results using the SAS 9.3 GLIMMIX procedure to determine reproductive differences between developmental stages of bisected embryos. The analysis considered the demi-embryos available after the bisection process.
[‡]Least-squares means and their respective standard error.

FIGURE LEGENDS

Figure 1. PRISMA information flow diagram through the different phases of a systematic review from the initial search to the selection of the studies included in the meta-analysis. The information was collected with different search engines and the results were screened to remove duplicated studies. The studies selected were those that reported the proportions of success and failure for each response variable.

Figure 2. Forest and funnel plot representation of the estimated odds ratio for the pregnancy rate of females receiving bisected embryos at different developmental stages.

Figure 3. Graphical representation of sensitivity analysis excluding one study at a time in the joint estimation of the meta-analysis for the pregnancy rate of females receiving bisected embryos at different developmental stages.

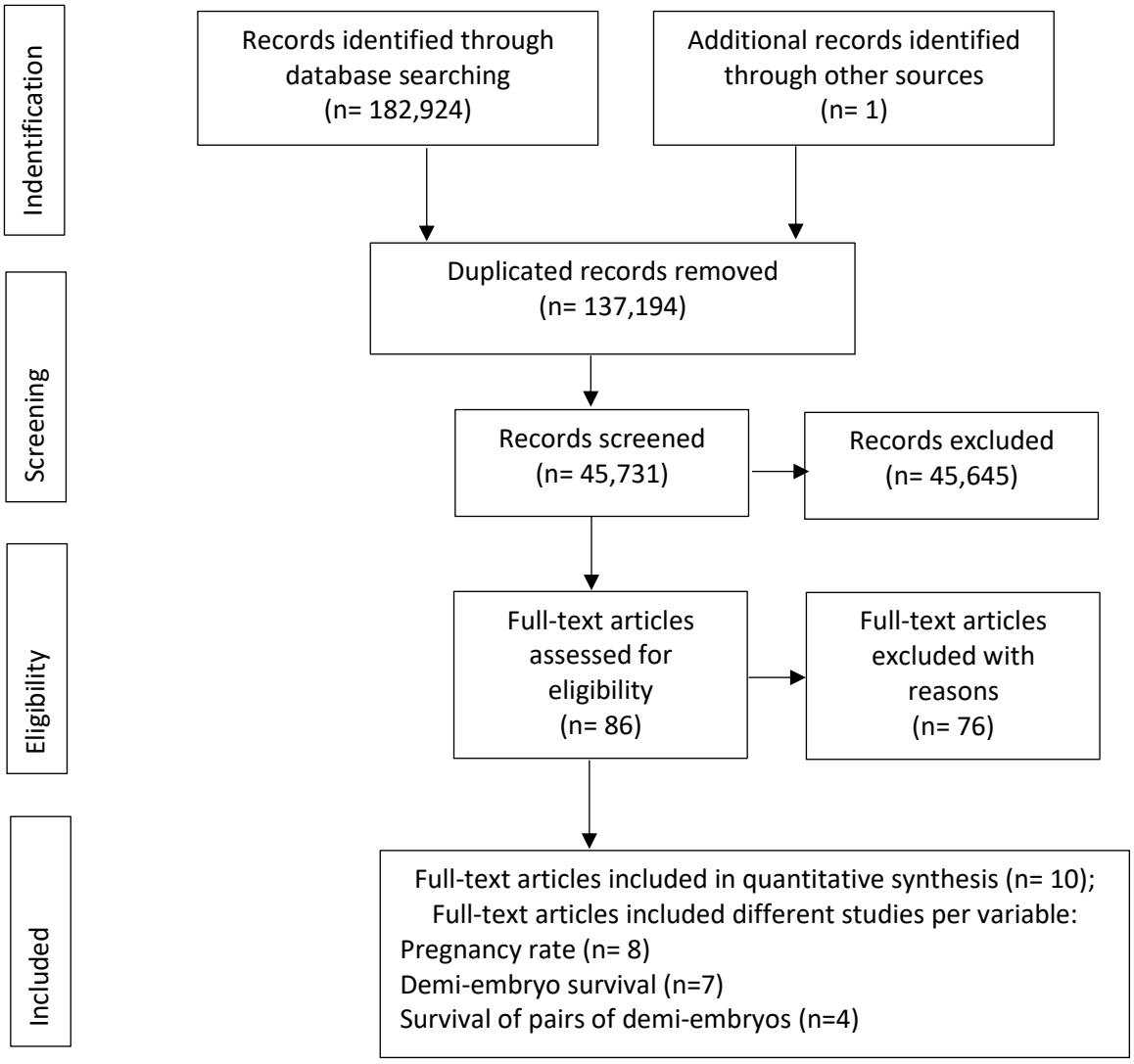
Figure 4. Forest and funnel plot representation of the estimated odds ratio for the survival of demi-embryos produced by bisected embryos at different developmental stages.

Figure 5. Graphical representation of sensitivity analysis excluding one study at time in the joint estimation of the meta-analysis for the survival of demi-embryos from bisected embryos at different developmental stages.

Figure 6. Forest and funnel plot representation of the estimated odds ratios for the survival of pairs of demi-embryos produced by bisected embryos at different developmental stages.

Figure 7. Graphical representation of the sensitivity analysis excluding one study at a time in the joint estimation of the meta-analysis for the survival of pairs of demi-embryos from bisected embryos at different developmental stages.

FIGURES



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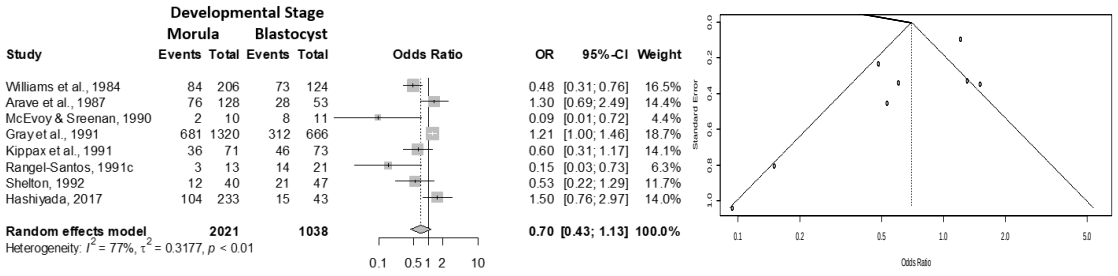

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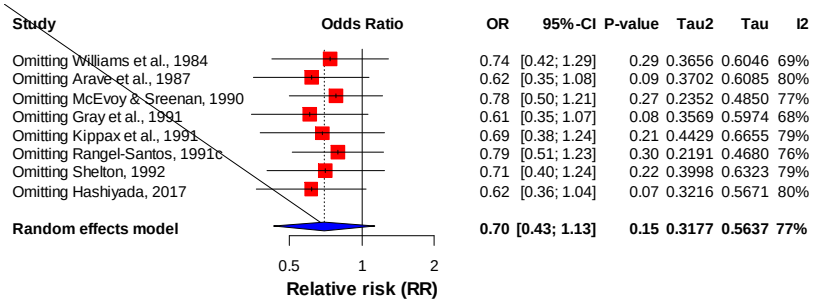

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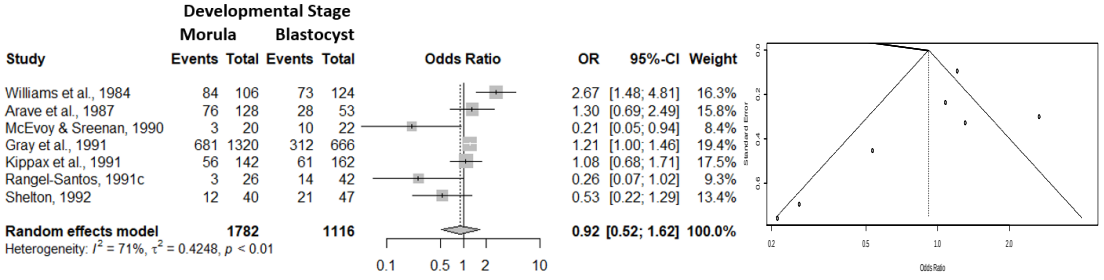

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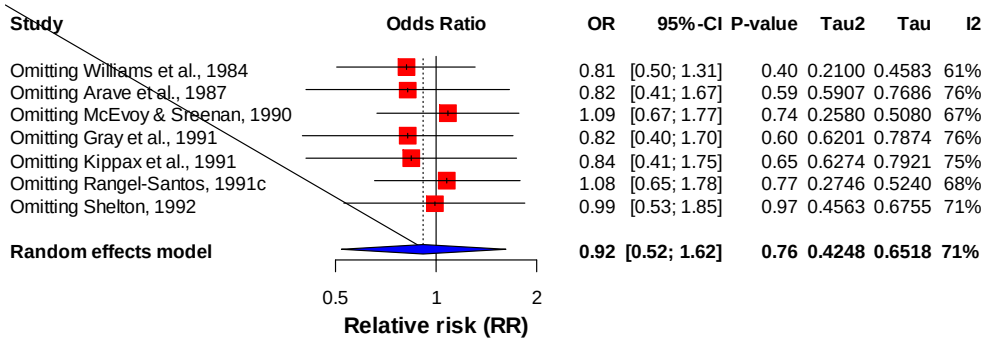


Figure 5. Graphical representation of sensitivity analysis excluding one study at the time in the joint estimation of the meta-analysis for the survival of demi-embryos from bisected embryos at different developmental stages.

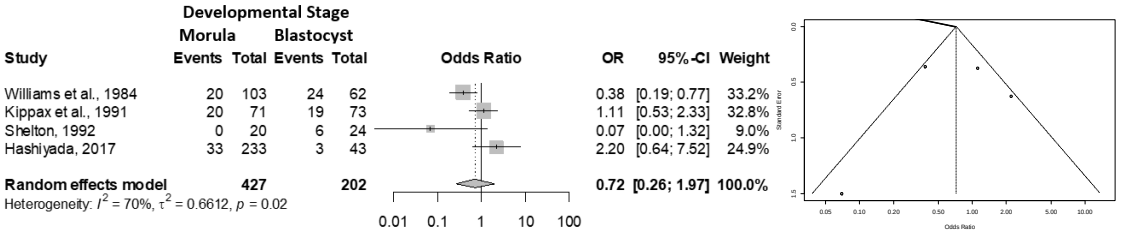
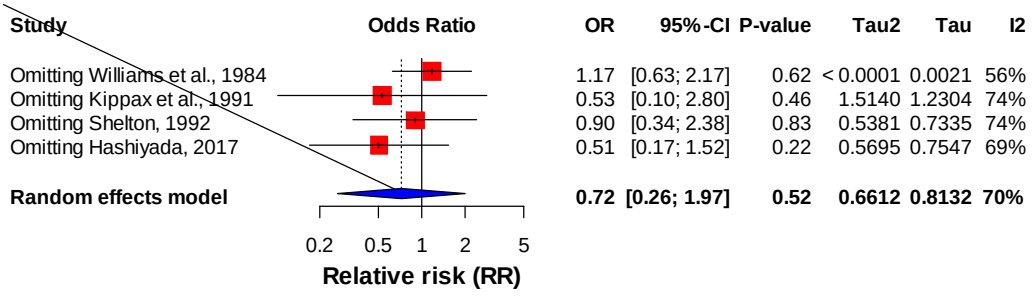


Figure 6. Forest and funnel plot representation of the estimated odds ratios for the survival of pairs of demi-embryos produced by bisected embryos at different developmental stages.



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Este capítulo es un artículo enviado a Italian Journal of Animal Science

5. Co-culturing low-quality ovine cumulus-oocyte complexes with denuded oocytes during *in vitro* maturation

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Dear Raymundo Rangel-Santos,

A manuscript has been submitted on your behalf.

Submission ID	**227670928**
Manuscript Title	**Co-culturing low-quality ovine cumulus-oocyte complexes with denuded oocytes during in vitro maturation**
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Abstract

Low-quality cumulus-oocyte complexes (COCs, <4 cumulus cell layers) and denuded oocytes (DOs) are frequently discarded in *in vitro* embryo production (IVEP) programs. Co-culturing could improve low-quality COCs competence. The present study evaluated the co-culturing low-quality COCs with DOs during *in vitro* maturation and its effect on embryo development. COCs were collected from slaughterhouse ovaries and morphologically classified. Only COCs with <4 cumulus cell (CCs) layers and homogeneous cytoplasm were considered. The experimental design was completely randomized. The COCs were assigned to one of two initial treatments, T1 (control)= COCs (n=323, without DOs); T2 (co-culture) = COCs + DOs (n=270+135, respectively). Gametes were matured in TCM-199 medium with 10% FBS and hormones for 24 h, at 5% CO_2, 38.5°C, and saturated humidity. Some DOs from the co-culture (T3, n=40) were independently fertilized and cultured, as well as a group of DOs without co-culture (T4 control, n=40). Fertilization was performed with fresh semen (1×10^6 mL^{-1}). The zygotes were cultured in a sequential medium for 72 and 96 h up to the blastocyst stage. SAS was used for data analysis. Co-culturing increased ($p<0.05$) by 10% morulae and blastocysts yield, and 22 µm the blastocyst diameter in the low-quality COCs group compared to the control. In the DOs groups, there was no significant difference ($p>0.05$) in the analyzed variables. Co-culturing low-quality COCs with DOs during *in vitro* maturation, increased the morula and blastocyst rates, as well as the blastocyst diameter.

Keywords: co-culture, cumulus-oocyte complex, embryo development, and denuded oocyte.

Highlights

- Low-quality COCs and denuded oocytes are generally discarded for IVEP.
- Co-culturing with denuded oocytes improved the low-quality COCs competence.
- Oocyte culture had a positive effect on embryo yield.

Introduction

The *In vitro* embryo production (IVEP) technique depends on obtaining cumulus-oocyte complexes (COCs) from live females (by ovum pick-up) or ovaries obtain from slaughterhouse. COCs selection is a common practice that is often based on morphological criteria and influences blastocyst production (De Bem et al. 2014). COCs are classified as grade I: >4 layers of cumulus cells (CCs), compact cumulus, and homogeneous cytoplasm; grade II: 3 or 4 layers of CCs, compact cumulus, and homogeneous cytoplasm; grade III: 1 to 3 layers of CCs and homogeneous cytoplasm; and grade IV: denuded oocytes (DOs, without CCs) (Davachi Kohram et al. 2012; Widyastuti et al. 2017). The CCs are important because they play an essential role in oocyte maturation and, consequently, in embryo development (Grive and Sauerbrun-Cutler 2021).

The oocyte depends on CCs to take up nutrients through cytoplasmic projections (Baena and Terasaki 2019), due to its limited ability to metabolize glucose, cholesterol, and amino acids (Richani et al. 2021). Glucose is metabolized to pyruvate in CCs via glycolysis, and pyruvate is used by the oocyte via oxidative phosphorylation (Richani et al. 2021). On the other hand, the oocyte seems to promote glucose metabolism in CCs by influencing their transcriptome, secreting several growth factors, such as Growth Differentiation Factor 9 (GDF9), Bone Morphogenetic Protein 15 (BMP15), and Fibroblast Growth Factor 8 (FGF8) (Richani et al. 2021). In most IVEP studies, grade I COCs are selected to achieve greater embryo production (Dey et al. 2012; Bunel et al. 2015). However, the quality of oocytes collected is variable, obtaining up to 49% of oocytes with few layers of CCs or completely denuded, which are generally not considered for IVEP (Davachi Kohram et al. 2012; De Bem et al. 2014).

Co-culturing viable COCs (>3 layers of CCs) and DOs increase the percentage and blastocyst quality, due to cumulative synergy in nuclear and cytoplasmic maturation, fertilization, and regulation of oxidative stress in oocytes (Dey et al. 2012). The factors GDF9 and BMP15 secreted

by the oocyte promote the proliferation and differentiation of CCs (Alam and Miyano 2020). Therefore, this study hypothesized that the endogenous factors secreted by the DOs could have a greater influence in supporting the low-quality COCs competence (<4 layers of CCs). This could allow the use of oocytes that are generally discarded in IVEP programs. This study aimed to evaluate co-culturing low-quality COCs with DOs during *in vitro* maturation and its effect on embryo development.

Materials and methods

Ethics statement

The ovaries were collected from adult ewes slaughtered in a local slaughterhouse based on the Official Mexican Standard NOM-033-ZOO-1995, Humanitarian Slaughter of Domestic and Wild Animals (Mexican official norm 1995), from January to April 2022. The entire experiment was conducted according to the guidelines of the Ethical Committee (No. CECBS1913) of Universidad Autónoma Metropolitana, Mexico. The ovaries were stored and transported to the laboratory in saline solution (0.9% NaCl) supplemented with 50 µg of gentamicin sulfate at 35 °C, for 1 h after collection.

COCs recovery and in vitro maturation

The COCs from eleven replicates were collected by follicular puncture. The punctured follicles were 2 to 6 mm in diameter, using an 18-gauge needle (18×38 mm) and a hypodermic syringe with TCM-199 medium plus HEPES, 50 µg mL^{-1} gentamicin sulfate, and 50 IU heparin mL^{-1} (Crocomo et al. 2016). The COCs were evaluated with a stereomicroscope (Leica, S8APO, Wetzlar, Germany) and classified into four categories according to the number of CCs layers (Davachi Kohram et al. 2012; Widyastuti et al. 2017). Grade I: >4 layers of CCs, compact cumulus, and homogeneous cytoplasm; grade II: 3 or 4 layers of CCs, compact cumulus, and homogeneous cytoplasm; grade III: 1 to 3 layers of CCs and homogeneous cytoplasm; and grade IV: denuded

oocytes (without layers of CCs) (Table 1). Grade II and III COCs were considered low-quality COCs and were used for this experiment. Grade IV oocytes were used for co-culturing low-quality COCs. Grade I COCs were not considered in this experiment.

Table 1. Classification and number of cumulus-oocyte complexes (COCs) collected from slaughterhouse ovaries (eleven replicates).

After sorting, the COCs were washed and cultured for 24 h in groups of 30-40 per well (Figure 1) (Nunc, Roskilde, Denmark). The maturation medium was TCM-199 (500 µL) supplemented with 10% (v/v) SFB (Biowest, Mayimex, Mexico City, Mexico), 5 µg mL^{-1} FSH (Folltropin-V, Bioniche, Ontario, Canada), 5 IU mL^{-1} hCG (Chorulon, Merck Shar, New Jersey, United States), and 1 µg mL^{-1} 17-β estradiol (Sigma Aldrich, Mexico City, Mexico) (Paramio and Izquierdo 2016), under 200 µL mineral oil (Sigma Aldrich, Mexico City, Mexico). The incubation conditions throughout the experiment were 38.5ºC, 5% CO_2, and saturated humidity (Paramio and Izquierdo 2016).

Figure 1. General description of co-culturing low-quality cumulus-oocyte complexes (grade II and III COCs) with denuded oocytes during *in vitro* maturation.

After 24 h, the oocyte maturation of both groups (control and co-culture) was evaluated according to the degree of maximum cumulus expansion and homogeneous cytoplasm (Romão et al. 2013). In the case of denuded oocytes (control and co-culture), they were evaluated by extrusion of the first polar body (Rao et al. 2002) using an inverted microscope at 200x (Nikon Eclipse TS100, Tokyo, Japan).

In vitro fertilization

Fertilization was performed with fresh semen collected from a Katahdin ram of known fertility using an artificial vagina. The semen sample remained at 22°C for 90 min (Romão et al. 2013). Then, 100 µL of semen plus 300 µL of commercial fertilization medium (Modified Tyrode, In vitro

SA, Mexico City, Mexico) were centrifuged at 225×g for 3 min. Sperm capacitation and selection were performed using the Swim-up technique (Younglai et al. 2001). Briefly, 100 µL of centrifuged semen were layered gently under 1 mL of fertilization media. The tube was slanted and incubated for 5 min. The swim-up sperm fraction was used for *in vitro* fertilization. Matured COCs were washed and placed in wells with 500 µL of fertilization medium plus 200 µL of mineral oil and incubated with 1×10^6 spermatozoa mL^{-1} for 22 h (Crocomo et al. 2016).

In vitro embryo culture

The presumed zygotes were washed and denuded by gentle pipetting. Groups of 30 to 40 zygotes were cultured in wells with 500 µL of cleavage medium (Cook IVF, Brisbane, Australia) plus 200 µL of mineral oil for 72 h. The cleavage rate was evaluated 30 h after the start of fertilization. After 72 h of culture, the morula rate was determined and the embryos were cultured in 500 µL of blastocyst medium (Cook IVF, Brisbane, Australia) plus 200 µL of mineral oil (Cocero et al. 2011) for 96 h. At the end of culture, the blastocyst rate was evaluated according to morphological criteria using an inverted microscope (Ushijima et al. 2009). Briefly, embryos at the blastocyst stage were considered those with a clear visual differentiation between the inner cell mass (ICM) and trophectoderm, dark and compact ICM, without visible defects in the trophectoderm and with intact zona pellucida.

Number of blastocyst cells

After sequential embryo culture (168 h), a random sample of blastocysts obtained from co-culturing low-quality COCs (n=20) and its respective control (without co-culturing, n=20) were selected. Blastocysts from both groups were stained for cell counting using the methodology described by Zabihi et al. (2019) with minimal modifications. Briefly, all blastocysts were fixed in 4% paraformaldehyde for 5 min at room temperature. Afterward, they were washed three times in PBS and stained with 0.1 mg mL^{-1} of bisbenzimide (Sigma Aldrich, Mexico City, Mexico) for 5 min.

Each blastocyst was placed separately on slides and the blue-stained nuclei were counted with an epifluorescence microscope (Nikon Eclipse E600, Tokyo, Japan) equipped with a UV-2A filter (wavelength: 330-380 nm).

Experimental design

The experimental design was similar to that used by Dey et al. (2012), described in Figure 1. Where the COCs were randomly matured under different culture conditions: T1 (Control)= low-quality COCs, T2= co-culture low-quality COCs + DOs (2:1, respectively). Fertilization and *in vitro* culture of the DOs from co-culture were performed independently, obtaining two more groups: DOs from the co-culture with its control (without co-culture).

Statistical analyses

Data were analyzed according to Gbur et al. (2012) with the SAS program (SAS 2021). The number of matured and fertilized low-quality COCs, as well as the number of morulae and blastocysts, were analyzed with the GENMOD procedure, assuming a binomial distribution with the Logit function. The diameter and number of blastocyst cells were analyzed with the GLM procedure, assuming a normal distribution. The statistical model was:

$$y_{ij} = \mu + T_i + \mathcal{E}_{ij}$$

where:

y_{ij}= diameter or number of blastocyst cells in the i^{th} maturation condition, μ= general mean, T_i= fixed effect of the i^{th} maturation condition, i= low-quality COCs (control) and co-culturing low-quality COCs + DOs; DOs (control) and DOs from co-culture. $\mathcal{E}_{ij}$= random error associated with the j^{th} diameter or number of blastocyst cells in the i^{th} maturation condition, assuming $\mathcal{E}_{ij}$~NIID (0, σ^2_e).

The ESTIMATE option was used for categorical variables and the TUKEY test for continuous variables to test differences between treatments. Results with $p<0.05$ were considered significantly

different. Least-square means and their respective standard error (LSM ± SE) are reported throughout the document.

Results

Table 1 shows the classification of 1,549 oocytes collected from 261 slaughterhouse ovaries. From the total, 40.4% of COCs were considered of low quality (grade II and III) and 16.1% as DOs (without cumulus). Grade I COCs (43.5%) were not included in this experiment. No differences (p>0.05) were found between co-culturing and the control for the maturation or cleavage embryo rate. The groups of DOs from the co-culture and its control did not show significant differences (p>0.05) for the mentioned variables (Table 2).

Table 2. *In vitro* maturation and cleavage embryos from co-culturing low-quality cumulus-oocyte complexes (COCs) with denuded oocytes (DOs).

Co-culturing low-quality COCs + DOs was higher with more than 10% (p<0.05) in the morula and blastocyst rate compared to the control group (Table 3). The DOs from co-culturing did not show a significant difference (p>0.05) when compared to its control group (6.5 to 9.6%).

Table 3. Morula and blastocyst rate from co-culturing low-quality cumulus-oocyte complexes (COCs) with denuded oocytes (DOs).

The blastocyst diameter was higher (p<0.05) in co-culturing low-quality COCs + DOs with 22 µm compared to the control group, while there was no difference in the number of blastocyst cells (p> 0.05) (Table 4). The blastocyst diameter of the DOs from the co-culturing was similar (p>0.05) to its respective control group.

Table 4. Diameter and number of blastocyst cells from co-culturing low-quality cumulus-oocyte complexes (COCs) with denuded oocytes (DOs).

Discussion

Co-culturing viable COCs (>3 layers of CCs) with DOs increases the embryo rate, due to the

cumulative synergy of endogenous factors secreted in the maturation medium (Hussein et al. 2006; Dey et al. 2012). In the oocytes recovered from slaughterhouse ovaries, 49% were low-quality COCs (0-3 layers of CCs) (Davachi Zeinoaldini et al. 2012). Low-quality COCs and DOs are generally discarded for IVEP programs (De Bem et al. 2014). These oocytes could be cultured together to take advantage of co-culturing. The present study evaluated the co-culturing low-quality COCs with DOs during *in vitro* maturation and its effect on embryo development. Co-culturing increased 10% morula and blastocyst yield and the blastocyst diameter in the group of low-quality COCs. Our results confirm previous reports by Hussein et al. (2006) and Dey et al. (2012) who found beneficial effects using co-culturing on *in vitro* maturation, reporting 13 to 17% more embryo yield. These authors used a ratio of 5 DOs for each COC during maturation. In the present study, a ratio of 1 DOs for every 2 COCs in the maturation medium was used (Figure 2). The main reason for using a lower proportion of oocytes was to take advantage of the DOs and low-quality COCs (<3 GC layers) that are normally recovered in ewe oocyte collection sessions (16.1 and 40.4%, respectively) (Table 1). However, 1 DO for every 2 low-quality COCs had a positive effect on low-quality COCs competence and their subsequent development (Figure 3).

Figure 2. Co-culturing low-quality COCs with DOs during *in vitro* maturation (×200).

Figure 3. Stained blastocyst (7 d) from co-culturing low-quality COCs with DOs. (×200).

Blastocysts diameter from the low-quality COCs was 22 µm greater when co-culture was used, which could be an indicator of good quality, because it is related to the number of embryo cells (Mori et al. 2002) and, consequently, with maternal recognition (Goff 2002). However, the number of blastocyst cells was similar (p=0.1) between treatments, contrary to reports in bovine embryos (Dey et al. 2012), where there was a significant difference. The endogenous factors secreted by the DOs in the maturation medium support the COCs competence (Hussein et al. 2005; Dey et al. 2012). The role of paracrine factors secreted by the oocyte is widely recognized in multiple

processes, such as cumulus expansion (Dragovic et al. 2005; Alam and Miyano 2020). The GDF9 protein is synthesized by the oocyte during folliculogenesis and induces cumulus expansion by stimulating hyaluronan synthase 2, cyclooxygenase 2, steroidogenic acute regulatory protein mRNA expression, and progesterone synthesis (Elvin et al. 1999; McNatty et al. 2005). Endogenous factors secreted by the oocyte can increase the expression of the steroidogenic acute regulatory protein (STAR) and glutathione peroxidase 1 genes in cumulus cells (Dey et al. 2012). The ability of oocytes to regulate CCs proliferation appears to be mediated by the developmental stage (Eppig 2001). The GDF9 and BMP15 mRNA proteins are expressed in the oocyte into the primary follicle with only one layer of CCs (McGrath et al. 1995; Dube et al. 1998) and participate as mitogenic factors in the regulation of cell proliferation (McNatty et al. 2005). Therefore, possibly these proteins secreted by DOs could have influenced the proliferation and expansion of the cumulus in COCs with a low number of CCs layers. This is important because glucose, cholesterol, and amino acids are metabolized in the cumulus, and it is essential for meiotic and cytoplasmic maturation (Richani et al. 2021). Unfortunately, molecular determinations to evaluate cumulus cell proliferation after co-cultivation were beyond our reach. However, even when no effect was found on the maturation rate, *in vitro* embryo development provides information about the improvement in the competence of low-quality COCs using co-culturing. Therefore, it is suggested to carry out molecular studies focused on improving the oocyte quality with a few layers of CCs.

On the other hand, the DOs from co-culturing underwent the entire IVEP process. However, there were non-significant differences for any of the variables evaluated. Contrary to results found by Dey et al. (2012), who observed an improvement in the DOs competence used in the co-culture (10% more blastocyst yield). It is possible that, due to the smaller number of cumulus layers (<4) of the COCs used in the present study, the secretion of factors able to stimulate DOs competence

was not sufficient. The embryo production results confirm the low developmental competence of DOs (<10%) (Tanghe et al. 2003; Stamperna et al. 2020). Taken together, co-culturing low-quality COCs and DOs could be used to increase the number of embryos produced.

Conclusion

Co-culturing low-quality cumulus-oocyte complexes with denuded oocytes during *in vitro* maturation, increased 10% morula and blastocyst yields and blastocyst diameter, under the conditions of this study.

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Declaration of interest statement

None of the authors have any conflict of interest to declare.

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Tables

Table 1. Classification and number of cumulus-oocyte complexes (COCs) collected from slaughterhouse ovaries (eleven replicates).

Categories	COCs (%)
Grade I	674 (43.5)
Grade II	322 (20.8)
Grade III	303 (19.6)
Grade IV	250 (16.1)
Total	1,549

Classification of COCs from slaughterhouse ovaries according to the number of cumulus cell layers (Davachi Kohram et al. 2012; Widyastuti et al. 2017).

Table 2. *In vitro* maturation and cleavage embryos from co-culturing low-quality cumulus-oocyte complexes (COCs) with denuded oocytes (DOs).

Treatment	COCs (n)	Maturation rate n (LSM ± SE, %)[1]	Cleavage rate n (LSM ± SE, %)[1]
COCs control	323	285 (88.24 ± 1.79)	223 (69.04 ± 2.57)
COCs + DOs[2]	270 + 135	270 (87.04 ± 2.04)	195 (72.22 ± 2.72)
DOs control	40	18 (45.00 ± 7.86)	15 (37.50 ± 7.65)
DOs[2]	40	17 (42.50 ± 7.81)	13 (32.50 ± 7.40)

[1]Least-squares means ± standard error.

[2]Denuded oocytes from co-culturing.

Table 3. Morula and blastocyst rate from co-culturing low-quality cumulus-oocyte complexes (COCs) with denuded oocytes (DOs).

Treatment	COCs (n)	Morula rate n (LSM ± SE, %)[1]	Blastocyst rate n (LSM ± SE, %)[1]
COCs control	323	191 (57.22 ± 4.92)[a]	52 (14.23 ± 2.53)[a]
COCs + DOs[2]	270 + 135	189 (69.71 ± 4.44)[b]	70 (24.40 ± 3.56)[b]
DOs control	40	14 (25.98 ± 10.29)[A]	2 (6.50 ± 4.73)[A]
DOs[2]	40	11 (33.21 ± 11.01)[A]	3 (9.68 ± 5.97)[A]

[1]LSM ± SE, Least-squares means ± standard error within a column without a common superscript (capital or lowercase letters) differ significantly ($p < 0.05$).

[2]DOs, denuded oocytes from co-culturing.

Table 4. Diameter and number of blastocyst cells from co-culturing low-quality cumulus-oocyte complexes (COCs) with denuded oocytes (DOs).

Treatment	Blastocyst diameter, µm (LSM ± SE)[1]	No. of blastocyst cells (LSM ± SE)[1]
COCs control	203.78 ± 6.43[a]	87 ± 2.13[a]
COCs + DOs[2]	225.89 ± 5.47[b]	91 ± 2.07[a]
DOs control	193.78 ± 32.84[A]	
DOs[2]	208.94 ± 26.72[A]	

[1]LSM ± SE, Least-squares means ± standard error within a column without a common superscript (capital or lowercase letters) differ significantly ($p < 0.05$).

[2]DOs, denuded oocytes from co-culturing.

Figures

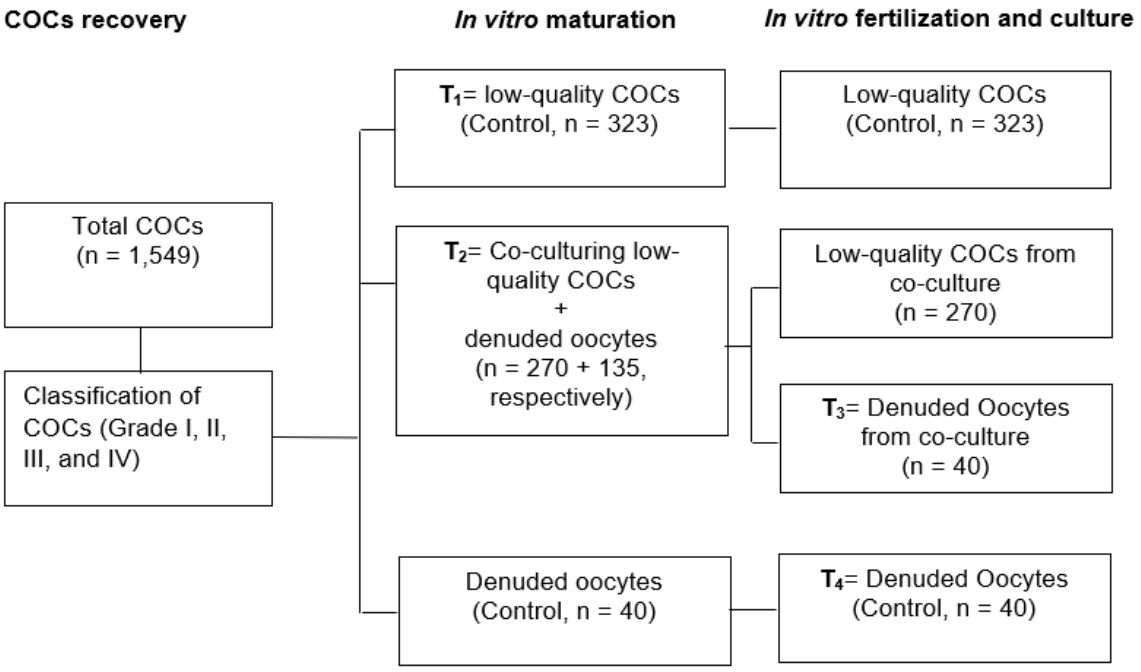

Figure 1. General description of co-culturing low-quality cumulus-oocyte complexes (grade II and III COCs) with denuded oocytes during *in vitro* maturation.

Figure 2. Co-culturing low-quality COCs with DOs during *in vitro* maturation (×200).

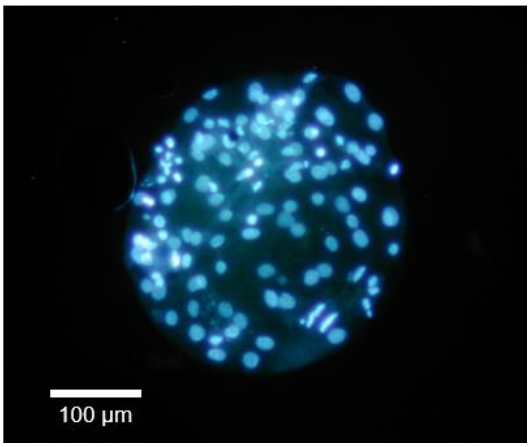

Figure 3. Stained blastocyst (7 d) from co-culturing low-quality COCs with DOs. (×200).

Figure captions

Figure 1. General description of co-culturing low-quality cumulus-oocyte complexes (grade II and III COCs) with denuded oocytes during *in vitro* maturation.

Figure 2. Co-culturing low-quality COCs with DOs during *in vitro* maturation (×200).

Figure 3. Stained blastocyst (7 d) from co-culturing low-quality COCs with DOs.

6. CONCLUSIONES GENERALES

La calidad del embrión bisectado es importante para el éxito de la reconstitución y sobrevivencia de los semi-embriones ovinos.

La eficiencia de la producción de embriones *in vitro* fue mayor cuando se utilizaron ovocitos de ovejas adultas no gestantes.

La bisección podría ser factible en embriones en etapa de mórula y blastocisto, sin influir negativamente en la respuesta reproductiva de las hembras receptoras de semi-embriones ovinos y bovinos.

El co-cultivo de complejos cúmulo-ovocito de baja calidad con ovocitos desnudos, durante la maduración *in vitro,* mejoró la producción de embriones ovinos.

La eficiencia de la producción de embriones se mejoró involucrando biotecnologías como la bisección. Además, se debe considerar la procedencia de los ovocitos sometidos al sistema *in vitro.*