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Ovum Pick Up (OPU) and *in vitro embryo* production in pregnant cows (*Bos Indicus*) at the Bachigualatito rancho, la Trinitaria, Chiapas

Aspiración Folicular (OPU) y producción *in vitro* de embriones de vacas gestantes (*Bos Indicus*) en el rancho Bachigualatito, la Trinitaria, Chiapas

NOGUEZ-ESTRADA, Juan†*, CORNEJO-CERVANTES Judith, VARGAS-MONTER, Jorge and SEBASTIAN-MEDINA Octavio

Universidad Politécnica de Francisco I Madero

ID 1st Author: *Juan, Noguez-Estrada* / **ORC ID**: 0000-0002-0493-8843, **Research ID Thomson**: X-4052-2018

ID 1st Co-author: *Judith Cornejo-Cervantes* / **ORC ID**: 0009-0000-9708-9213

ID 2nd Co-author: *Jorge, Vargas-Monter* / **ORC ID**: 0000-0001-9845-2598

ID 3rd Co-author: *Sebastián, Medina-Octavio* / **ORC ID**: 0009-0006-0451-5687

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Abstract

With the objective of determining the production, quality and development of bovine embryos (*Bos Indicus*), oocytes were collected from 14 females distributed in two groups: empty females (T1; n=7) and an experimental group of pregnant females (T2; n=7). The two groups were subjected to Ovum Pick Up (OPU) using disposable 18-gauge needles and a vacuum pressure of 100 mmHg. to later carry out in vitro fertilization (IVF). The study variables were the number of oocyte aspirated and fertilized and the expected embryos. A Wilcoxon test was performed for non-parametric data between two groups, finding no significant differences between treatments P> 0.05. 250 oocytes were recovered by fertilizing out of 190, obtaining a fertilization rate of 77%, reaching a transferrable blastocyst rate of 24% (45/190). In empty females (7), 113 oocytes were obtained, reaching a fertilization rate of 75% (81/113) and 25% transferable blastocysts (18/81). In the 7 pregnant females, 137 oocytes were collected, reaching a fertilization rate of 83% (109/137) and 23% transferable blastocysts (27/109). Follicular aspiration for *in vitro* fertilization of embryos from pregnant and empty cows is viable without differences between them, but being more variable in pregnant cows.

Bovine, Blastocyst, Oocytes, *In Vitro*

Resumen

Con el objetivo de determinar la producción, calidad y desarrollo de embriones bovinos (*Bos Indicus*), se recolectaron ovocitos de 14 hembras distribuidas en dos grupos: hembras vacías (T1; n=7) y hembras gestantes (T2; n=7). Los dos grupos fueron sometidos a aspiración folicular (OPU) utilizando agujas descartables de calibre 18 y una presión de vacío de 100 mmHg. para posteriormente llevar a cabo la fertilización in vitro (FIV). Las variables de estudio fueron número de ovocitos fertilizados y la previsión de embriones. Se realizó una prueba de Wilcoxon para datos no paramétricos entre dos grupos, no encontrando diferencias significativas entre tratamientos P>0.05. Se recuperaron 250 ovocitos, fertilizando 190, obteniendo una tasa de fertilización del 77%, con una tasa de blastocistos trasferibles del 24% (45/190). En hembras vacías (7), se obtuvieron 113 ovocitos, una tasa de fertilización del 75% (81/113) y 25 % de blastocistos transferibles (18/81). En las 7 hembras gestantes, se recolectaron 137 ovocitos, una tasa de fertilización del 83% (109/137) y un 23 % de blastocistos transferibles (27/109). La aspiración folicular para la fertilización *in vitro* de embriones provenientes de vacas gestantes y vacías es viable sin diferencias estadísticas entre ellas, pero con tendencias favorables con vacas gestantes.

Bovino, Blastocisto, Ovocitos, *In Vitro*

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† Researcher contributing as first author.

Introduction

Embryo transfer (ET) maximises herd reproduction through genetic improvement and the ability to ensure the birth of a greater number of offspring of the desired sex. In general, embryos are more resistant to heat stress than gametes, representing an advantage over artificial insemination (AI) and fixed-time artificial insemination (FTAI). ET together with the benefits of sexed semen application and *in vitro* embryo production (IVPP) are some of the main strategies to improve the livestock sector. (Zangirolamo, y otros, 2018).

IVPP and applied reproductive technologies in livestock have shown significant progress in recent years. The combination of IVPP with sexed semen (SS) and genomic selection (GS) is proving successful and widely used in countries all over the world (Ferré, y otros, 2020). The use of *in vitro* fertilisation (IVF) in bovine embryo production has increased globally to accelerate the selection of cows with high genetic values. The selection of embryos with high implantation potential is a critical factor in establishing gestation (Magata, 2023).

There are still unresolved aspects of IVEP that limit wider implementation of the technology, including low fertility due to the use of SS, reduced oocyte quality after *in vitro* maturation and low cryo-tolerance, which reduces gestation rates compared to in vivo produced embryos (Ferré, y otros, 2020).

In addition, the climatic and management conditions of the recipient females on the different farms influence the gestation rate of the transferred embryos (Pérez-Mora, Segura-Correa, & Peralta-Torres, 2020).

IVEP is a technique that makes it possible for unfertilised eggs to mature, fertilise and develop under laboratory conditions. Unfertilised oocytes and semen from selected bulls are the raw material for this technique. Under optimal conditions, about 95% of the oocytes can reach maturation, and of these, about 80% can reach the two-cell stage; however, less than half (30-50%) reach the blastocyst stage seven days after fertilisation (Ferré, y otros, 2020).

Oocyte maturation is a complex process involving nuclear and cytoplasmic modulations, during which oocytes acquire their ability to fertilise and support embryonic development. The oocyte is apparently "primed" for maturation during its development in the dominant follicle (Razza, y otros, 2019).

For best results, the technical components of transfer must be improved: nutrient quality and composition, synchronisation protocols, freezing and handling during embryo transfer. The conscious minimisation of these factors has important economic implications, favouring the efficiency of the transfer TE (Wieczorkiewicz, Jaśkowski, Wichtowska, Olszewska Tomczyk, & Jaśkowski, 2021).

The causes of low fertility in dairy cattle are complex and multifactorial, and may be due to compromised follicular development affecting oocyte quality, a sub-optimal reproductive tract environment unable to support normal embryo development, or a combination of both. (Lonergan & Sánchez, 2020).

The size of the CL influences pregnancy rate, with a higher likelihood being observed when transferring the embryo into the horn ipsilateral to a CL3 and 2, and less likely when transferring into a recipient with a CL1 < 15 mm in diameter. In addition, there is a greater likelihood of achieving pregnancy by transferring embryos at the BX expanded blastocyst and BL blastocyst stage of development, compared to BI early blastocyst and hatching developments BN (Valencia Ocampo, Rodríguez Colorado, & Mantilla, 2023).

Progesterone (P4) plays a key role in reproductive events associated with the establishment and maintenance of pregnancy through its effects on oocyte quality and its action on the uterine endometrium. Reduced P4 concentrations during ovulatory follicle growth are associated with lower fertility, and low circulating P4 concentrations after ovulation have been associated with lower pregnancy rates in cattle (Lonergan & Sánchez, 2020).

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In determining the effect of plasma progesterone (P4) on oocyte retrieval, oocyte quality and early *in vitro* embryo development in *Bos indicus* cows, the presence of higher P4 has a positive effect on oocyte retrieval, oocyte quality and early *in vitro* embryo production (IVEP) outcomes (Saad , y otros, 2019).

The efficiency of producing embryos using *in vitro* technologies in livestock species rarely exceeds the 30% to 40% threshold, indicating that the proportion of oocytes that fail to develop after fertilisation and *in vitro* culture is considerably large. The presence of cycle-related structures in the ovaries, follicle size between 6 and 10 mm, large number of cumulus cells, large oocyte diameter (>120 microns), quality of cytoplasm, structure of the perivitelline space, zona pellucida and polar corpuscle morphology have been associated with better quality. Sorting and selection of oocytes in livestock species for *in vitro* embryo production and micromanipulation techniques may be one of the most important steps in achieving superior embryo development and quality (Aguila, y otros, 2020).

In Holstein cattle, the combination of low oocyte recovery, young donor age and milk production status negatively influences IVEP. Oocyte quality is a key factor in obtaining a live calf from an in vitro embryo. Follicular wave synchronisation and the use of follicle stimulating hormone (FSH), improve oocyte quality and thus embryo production. Quality control in the laboratory and the use of high quality inputs are essential to reduce variability in production (Demetrio, y otros, 2020).

Embryo production consists of three stages, in vitro maturation (IVM) of oocytes, in vitro fertilisation (IVF) and in vitro culture (IVC) of potential zygotes, seeking to obtain quality blastocysts for greater reproductive efficiency (Salgado Cruz & Lopera Vásquez, 2020). A topic of discussion over the years is the viability of oocytes recovered by means of the Ovum Pick Up (OPU) technique from pregnant *Bos indicus* females versus oocytes obtained from empty females, because it is believed that the stimulation of progesterone produced by the corpus luteum increases oocyte viability, however, at the field level no difference has been observed in terms of in-vitro production (IVP).

Due to the limited information reported in Mexico on this subject, it is not possible to determine whether progesterone actually influences the improvement of oocyte quality and thus to have a better picture of embryo production. It is of utmost importance for the farmer to be aware of the advantages and disadvantages of subjecting pregnant females to follicular aspiration in order to be able to make decisions at crucial moments, in addition to the fact that this research can support a more accepted theory on this subject in Mexico. Therefore, in the present study the fertilisation and production of *in vitro* embryos obtained by follicular aspiration of pregnant cows (*Bos indicus*) in the Bachigualatito ranch, La Trinitaria, Chiapas, was evaluated.

Methodology to be developed

Location of the study area

The study was carried out at the Bachigualatito S.P.R. de R.L. ranch, located in La Trinitaria, Chiapas; with an altitude of 1540 m.a.s.l. (Figure 1). Fourteen *Bos Indicus* heifers were used in random stages of their estrous cycle with a Body Condition (CC) of 3 to 3.5 in a scale from 1 to 5, kept in grazing and with mineral salts supply, which were randomly distributed in two treatments: T1 empty cows and T2 Pregnant cows. Embryo production in the state of Chiapas, Mexico.

Figure 1 Batch of donor females (*Bos indicus*)

Follicular aspiration

The follicular aspiration process (Figure 2) started with epidural anaesthesia, 5 ml of 2% Lidocaine to minimise peristaltic movements and to have a better manipulation of the ovary. The perineal region was washed and disinfected and by means of a Mindray ultrasound equipment with a 5 mHz microconvex transducer which was adapted to a guide that was introduced into the vagina up to the fornix locating the ovaries via the rectum.

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With the help of the mandrel the aspiration was performed using 18 gauge needles that goes from a system to a falcon tube (corning 50 ml.) with 10 ml. of commercial flushing medium. A vacuum pump with a foot pedal was used throughout the process, applying a suction of 100 mmHg.

The aspirated cumulus-oocyte complexes (COCs) were between 2 and 10 mm in diameter.

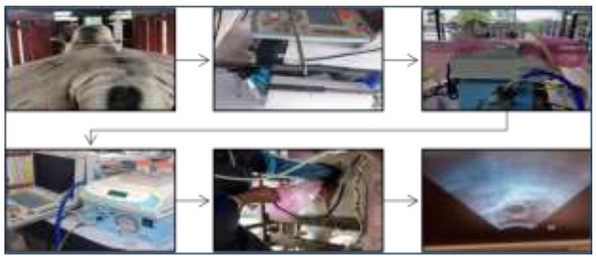

Figure 2 Methodology for follicular aspiration
Own Source

Selection and sorting

For the search, selection and sorting process, the laboratory equipment was placed in a contamination-free, sanitised area (Figure 3). All materials (pipettes, Petri dishes, falcon filter, serological pipette, 20 ml syringe) were placed on a heat platen at 37°C. At the end of oocyte aspiration, two washes were performed, tilting the collection tube at 45°, using a 20 ml flushing syringe and using a Falcon filter, placing the oocytes with the help of a serological pipette in a 30 x 40 ml Petri dish for searching with an EtScope stereoscope at 100X magnification, with a 10 µl pipette. COCs were selected and classified according to their morphology according to String fellow and Givens (2010):

Grade I are oocytes with more than three layers of compact cumulus cells and homogeneous, uniformly granular cytoplasm where the ooplasm filled the interior of the zona pellucida. Grade II are oocytes with less than three layers of cumulus cells and generally homogeneous cytoplasm. Grade III are oocytes with a single layer of cumulus cells and irregular looking cytoplasm with dark areas, the ooplasm is contracted, with space between the cell membrane and zona pellucida, irregularly filling the perivitelline space. No Cumulus bare oocytes, cytoplasm with abnormal colour and granulation and apoptotic cells, expanded are oocytes with expanded, degenerated cumulus that started their hatching process.

Oocytes were transported in 12 x 16 test tubes with medium in a TREO incubator at a temperature of 37.8 ° C.

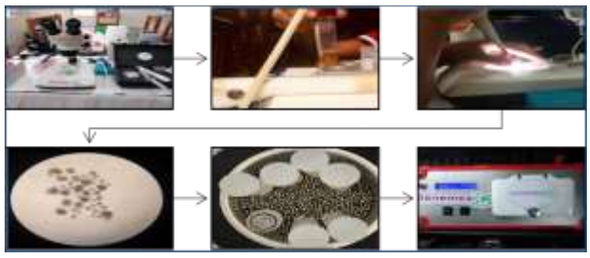

Figure 3 Oocyte search, selection and classification
Own Source

Oocyte maturation

For the in-vitro maturation process (IVM), only Grade I, Grade II and Grade III oocytes were selected, those with granular, homogeneous cytoplasm, without dark spots or clear spaces. The selected oocytes were transferred to culture plates containing HTF maturation medium, supplemented with 10% foetal bovine serum, oestradiol and follicle stimulating hormone (FSH), covering each cell with mineral oil, then placed in a NUAIRE incubator for 24 hours to be equilibrated in an atmosphere with 5% CO2, 5% O2 and 90% N2, at a temperature of 39ºC and maximum humidity (Figure 4). The theory of maturation is that the oocyte completes meiosis in response to the ovulatory LH surge. *In vitro* maturation requires 24 hours for the oocyte to complete nuclear maturation and reach the MII stage. It is also very important that there is cytoplasmic maturation, as this prepares the oocyte to support fertilisation and provide the nutrients required for embryo development.

In vitro fertilisation

Once the oocytes were matured for 24 h, conventional sperm capacitation was performed. Motile spermatozoa were obtained by centrifugation through a discontinuous gradient of Percoll 350 µl by adding 5 µl of the prepared semen in a 1 ml microcentrifuge tube at 3000 rpm for 5 minutes (Figure 4), to subsequently remove the seminal plasma components, cryoprotectants and dead or low vitality spermatozoa. The most motile fraction is enabled to penetrate the zona pellucida of the oocyte. Semen was added to each of the fertilisation cells with a pipette.

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Fertilisation plates and media were prepared prior to the start of semen capacitation. Approximately 1 million sperm per ml of media were added to the fertilisation plates (Figure 4) within the first minute after capacitation and placed in the incubator at 39ºC. Equilibrated to 5% CO2, 5% O2 and 90% N2, at maximum humidity, and cultured for a period of 20 hr.

Figure 4 *In vitro* fertilisation
Own Source

The embryo cultures were removed from the fertilisation plates and washed in new Syntetic Oviductal Fluid (SOF) medium, evaluated and the fertilised oocytes were transferred to the culture plates and placed in the incubator at 38.8ºC, equilibrated with 5% CO2, 5% O2 and 90% N2, at 100% relative humidity. On the fourth day, an evaluation of the embryos was carried out with the stereo microscope, supplementing the medium with pyruvate.

The number of blastocysts obtained was assessed at 7 days after IVF (Figure 5).

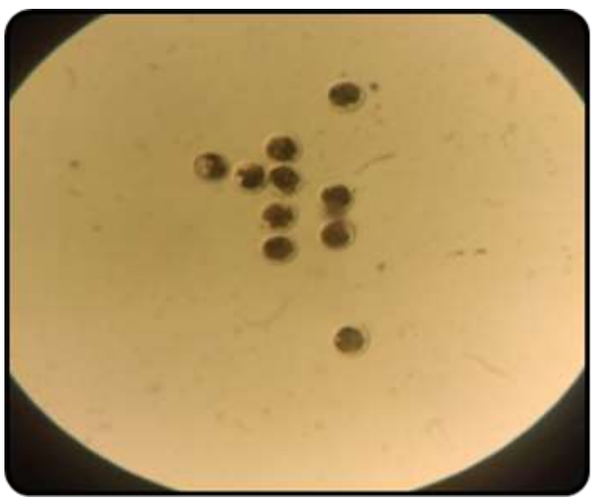

Figure 5 Blastocyst evaluation (in italics)

On day 7 of IVF, embryo transfer (Figure 6) was performed in the pre-implantation stage in the uterus of 45 recipient females.

Rectal palpation and ultrasound evaluation was performed to locate the ovary and state of the CL (corpus luteum), being in the horn ipsilateral to the C.L. where the embryo was transferred, in the lower third of the uterine horn. A TE gun was used, taking the straw by the end with a cotton seal, with the A.I. technique.

The TE gun was fitted with a sterile plastic sleeve to avoid introducing foreign bodies and microorganisms into the cow's reproductive tract. Epidural anaesthesia (5ml Lidocaine 2%) was applied between the sacral joints and the first coccygeal vertebra to block peristaltic movements and the anal sphincter muscle.

Figure 6 Methodology for embryo transfer
Own Source

Variables to be measured

The variables Oocytes aspirated, fertilised oocytes and transferable blastocysts were evaluated.

Statistical analysis

A completely randomised design was used, with the experimental unit being 14 cows.

$$Y_{ij} = \mu + Ti \quad + \varepsilon ij$$

Data were analysed by analysis of variance and comparison of means with Tukey's test ($\alpha \leq 0.05$) using SAS statistical software version 9.0. Data for all variables were analysed with the statistical package R Development Core Team (2019). R: A language and environment for statistical computing. A Wilcoxon test was performed for non-parametric data between two groups.

Results

250 oocytes were retrieved, fertilising 190 and obtaining a fertilisation rate of 77%, achieving a transferable blastocyst rate of 24% (45/190) (Table 1), of which 113 oocytes were retrieved from 7 empty females achieving a fertilisation rate of 75% (81/113) and a 25% transferable blastocyst rate (18/81).

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From the 7 pregnant females 137 oocytes were obtained achieving a fertilisation rate of 83% (109/137) and 23% transferable blastocysts (27/109). Similar fertilisation values of 733% and an embryo development rate of 35.1% were found in *in vitro* studies of oocytes collected at slaughterhouses (Fernández, Díaz, & Muñoz, 2007). In Fleckvieh heifers under tropical conditions, a blastocyst production rate between 41 and 58% is reported, with different aspiration frequencies in tropical climates (Solís-Corrales, Reinaldo, Morales, Ferrante, & Denis García, 2020).

Number	Donor Identification	Oocyte aspirate	Fertilised Oocytes	% Fertilised	Embryo forecasting	% Donor production
1	004/01	12	9	75	3	33
2	372	13	10	77	3	30
3	647	3	3	100	2	67
4	19	26	13	50	3	23
5	393	7	4	57	1	25
6	273	27	20	74	2	10
7	20	25	22	88	4	18
8*	639	15	14	93	4	29
9*	323	35	30	86	7	23
10*	509	16	15	99	5	33
11*	272	20	13	65	2	15
12*	267	35	27	77	5	19
13*	618/8	6	5	83	3	60
14*	1057	10	5	50	1	20
Total		**250**	**190**	**77**	**45**	24

Table 1 *In vitro* embryo production. Rancho Bachigualatito S.P.R. de R.L. *Pregnant females

Figure 7 shows the total mean number of fertilized oocytes for each treatment, being in cows with an average of 11.57 fertilized oocytes while, in pregnant cows, the average was 15.57 oocytes without significant differences (P>0.05), observing a greater dispersion of the data in pregnant cows.

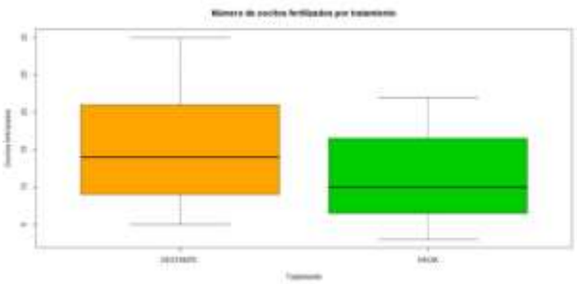

Figure 7 Number of fertilised oocytes per treatment

Figure 8 shows the total mean number of transferable blastocysts per treatment. In empty cows an average of 2.57 transferable blastocysts were obtained while in pregnant cows the average was 3.85 blastocysts.

Similar studies report 3.5 viable oocytes and 1.1 blastocysts per cow (Quispe E., Ancco G., Solano A., Unchupaico P., & Mellisho S., 2018). In OPU collection, an average of 4.3 viable oocytes is reported in Bos Taurus (Anchordoquy, y otros, 2013). In general, oocytes recovered in vivo after OPU develop better to the blastocyst stage than those obtained from ovaries in traces (Karadjole, y otros, 2010).

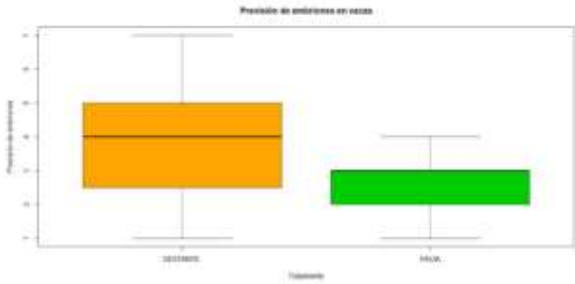

Figure 8 Number of transferable blastocysts

In studies of aspiration, procurement, selection and maturation of oocytes in vitro, it is reported that only 40% reach the blastocyst stage and of these, between 5 and 20% will produce pregnancies (Mayes M. & Sirard M., 2001). In the production of embryos, the aim is to make *in vitro* fertilisation more efficient and to mature as many oocytes as possible from in vivo aspiration in order to make the best use of donors with high genetic value without affecting their pregnancy status.

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Conclusions

Follicular aspiration for *in vitro* fertilisation of embryos from pregnant and empty cows is feasible without differences between them, however, the variability indicates that there are more factors that may be interfering and that need to be controlled and studied. The results are based on the methodology and experience to improve the embryo production programme in Zebu breeds.

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