In Vitro Production and Transfer of Buffalo Embryos (*Bubalus bubalis*) in Argentina

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Abstract: *Objective*: This study aimed to evaluate different hormonal stimulation protocols over follicular development, oocyte quality, embryo production, and embryo transfer outcome.

Methods and Materials: This study was performed in Argentina. Fifty-three non-pregnant females with proven fertility and good body condition were selected to produce embryos *in vitro*. Three ovarian stimulation protocols were tested: On Day 0, animals received a progesterone intravaginal device plus Estradiol Benzoate. On Day 4: TRT1 did not receive any treatment, TRT2 FSH (Folltropin-V®), TRT3 recombinant eCG (FoliRec®) TRT 4 serum eCG (Ecegon®), on day 7 oocyte retrieval was performed using ultrasonography. Embryos were produced, vitrified, and transferred to synchronized recipients using standard protocols. The number and quality of oocytes, number and size of follicles, embryo production, pregnancy rates, and Antimullerian hormone (AMH) levels were determined. Quantitative variables were compared, and an α level of 5% was considered significant.

Results: The total number of oocytes, follicles, and zygotes did not differ across the treatments. Better oocytes were obtained in TRT2 and TRT4 ($p<0.05$). Embryo production was highest in TRT2 (1.5 embryos/buffalo/OPU), and cleavage was higher in TRT3 ($p<0.05$). Forty-two embryos were transferred, and 12 live births were obtained. All were males.

Conclusions: The stimulation protocols used before OPU don't improve the number of ovarian follicles; some treatments produce higher-quality oocytes and embryo production. AMH dosage can help in selecting animals to increase the efficiency of IVEP. Reasonable results were obtained from the transfer of vitrified embryos, and the subsequent birth of live animals demonstrates the viability of this technique.

Keywords: Biotechnology, ovarian stimulation, oocyte.

1. INTRODUCTION

The domestic buffalo (*Bubalus bubalis*) was introduced to Argentina at the beginning of the 20th century through the importation of animals of Mediterranean, Murrah, and Jafarabadi breeds [6]. Currently, the country has a stock exceeding 200,000 buffalo heads [22], showing an annual growth rate varying between 9% and 13% over the past 25 years [5]. This increase demonstrates the current potential of buffalo farming as an alternative livestock activity in our country. Buffalo production is a valuable business option primarily for the beef industry and, in Argentina, to a lesser extent, for dairy production [22]. In the near future, food security will be a priority due to population growth, economic development, significant changes in consumption patterns, and increased consumption of livestock products. Global food needs are expected to more than double in the next 30 years; Argentina has about eight million hectares that are unexploited or inefficiently exploited, particularly low and flood-prone areas. These areas are unsuitable for traditional cattle but are suitable for buffalo, supporting one buffalo per two hectares. This could allow for a population of four million buffalo, which would not compete with traditional cattle, which have low productivity in these "marginal" areas [6].

The growth in buffalo production requires genetic improvement, particularly through the application of reproductive biotechnologies such as artificial insemination (AI), multiple ovulation and embryo transfer (MOET), and ovum pick-up (OPU) combined with in vitro embryo production (IVEP). MOET is not commercially viable in water buffalo, unlike in cattle, due to the low number of embryos recovered per session [3, 34]. In vitro fertilization is a highly impactful

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tool for genetic progress, particularly in species where embryo production and transfer present implementation difficulties [44].

In vitro fertilization and production of embryos (IVF/IVP) is currently one of the most promising tools for increasing the number of transferable embryos per donor [35]. However, one of the main limitations for establishing commercial IVF/IVP programs in buffalo is the low number and poor competence of oocytes derived from transvaginal follicular aspiration [13]. Certain parameters, such as anti-Müllerian hormone (AMH) concentrations, indirectly reflect the total number of morphologically healthy ovarian follicles and, therefore, the ovarian reserve [19]. Plasma concentrations of AMH have shown a strong positive correlation with the number of 3 to 7 mm antral follicles in primiparous dairy cows [31]. Another study found a positive association between the antral follicle population and circulating AMH concentrations in Murrah heifers (Bubalus bubalis), Holstein (Bos taurus), and Gyr (Bos indicus) breeds [1].

Stimulation with follicle-stimulating hormone (FSH) increases the number of follicles available for OPU and enhances embryo productivity in cattle [9, 17]. Although the literature on this topic in buffalo species is limited, recent studies have shown that different FSH stimulation treatments prior to OPU positively affect the competence of oocytes obtained in IVEP. This is possibly due to improved oocyte quality, as stimulation leads to increased growth in both the number and size of ovarian follicles, resulting in higher cleavage and blastocyst rates compared to control groups [8, 29, 32]. Porcine FSH (p-FSH) and equine chorionic gonadotropin (eCG) can be used to promote follicular growth and enhance the efficiency of OPU/IVP treatments in cattle. Although there is substantial variability in the super-stimulatory response to gonadotropins, the use of p-FSH provides consistent results compared to eCG. However, due to the higher economic cost, relatively shorter biological half-life, and consequently, the need for multiple administrations when using p-FSH, eCG presents itself as an alternative [30]. Gonadotropins (FSH and LH) used in animal reproduction are obtained through extraction and purification from pituitary glands derived from slaughterhouses. Additionally, chorionic gonadotropins are extracted from the blood of pregnant mares (eCG) [2]. It is essential to note that the glycosylation profile of eCG significantly impacts its half-life and effectiveness, and this profile can vary between mares and at different stages of gestation [25].

Recombinant hormones are produced using genetic engineering techniques [23]. The use of recombinant hormones has thus revolutionized large-scale production of animal protein-free products characterized by remarkable purity and consistent composition. This advancement allows for diverse applications across industries without variability [20].

To our knowledge, studies about stimulation treatments before OPU have mainly focused on FSH response, with limited information about how other hormonal combinations might impact oocyte collection and embryo development. The hypothesis of this work was that hormonal stimulation and animal selection criteria before OPU would improve embryo production in buffaloes as a consequence the objective of this study was to evaluate the response of buffalo donors to other hormonal stimulation treatments (FSH and eCG), on oocyte quality, embryo production and embryo transfer outcome.

2. MATERIALS AND METHODS

2.1. Animals

Procedures involving animals were conducted in accordance with the guidelines of the Ethics and Biosafety Committee of the Faculty of Veterinary Sciences at UNNE, Resolution No. 459/2013-CD. The field study was carried out at the Pedro Antonio Silva (h) establishment, located in the General Paz department, Corrientes Province (27º 20' 33" S latitude and 58º 08' 27" W longitude), under a working agreement with the Faculty of Veterinary Sciences at UNNE. All animals were evaluated at the beginning of the experiments, non-pregnant buffaloes without genital tract pathologies, proven fertility and good body condition were selected, grazing on natural pasture and provided with water *ad libitum*, with an average age of 6.73 ± 1.03 years and an average weight of 658 ± 67.7 kg. A total of 60 ovum pick-up (OPU) procedures were performed on Murrah and Mediterranean buffaloes,

Four ovarian stimulation treatments were tested prior to OPU, treatment groups were as follows:

Before each OP, antral follicle count was performed using a portable ultrasound with a linear probe (Mindray, DP30-Vet). The follicles were classified into small (≤3 mm Ø), medium (4-8 mm Ø), and large (>8 mm Ø) categories. Aspiration was performed using an ultrasound with a micro convex probe equipped with a 60 cm-long OPU handle and follicular aspiration guide

TRT 1 (Control, n=20)	Day 0	Intravaginal progesterone (DIV) insertion + 2 mg of estradiol benzoate (IM)
	Day 4	—
	Day 7	OPU performed without hormonal stimulation
TRT 2 (n=10)	Day 0	Intravaginal progesterone (DIV) insertion + 2 mg of estradiol benzoate (IM)
	Day 4	160 mg of FSH (Folltropin-V®) IM over two days (50 mg, 50 mg, 30 mg, 30 mg)
	Day 7	OPU performed 36 hours after the last FSH administration
TRT 3 (n=20)	Day 0	Intravaginal progesterone (DIV) insertion + 2 mg of estradiol benzoate (IM)
	Day 4	1050 IU of recombinant eCG (FoliRec®) administered IM
	Day 7	OPU performed 72 after eCG
TRT 4 (n=10)	Day 0	Intravaginal progesterone (DIV) insertion + 2 mg of estradiol benzoate (IM)
	Day 4	2500 IU of serum eCG (Ecegon®) was administered IM
	Day 7	OPU performed 72 hours after eCG

(WTA) with 18 G needle, connected to a vacuum pump (Wta, BV-003D) at a constant pressure of 60 mmHg. The follicular fluid was collected in a 50 ml tube maintained at 37°C using a portable tube warmer (WTA). The aspirate was immediately filtered (60 µm filter, WTA), and blood residues were removed through continuous washes in fresh buffered saline solution (PBS, DIPIA-Flush Plus). The fluid was then transferred to a Petri dish for cumulus-oocyte complexes (COCs) identification and evaluation, under a binocular microscope. COCs were classified based on the number of layers of cumulus cells according to the International Embryo Transfer Society method (IETS Manual, IL): Grade 1 - three or more layers of cumulus with uniform cytoplasm, Grade 2 - one to two layers of cumulus present, Grade 3 - only corona radiata or granulated, pyknotic, or irregular cytoplasm, Grade 4 - completely denuded oocyte. They were then placed in 1.5 ml tubes containing an in vitro maturation medium (Biok MIV /Bioklone®) and transported to the laboratory in an oocyte transporter (Minitube).

Once in the laboratory (3 hours of transportation), the COCs completed their maturation in an incubator at 38.5°C and 5% CO2 in humidified air. After 24 hours of in vitro maturation, in vitro fertilization (IVF) was conducted using frozen-thawed buffalo semen of proven fertility. A sperm-rich fraction were isolated by placing 500 ul semen in upper gradient media of Percoll (45%) 800ul and centrifuged at 9,000 rpm for 5 min, the pellet were resuspended in 800 µl of IVF medium, centrifuged again for 5 min at 800 rpm. The supernatant was removed, and sperm motility and concentration were evaluated.

Fertilization was performed in 90 µl drops of IVF medium covered with mineral oil, with an approximate concentration of 1 x 10^6 motile sperm/ml per drop, and placed in an incubator at 38.5°C, 5% CO2 in humidified air. After 24 hours, presumptive zygotes were mechanically denuded, washed in culture medium (Biok SOF /Bioklone®), placed in 90 µl drops covered with mineral oil, and incubated in an oven at 38.5°C, with an atmosphere of 5% CO2, 5% O2, and 90% N2, in humidified air. At 48 hours post-IVF, 50% of the culture medium was replaced (first feeding). The cleavage rate was recorded on day 5, and a second feeding change with medium were performed. On day 6, the embryos were evaluated, blastocyst rate (total number of blastocysts divided by the number of cultured oocytes) was recorded. Only grade 1 embryos were vitrified (Grade 1: excellent, 2: fair, 3: poor, 4: degenerated [28]. For vitrification, 0.25 ml thin straws, also known as OPS (Open Pulled Straw) [41], were used, with a maximum of 5 embryos per straw and stored in liquid nitrogen tanks (-196°C) (-Biok VITRI /Bioklone®).

2.2. Anti-Müllerian Hormone and Follicular Population in Buffaloes

To determine the relationship between the follicular population (FP) and anti-Müllerian hormone (AMH). 30 buffaloes of Mediterranean and Murrah breeds were used. Animals were scanned ultrasonographic (DP30-Vet ultrasound) at a random point in their estrous cycle. The follicular population at the ovarian level was recorded by counting the total number of antral follicles (≥3 mm Ø), and blood samples were taken for AMH measurement. Blood was obtained by jugular vein puncture, immediately refrigerated, and then centrifuged at 3000 rpm for 15 minutes to obtain the supernatant serum, which was stored at -20°C until analysis. The AMH analysis was performed using the chemiluminescence method (Beckman-Coulter UniCel DxI 800). Each donor was assigned to a high or low-concentration category based on the average AMH values. These donors were subsequently subjected to different ovarian stimulation treatments.

2.3. Transfer of Vitrified Embryos

For the embryo transfer, a reproductive ultrasound examination was conducted on potential recipients using a portable ultrasound device with a linear probe (Mindray, DP30-Vet). The evaluation included age, category, body condition score (BCS) (scale 1-5), and reproductive development grade (RDG). Buffaloes were classified as cycling if they had a corpus luteum (CL) present on their ovaries at the time of ultrasound (RDG 3), superficial anestrus if their ovaries had follicles ≥8 mm in diameter (RDG 2), and deep anestrus if their ovaries had follicles <8 mm in diameter (RDG 1) [18] then the transfer were performed during the favorable reproductive season to 58 suitable females.

At the time of evaluation, recipients with an RDG 3 animals received an intramuscular dose of 2 ml of prostaglandin (PGF) (Cloprostenol, Emefur®). Eleven days later, the synchronization treatment was initiated this day is considered day 0, an intramuscular dose of 2.5 ml of Gonadotropin-Releasing Hormone (GnRH) (Gestar, buserelin acetate, Over®) was administered; on day 7, an intramuscular dose of 2 ml of PGF (Prostal, Cloprostenol 0.0075g, Over®) and 400 IU of equine chorionic gonadotropin (eCG) (ECEGON®, Bago) was administered; on day 9, an intramuscular dose of 2.5 ml of GnRH (Gestar, buserelin acetate®, Over) was administered again. Seven days later, in the afternoon (day 6 of the cycle), the embryos were transferred to previously evaluated recipients as follows: a reproductive ultrasound to assess the presence or absence of a functional CL (recipient utilization rate).

Vitrified embryos were warmed, evaluated under a stereoscopic microscope, and then loaded into 0.25 ml straws with a holding medium and transported to the field in an embryo transporter (TE-240, TED®) (transit time under 6 hours). Seven days after the transfer, 400 IU of eCG (ECEGON®) was injected into the transferred recipients. Finally, ultrasonography was performed 50 days later to determine the pregnancy rate.

Data were recorded in a specially designed format, and quantitative variables included descriptive analysis and ANOVA, considering the independent variables, with Tukey's test at an α level of 5%. The pregnancy rate was analyzed using the $\chi 2$ distribution [10].

RESULTS

3.1. Ovum Pick-Up and Embryo Production

The total observed follicular population did not differ across the treatments ($p>0.05$). However, stimulation in

Table 1: Follicular Response to Stimulation Treatments

Treatment	No. of follicles	Follicular diameter (in mm)		
		≤3	4 a 8	>8
TRT 1	10.3 (a)	6.7 (b)	3.45 (a)	0.2 (a)
TRT 2	11.7 (a)	2.3 (a)	8.7 (b)	0.7 (a)
TRT 3	10 (a)	4.4 (ab)	5.3 (a)	0.8 (a)
TRT 4	11.2 (a)	2.1 (a)	8.3 (b)	0.3 (a)

Different letters within a column indicate significant differences ($p<0.5$).

Table 2: Oocyte Number and Quality according to the Treatment Group

Treatment	Number of oocytes	Oocyte quality (Grades 1-4)			
		G 1	G 2	G 3	G 4
TRT 1	5.85 (a)	0.55 (a)	0.75 (ab)	2.7 (ab)	1.9 (a)
TRT 2	6.4 (a)	1.4 (b)	0.7 (ab)	3.8 (b)	1.5 (a)
TRT 3	5.75 (a)	0.65 (a)	0.55 (a)	2.55 (ab)	2.2 (a)
TRT 4	7.5 (a)	1.5 (b)	1.1 (b)	2 (a)	2.9 (a)

Different letters within a column indicate significant differences (p<0.05).

TRT2 (FSH) and TRT4 (serum eCG) increased the proportion of medium-sized follicles (4-8 mm) available for OPU (p<0.05), which correlates with better quality, whereas the control group had the highest number of small follicles (≤3 mm) (Table **1**).

The number of oocytes collected per OPU did not differ between treatments (p>0.05), more grade 1 oocytes were obtained in TRT2 (FSH) and TRT4 (serum eCG) (p<0.05) (Table **2**).

Embryo production was highest in TRT2 (1.5 embryos/buffalo/OPU), followed by TRT3 (1.05 embryos/buffalo/OPU), and lowest in TRT4 and TRT1 (0.8 and 0.6 embryos/buffalo/OPU, respectively) (p<0.05). No difference was found in the number of zygotes (IVC) between treatments (p>0.05), with cleavage being higher in TRT3 (p<0.05). The values found are presented in Table **3**.

3.2. AMH and Follicular Population

The average AMH value was 0.15±0.09 ng/ml, low AMH animal levels were 0.10±0.02 ng/ml (n=16), and high levels were 0.22±0.09 ng/ml (n=14). The average follicular population was 11.47±3.13 follicles per

Table 3: Embryo Production Across Treatment Groups

Treatment	Embryo development		
	Zygotes	Cleaved	Embryo/female
TRT 1	4.95 (a)	24.7 (a)	0.6 (a)
TRT 2	7.3 (a)	25.3 (a)	1.5 (b)
TRT 3	5.45 (a)	31.1 (a)	1.05 (ab)
TRT 4	7.2 (a)	20 (a)	0.8 (a)

Different letters within a column indicate significant differences (p<0.5).

Table 4: Follicular Population (FP) according to Anti-Müllherian (AMH) Hormone Levels

	Mean (n=30)	High group (n=14)	Low group (n=16)
AMH level	0.15±0.09ng/ml	0.22±0.09ng/ml	0.10±0.02ng/ml
PF	11.47±3.13	13.38±0.68 (a)	9.63±0.61(b)

Different letters within a raw indicate significant differences (p<0.5).

animal, with 9.63±0.61 for low AMH and 13.38±0.68 for high AMH animals, resulting in a statistically significant difference ($p<0.05$).

It has been observed that high-level AMH animals showed no significant differences in total follicle count (TF) or in the number of small (≤3 mm) and large (>8 mm) follicles ($p>0.05$). However, there was an increase in the number of medium follicles (5-8 mm). In low AMH concentrations, no differences were observed in total follicle count or in the number of large follicles. The control group, TRT1, presented a higher number of small follicles compared to the others, while TRT2 and TRT4 showed an increased number of medium follicles compared to TRT1 and TRT3 ($p<0.05$). Ovarian stimulation improved follicular quality, as indicated by an increase in the number of medium follicles, regardless of AMH concentration.

3.3. Embryo Transfer

Recipients had a body condition score (BCS) of 2.97 (±0.34) and a reproductive development grade of 2.78 (±0.42) at the time of transfers. Synchronization was performed on 58 adult buffaloes, 50 responded to the treatment (86.2%), 43 exhibited quality 1 CLs (74.14%), and 7 had quality 2 CLs (12.07%). Eight buffaloes did not respond to the treatment and did not present CL (13.8%) at all. 42 animals were selected for transfer, resulting in 14 pregnancies (33.3%) at day 50. TRT 1 achieved a 25%, TRT 2 a 38% rate, and TRT 3 a 31% rate, with this difference being non-significant (P 0.7907) No data from TRT 4 was available for logistical reasons. A total of 12 births were obtained, all male individuals, with a pregnancy loss rate from 50 days of gestation to a term of 14.3%.

4. DISCUSSION

The findings of this study partially align with those reported by [8] in different categories of buffaloes. De Carvalho *et al*. [8] noted that FSH treatment increased the proportion of medium-sized follicles (6-10 mm; FSH = 36.3% and Control = 6.1%) available for OPU procedures and also found an increase in large follicles (>10 mm; FSH = 16.2% and Control = 2.0%). Additionally, FSH-treated buffalo donors had higher rates of viable oocytes, blastocyst rates, and embryo yields per OPU session. Petrovas *et al*. [29] obtained similar results by testing two FSH treatment variables, which increased the number of total and medium-sized follicles. Follicle size is positively associated with oocyte competence in cattle, with the highest embryonic production achieved by aspirating follicles 6 to 10 mm in diameter [4]. This may be due to a sequence of transcriptomic and molecular alterations during follicular growth that are related to the final developmental potential of the oocyte [37], indicating that the competence for oocyte development is gradually acquired during follicle growth. Sakaguchi *et al.* [32] noted that the number of follicles less than 3 mm in diameter was higher in the control group, while the number of follicles greater than 8 mm in diameter was higher in the super-stimulated group. They also obtained more oocytes covered by multiple layers of cumulus cells in the super-stimulated group than in the control group, indicating improved oocyte quality, similar to the present study. These authors suggest that the improvement in oocyte quality produced by super-stimulation is associated with the promotion of cell-to-cell connections between oocytes and cumulus cells and increases mitochondrial reorganization and ATP production, resulting in greater developmental competence of buffalo oocytes.

A meta-analysis of IVEP literature in buffaloes suggested that oocytes with more than three to five layers of cumulus cells recovered from large follicles resulted in maximum maturation rates and subsequent embryonic development [38]. Another important consideration is the deprivation time from the last application of the stimulating hormone to OPU. Petrova's [29] results suggest that a shorter FSH deprivation time is needed in buffalo to achieve a high proportion of medium-to-large follicles (28-32 hours). Since no further follicular growth was observed at later times, it is likely that final follicular growth in this species occurs sooner. Additionally, an extended incubation time of 64–68 hours has been shown to decrease the percentage of oocytes morphologically suitable for IVM (A + B + C), along with an increase in the percentage of discarded oocytes, i.e., those not used for embryo production. This suggests that buffalo oocytes may acquire competence more rapidly, aligning with the phenomenon of early oocyte aging described previously in this species [14]. In this study, no alterations in oocyte morphology were observed, but it may have been a factor influencing treatments with eCG, where the deprivation period was 72 hours.

Studies by Ribas *et al.* [30] on Indicus-cross cattle and Fernandez *et al.* [12] on zebu cattle, testing different doses of eCG demonstrated a positive effect of its use prior to OPU, it also improves average follicle size, the number of follicles available for aspiration, and oocyte quality. Odriozola *et al.* [27], aiming to improve

antral follicle counts in Holstein cows, evaluated follicle counts following stimulation with different doses of recombinant eCG, medium (1500 IU) and high (2000 IU) doses led to a trend of greater medium follicle populations compared to the Control and suggested that the ideal timing for OPU with medium/high doses would be on day 6 (48 hours post-reCG).

Baldrighi *et al.* [1], working with different genetic groups, determined that the follicular population (FP) was higher in Gyr heifers (Bos indicus) compared to Holstein (Bos taurus) and Murrah (Bubalus bubalis) heifers (p=0.01). There was a positive correlation between FP and plasma AMH concentration for Murrah heifers (r = 0.62; p<0.01), Holstein heifers (r = 0.66; p<0.001), and Gyr heifers (r= 0.881; p<0.001). The results found in buffaloes (Bubalus bubalis) showed an average of 25.6 for FP (low 22±1.8 and high 33.8±2.5) and for AMH 0.18 ng/ml (low 0.11±0.02 and high 0.32±0.06), similar to those obtained in this study. Additionally, studies demonstrate a positive correlation between AMH and OPU along with in vitro embryo production in Holstein cattle [42], beef cattle (Korean Hanwoo) [15], Bos indicus (Zebu) [16] and buffaloes [7]; we found that stimulation treatments improve follicle quality in both high and low AMH animals, selecting animals with high AMH concentrations allows for a larger follicular population, which translates to greater efficiency in IVEP programs, as demonstrated by Chello [7].

The embryo transfer results are similar to those presented by Silva *et al.* [36], who evaluated 193 recipients synchronized for fixed-time transfer, of which 165 (85.5%) had CL and were deemed suitable for embryo transfer. Vitrified (VT) and directly transferred frozen embryos (DT) were used, resulting in a pregnancy rate of 60 days of 30.4% (28/92) for VT embryos and 37.1% (29/70) for DT embryos. Saliba *et al.* [33] transferred 70 vitrified embryos, reporting pregnancy/embryo transfer (P/ET) rates at 30 days, 60 days, and at term, which were 37.1% (26/70), 31.4% (22/70), and 24.3% (17/70), respectively. The pregnancy loss between days 30 and 60 was 15.4% (4/26), and between day 60 and term, it was 22.7% (5/22), with the latter being higher than the loss observed in the present study.

In the present study, all the animals born from IVP, vitrified/warmed embryos were males. In a study by Mahmoud *et al.* [21] on the cryopreservation of in vitro buffalo embryos and the evaluation of the sex ratio of vitrified embryos, it was reported that the total proportion of male embryos increased significantly compared to the normally expected ratio (1:1). They also observed a higher number of live males compared to dead embryos after warming, although the difference was not significant. The higher survival rate of male blastocysts 24 hours after warming was likely attributed to the slower development of female blastocysts. For instance, it has been shown that glucose metabolism is higher in male blastocysts than in female ones [39]. In an in vitro fertilization study in cattle by Nadambale *et al.* [26], it was found that although the overall sex ratio remained close to 50:50, embryos that reached the blastocyst stage earlier were predominantly male, survived vitrification and subsequently hatched better than female blastocysts. The authors attribute this to the fact that early-cleaving embryos express a fast preimplantation development (Ped) gene, conferring a developmental advantage over late-cleaving embryos that express a slow Ped gene [11,45]. Additionally, blastocyst quality correlated with rapid development, as more blastocysts that appeared early were graded as excellent (C1), while more blastocysts that appeared later were graded as regular (C2). A similar influence may have affected the present study, as only grade 1 embryos were vitrified, and their higher survival rate after cryopreservation may have altered the sex ratio.

5. CONCLUSION

In summary, and based on the results of the present study, we can conclude that the different ovarian stimulation protocols presented prior to OPU improve the size of ovarian follicles, leading to higher quality oocytes and, consequently, a higher rate of embryo production. Additionally, we confirmed endocrine markers such as follicular population (FP) and anti-Müllerian hormone (AMH) can aid in selecting animals to enhance the efficiency of IVEP. The results from the transfer of vitrified embryos were acceptable, and the subsequent birth of live animals demonstrates the viability of this technique.

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DECLARATION OF COMPETING INTERESTS

The authors state that the research was conducted without commercial or financial relationships that could be construed as a conflict of interest.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

For this work, the authors used ChatGPT-3 to improve readability and correct grammatical mistakes. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the publication's content.

LIMITATION SECTION

The reduced number in some experiments is mainly due to logistical reasons, such as obtaining enough animals on the farm that meet the inclusion criteria. Additionally, more animals need more funds to finance the research.

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