In vitro production of buffalo embryos in Colombia – results of the addition of growth factors and antioxidants to defined culture medium

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Abstract

To increase the success rate of the in vitro production (IVP) system of embryos in buffalo, it is necessary to improve parameters. This field requires more research since its results are low compared to cattle. In this study carried out in Colombia, a modification of the methodology to produce buffalo IVP embryos is described, and 24 non-lactating females, aged 4 to 11 years old were included. Follicular wave was synchronised by ablation of the follicles within the ovary, seven days later all follicles were aspirated to obtain oocytes for maturation, fertilisation and embryo production. For all culture media used, animal protein was avoided. Culture media were supplemented with growth factors and other molecules associated with embryonic development. Special care was taken in adding supplements to protect the zygotes against the harmful effect of oxygen free radicals in the culture medium. Two aspiration series were performed 15 days apart. The average number of oocytes obtained by aspiration was 11.33, the number of blastocysts was 3.05, and the obtained blastocyst production rate (36.2%) was higher than literature reports for this species; we hypothesise that this increase is associated with the supplements added. It was possible to observe a female that produced 50 oocytes. Subsequent research is needed to evaluate the effect of the medium on embryo quality and pregnancy rates. A cost analysis of the new proposed medium is required, since all supplements must be imported and handled with a cold chain of up to -80°C, making it more expensive and restricting widespread use. Finally, it is shown that with three embryos per aspiration, at least one pregnancy can be successful after the aspiration, which is one of the objectives of the IVP system.

Key words: *embryos; IVP; buffaloes; growth factors; antioxidants*

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Introduction

Buffaloes are an exceptional species due to the quality of their meat and milk, and excellent raw materials for agri-business. Unfortunately, global production still does not meet market demands. Reproductive biotechnologies and genetic improvement are tools that help to achieve this goal, and one such method is embryo transfer. Due to the fact that the recovery of embryos after multiple ovulation is low (Baruselli et al., 2020), alternatives have been applied such as the *in vitro* production of embryos (IVP). To date, this is the best alternative to obtain embryos for transfer (Gasparrini, 2002), and together with genetic improvement programmes, allow for increase productivity. IVP parameters from buffaloes and cows are not the same. When compared, they have similar oocyte maturation rates (94 *vs*. 87%), but lower cleavage rates (65% *vs.* 84%) (Neglia et al., 2003), and blastocyst rates (20% *vs*. 35-48%) in buffaloes and cattle, respectively (Dubeibe, 2016). Despite these differences, *in vitro* production (IVP) is an alternative for increasing productivity in Latin America due to the great maternal effect in buffalo production systems.

The first birth derived from IVP in Colombia was in 2011 (Gamarra et al., 2015), and research since that time has continued towards improving parameters. However, its use has not yet become widespread due primarily to the poor embryo production rates, some of them derived from characteristics of the species (Bertoni et al., 2020).

Initially, IVP in buffaloes was based on the same media and culture conditions as developed for cattle (Galli et al., 2014), but very quickly researchers and practitioners found that these media and culture conditions were not optimal for buffaloes. The sensitivity of buffalo embryos to free oxygen radicals was observed, and supplementing the culture medium with cysteamine showed a favourable effect on embryonic development (Baruselli et al., 2018a).

From studies with cattle, it is known that the enrichment of culture media for oocyte maturation with promoters and growth factors increases the number of embryos. Additionally, supplementation of culture media for buffaloes with epidermal growth factor, β-mercaptoethanol, and with insulin, transferrin, and selenium (ITS) (Marin et al., 2019) has been reported. Sadeesh et al. (2016) evaluated three different media for embryo production, TCM-199, mCR2aa supplemented with 10% FBS, and a defined medium supplemented with PVA–myo-inositol–phosphate–EGF, and found no significant differences in stages of 2, 4, 8, 16 cells, though the defined medium produce better results in blastocyst and hatching rate.

Live-born offspring from different sources of IVP embryos: fresh, frozen or vitrified (Baruselli et al., 2020) revealed that pregnancy and birth rates are low compared to cattle (10.5% *vs.* 26.9% pregnancy and 10.0% *vs.* 25.0% live births) (Marin et al., 2019). The effect of oocyte quality on blastocyst production and pregnancy rates, synchronisation protocol, progesterone levels and reproductive season has been recognised (Saliba et al., 2020), showing that for bubaline species there are many factors to study in order to offer this technology to producers. This aim of this study was to provide evidence from the literature and contribute to the improvement of buffalo embryo production, especially in Colombia. This study outlines the results of an embryo production programme through the supplementation of culture media with insulin-transferrin-selenium (ITS) and insulin-like growth factor (IGF-1).

Materials and methods

The present study was performed at a buffalo farm located on the north coast of Colombia, during the reproductive season of 2017. In total, 28 buffaloes between 1 and 8 calvings, with more than 50 days postpartum and an average age of 5.3 ± 2.8 years were chosen. The animals underwent two follicular aspiration sessions (OPU) at an interval of 15 days, ablation of all follicles within the ovary was performed to synchronise the wave at the beginning of the experiment. All procedures with animals were approved by the Ethics Committee for animal care of the National University of Colombia (Act No CICUA-013, 2017).

The perineal area was cleaned and disinfected with 70% alcohol, and 3 mL 2% lidocaine (Lidovet R, Bravet) was administered for epidural anaesthesia. OPU was performed using a transvaginal probe connected to an ultrasound machine (Aloka 500 R, Tokyo, Japan, frequency 5 MHz). For aspiration, a system coupled to vacuum pump with negative pressure (WTA, Watanabe Applied Technology) was used. Follicles >3 mm were aspirated with an 18G needle, and 60–65 mmHg pressure (10 to 12 mL of water/minute) were used. Follicular fluid was collected in a 50 mL tube containing 1 mL solution (heparin-supplemented buffered saline (PBS) (5,000 IU/L; Parinex R, Hypolabor), and 0.1% amikacin antibiotic solution). After aspiration, the system was washed with PBS solution.

The follicular fluid was deposited in a 70 μm filter for cells (Falcon, Corning Brand, USA), which was washed to remove blood and debris. Subsequently, the cumulus-oocyte complexes (COCs) were identified and classified according to the number of granulosa cell layers and the homogeneity of the cytoplasm using a stereomicroscope (Nikkon, Tokyo, Japan), The medium used for oocyte manipulation was synthetic oviductal fluid (H-SOF) supplemented with HEPES. All COCs of each female were transferred to 500 μL maturation medium (MIV) in 5 mL culture tubes (Falcon, Corning Brand, USA), and cultured for 18–22 hours at 38.5°C and 5% CO_2 in air and 100% humidity. MIV composition was as follows: TCM-199 supplemented with epidermal growth factor (EGF) (6 ηg/mL), β-mercaptoethanol (50 μM), FSH (0.5 μg/mL), LH (5 μg/mL), ITS (10 μL/mL), sodium pyruvate (0.2 mM) and kanamycin (0.75 mg/mL).

For fertilisation, frozen semen from Mediterranean breed IVP proven bulls were used, thawed at 37°C during 45 seconds, after motility evaluation, the motile sperm fraction was obtained using discontinuous Percoll density gradient technique (90% and 45%) (Merck, Darmstad, Germany) after separation, motility was re-checked under the microscope (Nikkon, Tokyo, Japan), and oocytes were inseminated with 4 x10^6 sperm/mL in 50 μL droplets of TALP-IVF, fertilisation medium supplemented with 0.2 mM/mL penicillamine, 0.1 mM/mL hypotaurine and 0.01 mM/mL heparin and cultured for 18 hours. Presumptive zygotes were washed and transferred to a hyaluronidase solution to remove remaining cumulus cells and left in culture in 500 μL modified SOF medium with albumin (8 mg/mL) and hyaluronic acid (100 ng/mL) in 4-well culture plates (Nunc, Nunc Brand, Denmark). On the third and fifth day of culture, half of the culture medium was renewed. Evaluation of embryo cleavage and blastocyst rate was performed on days 3 and 6 of culture. For cryopreservation, only grade 1 embryos were chosen, according to the classification of the International Embryo Transfer Society (IETS).

An excel worksheet was designed to record the number of oocytes obtained and matured, cleaved embryos and blastocysts obtained and whether it was frozen or not. Before the comparisons of

embryo production, the age of donors, the effect of bulls and the results of oocytes from each OPU session were recorded and analysed. Descriptive statistics and comparisons between different data groups were performed using Tukey's test. A value of $P<0.05$ was considered significant.

Results

In the two OPU sessions, 28 animals were aspirated, of these, 12 were aspirated twice. In total, 40 OPU sessions were performed, 441 oocytes and 117 blastocysts were obtained, and 100 embryos were frozen. The general parameters of the two aspirations can be seen in Table 1. One animal (2.5%) did not produce oocytes, and 6 (15.38%) buffaloes did not produce blastocysts with sufficient quality for freezing. One buffalo produced 50 oocytes. Of the recovered oocytes, 85% (375) were placed in culture (9.64 cultivable oocytes/buffalo) for maturation. On the third day of culture, 187 embryos had 4 to 8 cells (49.86%) and on day 7, 117 blastocysts (32.33%) were observed. The results represent an average of 2.92 blastocysts/OPU. From the embryos obtained, 82% had sufficient quality for freezing.

High variation was observed in the number of oocytes obtained by OPU, ranging from 0 to 50, (mean ± standard deviation 11.13 ± 8.66) with statistical differences between individuals (P=0.0041). No significant differences were observed when the effect of session (P=0.731), age (P=0.6383) and of the bulls used (P=0.499) were evaluated, and their effects will not be considered in the analysis. When the number of oocytes was compared between buffaloes aspirated once or twice, significant differences (P=0.004) were found in the number of oocytes but not in the number and proportion of blastocysts (P=0.671). There were no differences in the cleavage rate (P=0.6711) and blastocysts obtained (P=0.055) between individuals.

Based on the mean number of oocytes per OPU (11.53 oocytes/OPU), animals were classified as high and low oocyte producers and the parameters of the IVP were compared. Significant differences ($P<0.05$) were found in the number of oocytes obtained, cleaved embryos and the number of blastocysts between groups, but when this comparison was based on proportionality, no significant differences were found in cleaved embryos and blastocyst production, ($P>0.0552$ and P=0.671), respectively.

Discussion

This paper outlines the various aspects of the evolution of buffalo embryo production in Colombia. Previous reports in the country (Berdugo-Gutierrez et al., 2017) reported 8.07 oocytes/OPU and 19% blastocysts compared to 11.01 oocytes/OPU and 32% blastocysts in this study, clearly showing the increase in the number of oocytes due to improvements in the aspiration technique. Other authors in the US have reported the obtention of

Table 1. General parameters of buffalo embryo production

Month (OPU)	Oocytes	Oocytes/ OPU (*n*)	Cultured/ OPU (*n*)	Fertilisation/ OPU (*n*)	Cleavage/ OPU D3(*n*)	Cleavage D3 %	Blast/ OPU (*n*)	Blast %	Frozen/ OPU (*n*)
August (22)	233	11.10	9.29	7.86	3.48	47.56	2.38	29.17	2.65
September (18)	208	11.56	10.00	9.89	6.33	63.91	3.72	36.02	3.06
Total (40)	441	11.33	9.64	8.87	4.9	55.73	3.05	32.60	2.85

10.2 oocytes/OPU (Baruselli et al., 2018a). There is a prominent range in the number of oocytes obtained per animal (from 0-50 oocytes), and this is the first report of the obtention of 50 oocytes from a buffalo female by OPU.

Additionally, the statistically significant differences in oocyte yield when comparing once *vs.* twice OPU was confirmed, as reported by others. From the results obtained here it is feasible to propose that the number of oocytes/animal could be used as a selection parameter (Bhardjwaj et al., 2016a; Marin et al., 2019). In this study, differences were seen in the parameters between high and low oocyte producers (number of oocytes, up to the production of blastocysts) after grouping animals according to the number of oocytes recovered. Thus, a strategy could be adopted by the technicians for oocyte production characterisation of each female.

It is very encouraging to observe that in spite of the individual variation, 97.5% of aspirated females produced oocytes for culture, giving practitioners the necessary support to establish genetic improvement programmes based on the use of the IVP. The effect of age and parity on the number of oocytes, as reported by other authors, could not be demonstrated in this research.

Suresh et al. (2009) described that the best way to produce an embryo in buffalo females: when an oocyte with the adequate number of granulosa cells is obtained, coming from a healthy developed follicle during the favourable season, it is cultured in TCM-199 medium, and supplemented with foetal bovine serum and cysteamine.

From the above, in this report we take two aspects for discussion. First, the quality of the oocytes is a limitation factor for IVP. Although it is true that cumulus-oocyte complex morphology is characteristic to each species, the concern remains about the possible effect of adopting other selection criteria in buffalo oocytes, other than the commonly used parameters for cattle. Considering the need to increase the efficiency of the technique in this species, there is still a lack of knowledge about the characteristics of a good quality buffalo oocyte and its response to culture conditions. The second is in relation to culture media: the scarce information available on the metabolic characteristics and biochemical requirements of the buffalo oocyte/embryo, have not allowed for the development of a specific culture medium for the species, and the most accepted is supplementation with the use of antioxidants. Some authors have reported that the buffalo embryo requires high concentrations of glucose (1.5 M) at the beginning of its development (Bhardawaj et al., 2016b). Other have reported the use of a single medium throughout the embryo culture, using SOF supplemented with foetal bovine serum (Marin et al., 2019). Sadeesh et al. (2016) reported significant differences ($P>0.05$) in the number of blastocysts obtained in the defined medium compared to the non-defined medium, however they did not observe differences in other stages of development. In the present study, a defined culture medium was used, supplemented with growth factors and ITS, and this gave satisfactory results in relation to the final production of blastocysts.

In cattle, the implementation of an ovarian stimulation protocol with follicle-stimulating hormone followed by a period of coasting has been reported, which has been shown to increase the quality of oocytes, evidenced by their better classification and an increase in the number of blastocysts (Khan et al., 2015). Its application in buffaloes has recently been reported with promising results (Baruselli et al., 2018b).

The results obtained allow us to suggest that proposed supplementation

of the culture media gives better results in embryo production. However, the proportion of oocytes that do not reach the blastocyst stage is still high, suggesting that the adjustment of other factors such as the clinical analysis of the animals, their reproductive potential, their behaviour in these programs, and feeding and management systems could make *in vitro* buffalo embryo production programmes the tool of choice in advancing improvements in this species.

In order to establish more efficient future IVP programs in buffaloes, it is necessary to include additional aspects related to animal management, studies that analyse the metabolic and biochemical characteristics of the animals and of the gametes and embryos (Ohashi et al., 2017), in addition to the application of markers of oocyte production potential such as anti-Müllerian hormone concentrations (Berdugo et al., 2017) and antral follicle count (Morotti et al., 2018).

In conclusion, a successful reproducible IVP program in buffaloes with high rates of blastocyst production using defined media supplemented with antioxidants, growth factors and intracellular signal inducers is outlined. The combination of these aspects with the clinical analysis of animals will be key for the continued advancement of efficiency of this biotechnological technique when applied to the buffalo species.

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In vitro proizvodnja zametaka bizona u Kolumbiji – rezultati dodavanja faktora rasta i antioksidansa definiranom mediju za uzgoj zametaka

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Za povećanje djelotvornosti sustava *in vitro* proizvodnje (IVP) zametaka u bizona, potrebno je poboljšati parametre. Budući da su rezultati slabi u usporedbi s govedima ovo područje zahtijeva više istraživanja. U ovom radu, provedenom u Kolumbiji, modifikacija metodologije za proizvodnju IVP zametaka bizona opisana je na sljedeći način: korištene su 24 ženke u dobi između 4 i 11 godina, a koje nisu bile u laktaciji. Folikularni val je sinkroniziran ablacijom folikula unutar jajnika, a nakon sedam dana obavljena je aspiracija svih folikula ženki za dobivanje oocista za dozrijevanje, oplodnju i proizvodnju embrija. Za sve rabljene podloge izbjegavan je životinjski protein. Podloge su obogaćene faktorima rasta i drugim molekulama povezanima s razvojem embrija. Posebna pozornost posvećena je pri dodavanju dodataka za zaštitu zigota od štetnih učinaka slobodnih radikala u podlozi. Obavljene su dvije aspiracijske serije u razmaku od 15 dana. Prosječni je broj oocista dobivenih aspiracijom bio 11,33, broj blastocista bio je 3,05, a dobivena je stopa proizvodnje blastocista (36,2 %) bolja je u usporedbi s onom koju su objavili drugi autori u državi, za istu vrstu. Pretpostavljamo da je ovo povećanje povezano s dodanim dodatcima, bilo je moguće promatrati ženku koja je proizvela 50 oocista. Naknadno je istraživanje potrebno za procjenu učinka podloge na kvalitetu zametaka i postotka gravidnosti; potrebna je i analiza troškova novog predloženog medija, jer se svi dodatci moraju uvesti u Kolumbiju i čuvati u hladnim uvjetima do minus 80 °C, što ih čini skupljima, a ograničava i njihovu široku primjenu. Na kraju našeg istraživanja uočeno je da s tri zametka po aspiraciji u ove vrste nije teško postići najmanje jednu gravidnost, a to je i jedan od ciljeva IVP-a.

Ključne riječi: *zametci, IVP, bizoni, faktori rasta, antioksidansi*