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EVALUATION OF THE MATURATION TIME OF ABATTOIR-SOURCED WATER BUFFALO (*Bubalus bubalis*) OOCYTES FERTILIZED *In vitro*

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. Authors IIAR and MBM designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors BDM, OEZG and MK managed the analyses of the study. Authors BDM and LTEI managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The purpose of this study was to compare the maturation, cellular division, and blastocyst production rates of water buffalo oocytes at 18, 21 and 24 hours of incubation. These oocytes were obtained through follicular puncture, assessed, and matured in BO-IVM medium at 38.5°C, and with a 5% of CO_2, at 18 (n=31), 21 (n=34) and 24 hours (n=33). Fertilization was done in a BO-IVF medium at 38.5°C, and with a 5% of CO_2, for 18 hours; the maturation rate was assessed a day after fertilization. The embryos were cultured in a BO-IVC medium, at 38.5°C, 5% CO_2, 7% O_2 and 88% N_2, and the cell division rate and number of embryos were assessed at 5 and 7 days after fertilization, respectively. The maturation rate was comparable ($P>0.05$) among the three groups, while blastocyst production and cell division rates were higher in the group of oocytes matured for 18 hours ($P<0.05$) than in the 24-hour group. The blastocyst production and cellular division rates in oocytes that were matured for 21 hours were comparable ($P>0.05$) to the other groups. After 21 hours of maturation, oocytes started to degenerate, which resulted in lower cellular division and blastocyst production rates.

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1. INTRODUCTION

Many Mexican livestock farmers have established water buffalo herds in their production systems, profiting from the characteristic hardiness, efficiency, and productivity of the species [1]. *In vitro* production of embryos is an indispensable tool to improve genetics and reproductive efficiency, which can be applied for water buffalos [2]; however, the lack of information about the required characteristics of gametes and embryos hinders the progress of its implementation [3]. One of the key points of *in vitro* fertilization (IVF) is oocyte maturation, a sequence of stages in which a primary oocyte restarts meiosis and reduces the number of its chromosomes to become a haploid cell and, subsequently, a mature oocyte during metaphase II. This process provokes changes in the nucleus and cytoplasm, allowing fertilization and embryonic development [4]. Maturation depends on certain factors, such as atmospheric gaseous composition, culture medium, temperature, and incubation time [5]. Extant studies about the kinetics of the maturation of these gametes are controversial in their estimation of the time needed for adequate maturation. Some early studies proposed periods of 24 to 32 hours for a precise process [6], while others found that a large proportion of gametes reached Meiosis II stage within 15 to 19 hours, simultaneously reporting the finding of degenerated gametes after that period [7].

It has been reported that degeneration may be present after 24 hours of maturation, characterized by changes in the structure of chromatin, compromising fertilization, and originating low embryonic production rates. These studies have proposed that buffalo gametes and embryos are more sensitive to incubation conditions than those of other species [8-9]. Moreover, fertility is an important component of both sexual and asexual reproduction, and it can be viewed as a direct or *an* indirect process [10-11]. Healthy and fertilized oocyte is a strong predictor of fecundity and explained a high percentage of variation in fertility among species [12]. The evolution of fertility is associated with fertilization of oocyte [13] and defective oocytes can decrease *fertility* [14].

Thus, the purpose of this study was to compare the maturation, cellular division, and blastocyst production rates of water buffalo oocytes at 18, 21 and 24 hours of incubation.

2. MATERIALS AND METHODS

Sourcing of ovaries: The ovaries used for this study were collected at a TIF 647 slaughterhouse, located in Highway Road of Golfo Km. 221.5, Acayucan, Veracruz State, Mexico. They were obtained after the slaughter of buffaloes (n=15), washed with a saline solution (0.9% NaCl), and stored in an isothermal container with a saline solution at 38.5 °C until their arrival to the lab.

Oocyte collection and classification: The ovaries were transported (3 ± 0.5 hours) to the lab, located in the city of Veracruz, state of Veracruz. Just the ovarian follicles with a size between 2 to 8 mm. were punctured and aspirated with needles (18G /1") [15], and the oocytes were classified, according to their morphology, as grade A, B and C [16]. Grade-A oocytes are round, and completely lined with more than three compact layers of cumulus cells and possess a dark and homogeneous cytoplasm. Grade-B oocytes are round, lined with, at least, one or two compact layers of cumulus cells, and a dark, regular, and homogeneous cytoplasm. Grade-C oocytes are completely devoid of cumulus cells, have an irregular cytoplasm or, otherwise, are lined with fibrin.

In vitro maturation: The oocytes were cultured in 20 µL drops of BO-IVM (IVF Bioscience®) maturation medium, 5 oocytes per drop, previously heated under a layer of mineral oil. They were matured in the incubator with 5% CO_2 at 38.5 °C in three different intervals: 18 hours (n=31), 21 hours (n=34) and 24 hours (n=33).

Sperm preparation and in-vitro fertilization: The oocytes were taken out of the culture medium, washed, and placed in 20 µL drops, 5 oocytes per drop, of fertilization medium, previously heated, gasified and covered with a layer of mineral oil.

The same semen was taken for all oocytes. Semen straws were defrosted at 37° C for 30 seconds, taking a semen sample to verify its suitability and motility. The semen was centrifuged in a 45% Percoll gradient medium, at 700G, for 3 minutes, and, subsequently, in BO-SemenPrep (IVF Bioscience®) medium, at 700G, for a minute. Sperm cells were introduced in the fertilization medium drops, with the oocytes, and both gametes were incubated at a temperature of 38.5°C, with a 5% of CO_2, for 18 hours.

Assessment of maturation: The oocytes were taken out the fertilization medium and washed in HTF medium with added hyaluronidase for 3 minutes; consequently, they were stripped of their cumulus cells with a Stripper® (Origio) device and observed in a stereoscopic microscope. The presence of a polar body indicates a correct maturation (Fig. 1).

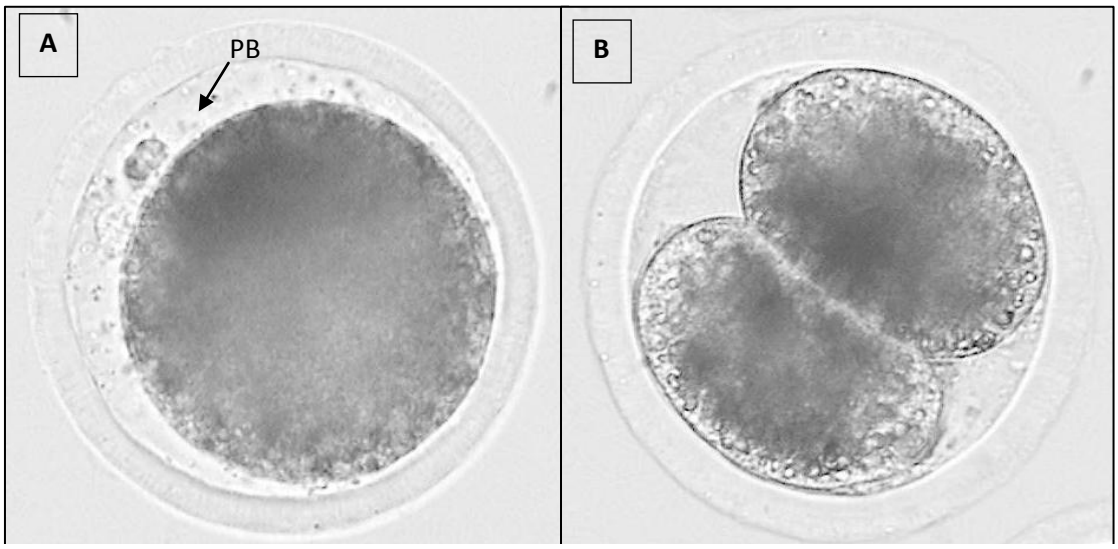

Fig. 1. Water buffalo oocytes at Day 1 post-fertilization, (A) showing a polar body (PB), and (B) at the first cell division (20X objective)

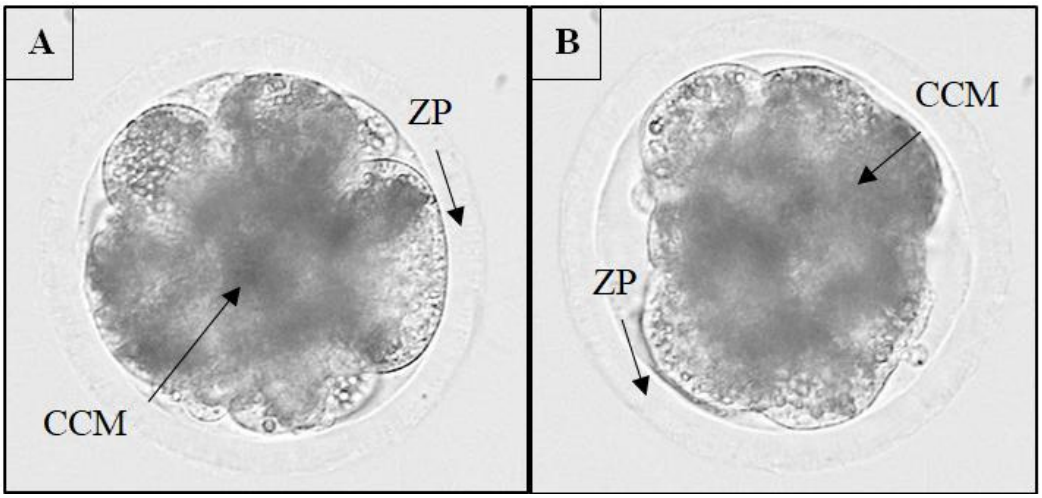

Fig. 2. Buffalo morulae during cell division (5 days after fertilization). A and B. Morula showing the zona pellucida (ZP) and compacted cell mass (CCM; 20X objective)

***Culture in vitro**:* After the fertilization period, the presumed zygotes were taken out of the fertilization medium and transferred to a BO-IVC (IVF Bioscience®) culture medium, previously heated, gasified and covered with a layer of mineral oil, and incubated in an atmosphere with 5% CO_2, 7% O_2, and 88% N_2, at 38.5°C for 5 days to observe those fertilized oocytes that underwent cell division (Fig. 2). The internal cells could not be observed due to the characteristics of buffalo morulae; thus, the non-fertilized structures were only separated and differentiated. The zygotes that underwent cell division were further incubated for 2 more days, and the quality of the structures that reached blastocyst stage was assessed at the 7th day after fertilization [16]. A-quality structures (Excellent) are those with a symmetrically spherical embryonal mass and uniform blastomeres. B-quality structures (Good) have few irregularities in their inner cell mass, which is intact and viable, and homogeneous blastomeres in their color and distribution. C-quality structures (fair) have between 25% and 50% of intact and viable cell mass, while D-quality structures (bad) are those with irregularities all over their body (Fig. 3). Only A-quality and B-quality structures were considered viable.

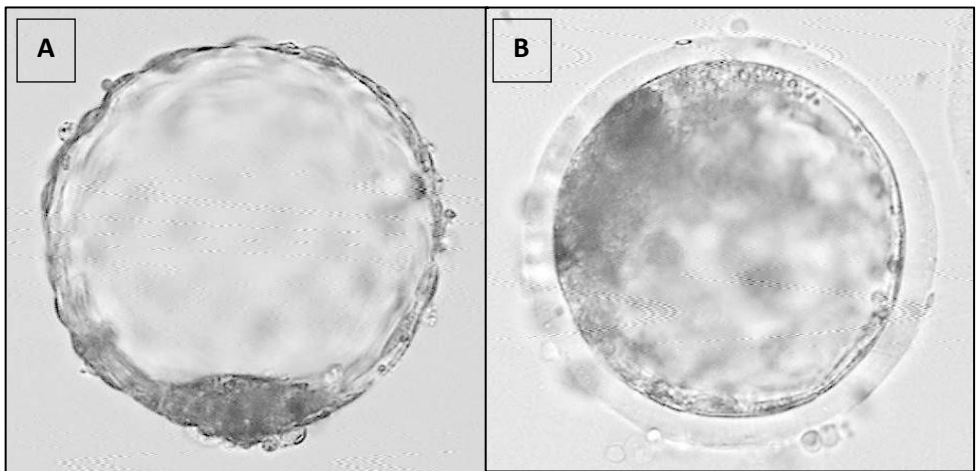

Fig. 3. Buffalo blastocysts at different development stages, on the 7th day after fertilization: A. Hatched blastocyst. B. Expanded blastocyst (20X objective)

***Statistical analysis**:* The statistical analysis was done using the Statistica v10.0 (StatSoft®) software and the Chi-squared test, considering $P<0.05$ as significant.

3. RESULTS AND DISCUSSION

A total of 123 buffalo oocytes were recovered (4.1 oocytes per ovary, and a total of 8.2 oocytes per buffalo). 98 oocytes were classified as viable, which means 3.26 viable oocytes per ovary, and 6.53 per buffalo (a viability rate of 79.67 %). The viable oocyte recovery rate was higher than in other studies although oocyte sampling was carried out during the period of least reproductive activity in buffalos. The average number of obtained oocytes per buffalo reported in this study was similar to that reported previously [17] in Colombia, where, as in Mexico, the reproductive biology of buffalo does not seem to be significantly influenced by photoperiods. Unlike in Italy [18] and Brazil [4], where oocyte viability was lower than in this study, due to the impact of the season changes in those countries [19]. There is evidence that oocyte viability, gamete competence and embryonic development are greater during reproductive seasons; thus, it is recommended to implement IVF protocols in seasons of high reproductive activity [20].

No significative statistic differences ($P>0.05$) were found in the maturation rates of buffalo oocytes upon comparison of the three maturation period groups (Table 1). The maturation rate in this study was assessed by the presence of a polar body, condition which would indicate that the oocyte has reached the Meiosis II stage [4], this is, that it is apt for fertilization; however, other authors [21], assess maturation on the basis of cumulus cell expansion, obtaining higher maturation rates, but lower cell division and embryo production rates than in this study. Cumulus cell expansion is not a trust-worthy marker of maturation, which has been proven by other authors who assessed maturation based on both criteria (cumulus cell expansion and polar body presence), comparing them with the presence of chromosomes in the Meiosis II stage. The proportion of oocytes that reached Meiosis II stage was similar to the proportion of oocytes with polar bodies, but lower than the proportion of oocytes with expanded cumulus cells [22].

Table 1. *In vitro* maturation rates of buffalo oocytes, using three maturation periods

Period group	Oocytes (n)	Mature oocytes (n)	%
18 h	31	22	70.97 ± 8.29 [a]
21 h	34	19	55.88 ± 8.64 [a]
24 h	33	19	57.57 ± 8.74 [a]

Note: Percentages values with different literals are different ($p<0.05$)

Has been reported that 12 hours may be a sufficient incubation period to reach Meiosis II [4]; on the other hand, other authors [7] had mentioned that most oocytes reach that stage after 15 to 19 hours of maturation, commencing a degenerating process after the 19th hour. The results of this study were different to other authors [6], who found that the first oocytes started to mature after 14 hours, reaching the best rates between 26 and 32 hours. On their part, has been found [23] that some oocytes start to mature at 17

Table 2. Cell division rates of buffalo oocytes fertilized *in vitro*, using three different maturation periods

Group	Oocytes (n)	Divided (n)	%	Divided/Mature (%)
18 h	31	19	61.29 ± 8.89 [a]	86.36 ± 7.48 [a]
21 h	34	14	41.18 ± 8.56 [ab]	73.68 ± 1.03 [a]
24 h	33	12	36.36 ± 8.50 [b]	63.15 ± 1.13 [a]

Note: Percentages values with different literals are different (p<0.05)

Table 3. *In vitro* production rates of buffalo blastocysts, using three different maturation periods

Group	Blastocysts n (%)	Blastocysts/Mature (%)	Blastocysts/Divided (%)
18 h	6 (19.35 ± 7.21) [a]	27.27 ± 9.71 [a]	31.57 ± 10.95 [a]
21 h	3 (8.82 ± 4.94) [ab]	15.78 ± 8.59 [a]	21.42 ± 11.38 [a]
24 h	1 (3.03 ± 3.02) [b]	5.26 ± 5.26 [a]	8.33 ± 8.32 [a]

Note: Percentages values with different literals are different (p<0.05)

hours, with most of them maturing between the 22 and 24 hours. These differences may be due to different oocyte collection protocols, collection time, incubation conditions, the addition of growth factors, hormones, and antioxidants [24].

The cell division rate in buffalo oocytes matured for 21 hours (P>0.05) was similar to that of oocytes matured for 18 or 24 hours. Nevertheless, the cell division rate in buffalo oocytes matured for 18 hours was higher (P<0.05) than in oocytes matured for 24 hours (Table 2).

No significant (P>0.05) statistic differences were found among the three maturation groups (Table 2) regarding the percentage of fertilized oocytes that attained cell division from the total number of mature oocytes (Divided/Mature).

The rate of blastocyst production in oocytes matured for 21 hours was similar (P>0.05) to the 18 and 24-hour group. However, the rate of blastocyst production in oocytes matured for 24 hours was significantly lower (P<0.05) than in the 18-hour group (Table 3).

The results of this study show that maturation time influences cell division and blastocyst production, in line with the results obtained in other investigation group [25], who state that the optimal maturation period length is 18 hours, with the cell division and blastocyst production rates increasing as the maturation period shortens. Results in this study suggest that, although there was no difference in the maturation rates of the 18, 21 and 24-hour groups, oocyte competence is reduced with time, and a degenerative process starts beyond 21 hours of maturation. However, competent oocytes cannot be differentiated from degenerated ones without cytoplasm assessment beyond its structure.

No significant (P>0.05) statistic differences were found among the three maturation groups (Table 3) upon assessment of the buffalo blastocysts obtained from mature oocytes (Blastocysts/Mature) versus the percentage of blastocysts obtained from fertilized oocytes that had undergone cell division (Blastocysts/Divided).

In this study, the oocytes were submitted to thermal shock to assess their maturation, while other studies forgo this step because buffalo gametes and embryos are more sensitive to temperature changes during the in vitro fertilization process, which can affect fertilization and embryonic development [26].

4. CONCLUSION

Buffalo oocytes are more sensitive to maturation time than previously thought. The optimal maturation period was found to be 18 hours, with the cell division and blastocyst production rates increasing as maturation periods got longer. Oocytes left to mature for more than 21 hours start to degenerate, which affects the production of embryos.

COMPETING INTERESTS

Authors have declared that they have no known competing financial interests or non-financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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