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Ovum pick-up interval in buffalo (*Bubalus bubalis*) managed under wetland conditions in Argentina: Effect on follicular population, oocyte recovery, and in vitro embryo development

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ABSTRACT

The excellent adaptation of water buffalo (*Bubalis bubalis*) to swampy environments means that animals are frequently managed in areas with restricted access for reproductive procedures. The objective of this study was to evaluate the effect of the ovum pick-up (OPU) interval on follicular population, oocyte recovery, oocyte quality and in vitro embryo production. Twelve Murrah buffaloes were subjected to two consecutive dominant follicle reductions, and randomly assigned to either 7-day (n = 6) or 14-day (n = 6) OPU interval groups. Although there was no significant difference in the average number of small (<3 mm) and large (>8 mm) diameter follicles available per OPU, a higher proportion of medium-sized follicles (3–8 mm) were observed in the 14-day interval group (5.129 vs 3.267; $p < 0.05$). The number of recovered oocytes per donor was also significantly higher (4.51 vs. 2.8; $p < 0.05$) in the 14-day interval group, although this was attributed to an increase in the proportion of lower quality oocytes (grades III and IV). After in vitro fertilization, embryo developmental competence from grade I and II oocytes was superior to that from grade III and IV oocytes, irrespective of OPU interval group. There was no significant difference in the proportion of grade I and II oocytes cleaved after sperm co-incubation; however, there was a higher proportion of blastocysts produced in 14-day interval group (28 vs. 6%, $p < 0.05$). No blastocysts were produced from grade III and IV oocytes. This study indicates it is possible to use a 14-day interval for oocyte collection in water buffalo; this approach could be considered as an alternative when access to animals is restricted.

1. Introduction

In domestic farm animal species, the technique of repeated ovum pick-up (OPU) allows for the continuous collection of competent oocytes, which in turn can be used for the production of in vitro-derived embryos. In 1988, Pietrese et al. reported the first repeated ultrasound-guided follicular puncture in cattle, and concluded that consecutive procedures could be conducted without detrimental effects to donor health. Boni et al. reported the first OPU in water buffalo in 1994. In that initial report, donors in deep anestrus were subjected to FSH priming followed by weekly oocyte aspirations; the average recovery rate was 31.9% for untreated

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controls and 44% for FSH-stimulated donors. Since then, this technique has become an integral part of assisted reproductive technologies for the production of in vitro-derived embryos, which has become a competitive alternative to superovulation for in vivo embryo recovery and transfer (Lambert et al., 1983; Gasparrini 2002; Presicce, 2007).

Optimal OPU interval is still a matter of debate in buffalo. OPU interval and its relation with follicular wave emergence, phase of follicular wave at the time of collection and oocyte developmental competence are key interacting elements that are not completely understood. Although consecutive OPU procedures have been reported using twice-weekly (Boni and Zicarelli, 1996; Sá Filho et al., 2009; Neglia et al., 2011; Di Francesco et al., 2012; Gasparrini et al., 2014), weekly (Gupta et al., 2006; Ferraz et al., 2007, 2015; Neglia et al., 2011) and bi-weekly (Gimenes et al., 2015; Ferraz et al., 2007, 2015) intervals, results are not consistent among studies and are sometimes difficult to extrapolate due to confounding variables such as breed, hormonal stimulation treatments and seasonal effects.

Neglia et al. (2011) indicated that a short, twice-weekly OPU interval was associated with a progressive decline in follicular population. In that study, which was the longest continuous OPU reported for buffalo to date, 18 OPU procedures were conducted every 3–4 days, followed by 16 procedures conducted every 7 days. Although no difference in oocyte recovery rate was observed, weekly OPU was associated with a reduction in the proportion of lower quality oocytes, higher cleavage and blastocyst rates and overall improved oocyte competence. This study contradicted earlier results from Boni et al. (1997) showing that an extension of the OPU interval resulted in larger diameter follicles and reduced oocyte quality.

The first OPU conducted at a 14-day interval was reported by Ferraz et al. (2007, 2015). The effect of weekly (7-day) versus bi-weekly (14-day) intervals, with or without bovine somatotropin (bST) treatment was evaluated in lactating Murrah x Mediterranean crossbreeds; follicular population, oocyte recovery and embryo production were analyzed. Bi-weekly interval resulted in an increase in the number of follicles available for aspiration (15.6 vs. 12.8; $p < 0.0001$) and the number of oocytes recovered (10 vs. 8.5; $p < 0.004$). An interaction between the 14-day OPU interval and bST treatment was reported, which resulted in an increased rate of oocyte degeneration and reduced embryo production. Noteworthy, experiments were conducted in lactating animals managed under twice-daily milking regimes, intensive management and high nutritional planes; results may not necessarily apply to animals managed under more extensive conditions.

The rusticity and excellent adaptation of water buffalo to swampy environments means that in Argentina, as in many other regions of the world, animals are often managed in wetland areas with restricted access. This presents a challenge for routine assisted reproductive procedures such as super ovulatory treatments, ultrasound screenings and ovum pick-up for in vitro embryo production. For this reason, there is a growing interest among local producers and practitioners to evaluate the efficacy of protocols that require less frequent access to animals. Therefore, the objective of this study was to evaluate the effect of 7- versus 14-day OPU intervals on follicular population, oocyte recovery, oocyte quality and in vitro embryo production.

2. Materials and methods

2.1. Animals and experimental conditions

The study was conducted during the buffalo reproductive season in the Southern Hemisphere (March to May). All experimental animals were part of a commercial herd managed under wetland conditions in the province of Corrientes, Argentina (-27.742859 latitude, −57.773611 longitude).

A total of twelve (n = 12) non-pregnant, 4- to 12- year old Murrah buffalo cows (*Bubalus bubalis*), with good body condition scores (2.97 ± 0.40 on 1–5 scale; Baruselli et al., 2001) and proven fertility of at least one live offspring recorded were available for this study (Fig. 1). All experimental animals were considered to be cyclic based on ultrasound detection of corpus luteum prior to initiation of treatments and consistent ovarian activity with the presence of multiple follicles per ovary.

Ultrasound-guided follicular ablation has been reported as a non-hormonal physical method to synchronize follicular wave emergence in water buffalo (Honparkhe et al., 2014). All animals in the present study were subjected to double, consecutive dominant follicle reduction (DFR) procedures 1 week apart in order to initiate a new follicular wave; they were then randomly assigned to

Fig. 1. a. Swampy, extensive management conditions of experimental animals, Little Punjab Ranch, Corrientes, Argentina. b. Animals available for this experiment.

OPU conducted at either weekly (7-day) or bi-weekly (14-day) intervals. The initial OPU was conducted either 7 or 14 days after the second DFR, according to the interval treatment proposed for each group. Time line in weeks for each treatment group is shown in Fig. 2.

2.2. Ovum pick-up procedure

All procedures were reviewed and approved by the Animal Welfare Committee, (Universidad Nacional del Nordeste, Corrientes, Argentina), and conducted in accordance with ethical standards approved by the Institution (Fig. 3).

In order to ensure immobilization and ease of manipulation in the palpation chute, animals were administered 2% xylazine (i.m.) (Rompun, 0.25 ml/100 kg body weight), followed by procaine (Procasel 2%, 5–7 ml) epidurally prior to follicular aspirations. Ovum pick-up was performed as previously described (Konrad et al., 2016). Briefly, OPU equipment consisted of a Mindray DP-30 Vet ultrasound with a 5 MHz micro convex scanner fitted with a 60-cm transvaginal guide and a disposable 17 G needle (0.9 × 70 mm, WTA, Brazil). Upon visualization of an ovarian follicle, vacuum pressure (range 40–60 mmHg) was maintained at a constant rate (WTA, Brazil) and follicular fluid was collected into 50-ml polypropylene conical centrifuge tubes (Corning® Life Sciences, MA, USA) pre-warmed and maintained at 37 °C. Between collections, the aspiration line (Cook Vacuum Line and filter, Cook Medical Australia,

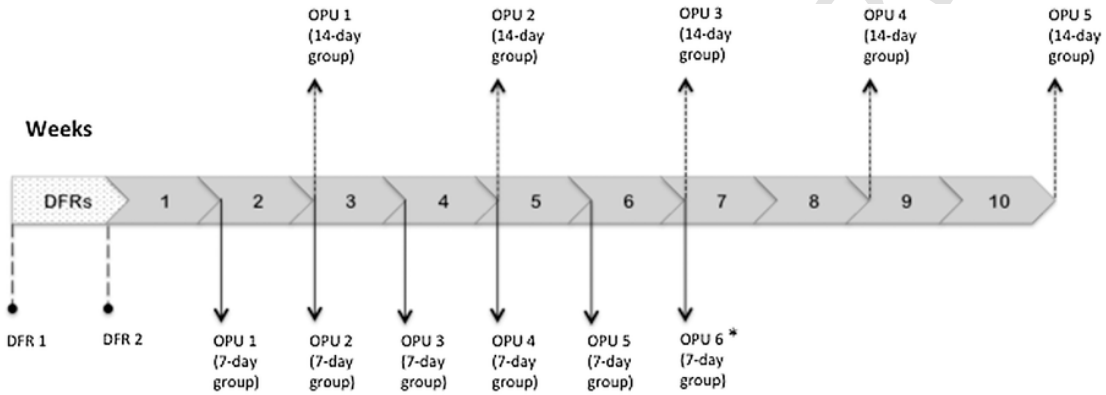

Fig. 2. Time line (in weeks) for DFRs and OPU intervals.

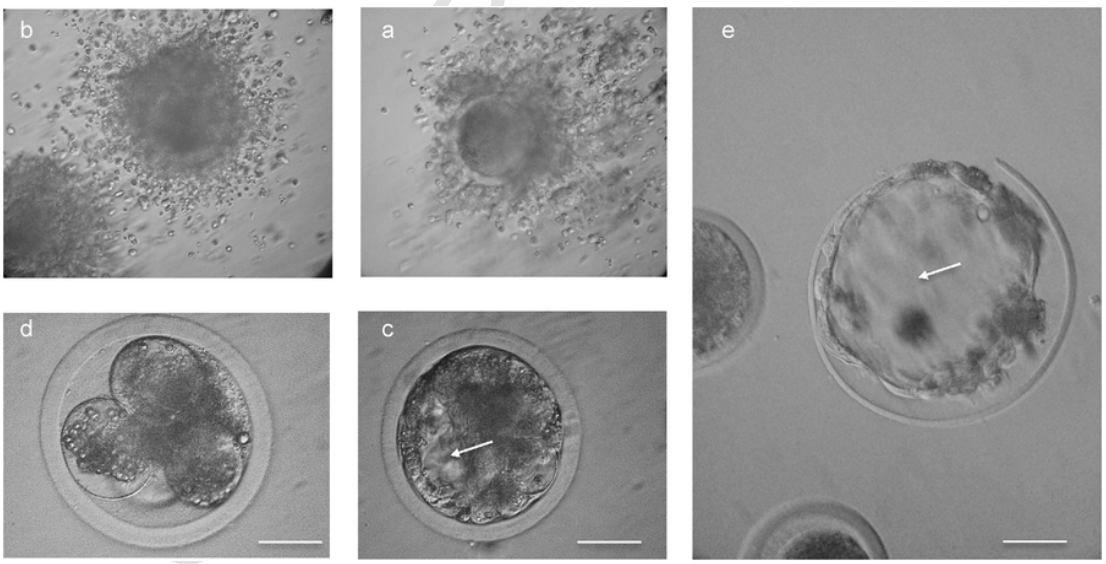

Fig. 3. Buffalo oocytes and embryos obtained with bi-weekly OPU regime. a, b. Grade I and II oocytes, 10X objective. c. Early blastocyst (day 7 post insemination) with arrow indicating blastocele cavity, 20X objective. d. Cleaved embryo (48 h post insemination), 20X objective. e. Hatching blastocyst (day 9 post insemination), 20X objective. Scale bar = 50 μm.

Queensland, Australia) was rinsed with aspiration medium consisting of Dulbecco's Phosphate Buffered Solution (DPBS, Serendipia Labs, Argentina) supplemented with 100 USP/ml units of heparin, 1% v/v fetal bovine serum (FBS, Natocor, Argentina) and penicillin-streptomycin. At the time of aspiration, all visible ovarian follicles were classified into three groups: small (diameter <3 mm), medium (diameter 3–8 mm) and large (diameter >8 mm), classification scale was based on previous observations of follicular distribution range in buffalo (Konrad et al., 2013, 2016). Follicular aspirates were filtered using a 75 um-pore filter (WTA, Brazil) and searched under a 50X stereomicroscope with a heated (37 °C) stage.

2.3. Oocyte classification and in vitro maturation

Unless otherwise stated, all chemicals were purchased from Sigma Argentina. Recovered cumulus-oocyte complexes (COCs) were washed and maintained in TCM 199 supplemented with Hank's salts, 10% v/v FBS (Gibco, Thermo Fisher Scientific, Argentina) and 0.1% v/v gentamicin (Gibco, Thermo Fisher Scientific, gentamicin reagent solution, 50 mg/ml) for classification according to quality. Cumulus-oocyte complex quality was classified according to IETS guidelines (quality I- highest to quality IV- poorest) and previous reports for buffalo (Di Francesco et al., 2011) based on the number of layers of compact cumulus investments and presence of homogenous cytoplasm. Once classified, the COCs were washed and transferred to a 35-mm Petri dish containing 3 ml of maturation medium consisting of TCM 199 with Earl's salts and 25 mM Hepes, 10% v/v FBS, 50 mM cysteamine, 5 μg/ml FSH (NIH-FSH-P1; Folltropin-V; Bioniche Animal Health, Belleville, Ontario, Canada) and 0.1% v/v gentamycin sulfate (Gibco, Thermo Fisher Scientific, gentamicin reagent solution, 50 mg/ml). They were then transferred to 1.8 ml Eppendorf tubes filled to the top with pre-equilibrated maturation medium and allowed to mature in transit to the IVF laboratory (8 h from farm) in a portable incubator (Minitube, Germany) set at 37 °C.

2.4. In vitro fertilization

After 15–18 h of in vitro maturation (Neglia et al., 2011), oocytes were removed from maturation medium, washed 3 times and placed in a defined insemination medium (Brackett and Oliphant, 1975). All inseminations were conducted using straws from one single buffalo bull of proven fertility and good in vitro performance. Sperm were washed by double spin-down centrifugation (400*g*, 5 min), and subsequently purified for selection of progressively motile spermatozoa by swim up procedure for 25 min at 38.5C and 5% CO_2 in BO-BSA medium. After swim-up, 200 μl of the upper layer were recovered and adjusted to a final concentration of 2×10^6 motile sperm/ml. Initiation of sperm and oocyte co-incubation was recorded as 0 h post-insemination. Groups of 8–10 oocytes were co-incubated for a total of 6 h, after which presumptive zygotes were washed in TCM 199 Hank's medium supplemented with 0.3% w/v fatty-acid free BSA. Remaining cumulus cells were removed by repeated pipetting in TCM 199 medium with Hank's salts supplemented with 0.1% w/v hyaluronidase (400–1000 units/mg). Finally, the presumptive zygotes were cultured (5 μl culture medium/presumptive zygote) under 5% CO_2, 5% O_2 and 90% N_2 at 38.5C in SOFaa-BSA medium (Holm et al., 1999) with 0.3% w/v fatty-acid free BSA for the first 3 days of culture. The medium was changed on day 4 of culture to SOFaa-BSA with the addition of 5% v/v FBS.

2.5. Statistical analysis

Continuous data were analyzed by analysis of variance analysis (ANOVA) using a repeated measures model and proportional data were analyzed by Chi square test using GraphPad Prism v.7 software. Differences were considered statistically significant at $p < 0.05$.

3. Results

A total of 61 OPU procedures were conducted at 7-day or 14-day intervals for a period of 10 weeks. Thirty OPU procedures were conducted at 14-day intervals while 31 OPU procedures were conducted at 7-day intervals (Table 1).

There was no difference in the average number of small and large-sized follicles per OPU procedure. However, there were a greater number of medium-sized ovarian follicles available for puncture when OPUs were conducted at 14-day intervals (5.129 vs 3.267; $p < 0.05$). In addition, the 14-day interval resulted in a higher COC recovery rate than the 7-day interval (51 vs. 31.5%; $p < 0.05$).

A total of 140 oocytes were recovered from 30 OPUs conducted at 14-day intervals, whereas 84 oocytes were recovered from 31 OPUs conducted at 7-day intervals (Table 2). The number of oocytes recovered per donor was significantly higher (4.51 vs. 2.8; $p < 0.05$) when OPU was conducted at 14-day intervals. Although there was no difference in the number of quality grade I and II oocytes recovered per donor, there was a significant increase in the number of quality grade III and IV oocytes per donor when OPU was conducted with 14-day intervals (3.71 vs. 2.27; $p < 0.05$)

The effect of OPU performed at 7 or 14-day interval on embryo development is shown in Table 3. After in vitro fertilization, embryo developmental competence from grade I and II oocytes was superior to the development from grade III and IV oocytes, irrespective of the interval group. The OPU interval did not have an effect on the proportion of cleaved embryos from grade I and II oocytes. However, there were a higher proportion of blastocysts from animals punctured every 14 days (28 versus 6%; $p < 0.05$). The result-

Table 1
Number of follicles and COCs recovered from OPU procedures conducted at 7 and 14-day intervals.

	Treatments	
	14-day OPU Interval	7-day OPU Interval
No. OPU procedures	30	31
No. total follicles aspirated	273	266
No. small (< 3 mm) follicles (mean ±SE)	3.681 ± 0.41	4.581 ± 0.39
No. medium (3–8 mm) follicles (mean ±SE)	5.129 ± 0.50[a]	3.267 ± 0.50[b]
No. large (> 8 mm) follicles (mean ±SE)	0.2903 ± 0.08	0.7333 ± 0.30
No. (%) COCs recovered[c]	140 (51%)[a]	84 (31.5%)[b]
CL-like structures[d]	yes	yes

[a,b]Different superscripts within rows indicate significant differences; $p < 0.05$.
[c] No. of COCs/No. of follicles aspirated.
[d] CL-like structures observed by ultrasonography in aspirated animals between OPU procedures.

Table 2
Recovery and quality grade of oocytes from OPU procedures conducted at 7 and 14-day intervals.

Trt	Total oocytes recovered (mean ±SE)	Grade I and II oocytes (mean ±SE)	Grade III and IV oocytes (mean ±SE)
14-day interval	140 (4.516 ± 0.49)[a]	25 (0.8065 ± 0.18)	115 (3.71 ± 0.44)[a]
7-day interval	84 (2.8 ± 0.45)[b]	16 (0.5333 ± 0.14)	68 (2.267 ± 0.37)[b]

[a,b]Different superscripts within columns indicate significant differences; $p < 0.05$.

Table 3
Embryo development after IVF according to oocyte quality grade, from OPU procedures conducted at 7 and 14-day intervals.

Oocyte grade	Grades I and II oocytes		Grades III and IV oocytes	
Trt	14-day OPU Interval	7-day OPU Interval	14-day OPU Interval	7-day OPU Interval
No. oocytes for IVF	25	16	75	68
No. (%) cleaved[c]	16 (64)[a]	7 (44)[a]	3 (4)[b]	1 (1)[b]
No. (%) blastocyst[b]	7 (28)[a]	1 (6)[b]	0	0

[a,b]Different superscripts within rows indicate significant differences; $p < 0.05$.
[d]No. blastocysts produced/No. of oocytes, development assessment conducted on day 7 post-insemination.
[c] No. of cleaved zygotes/No. of oocytes, development assessment conducted on day 3 post-insemination.

ing embryos were not available for extended culture to day 9 because donors were part of a commercial operation. Embryos were vitrified on day 7 and stored for later transfer by owner.

After follicular aspirations, animals were returned to the main herd and exposed to bulls. Although this study was not designed to evaluate effect of OPU interval on fertility, 83% (4 donors from 7-day interval group and 6 donors from 14-day interval group) were confirmed pregnant by rectal ultrasonography at 45 days.

4. Discussion

In the first OPU reported for buffalo by Boni et al. (1994), donors were aspirated every 7 days. Authors reported an average of 6.75 and 4.17 follicles available and oocyte recovery rates of 44.4 and 31.9% for FSH-primed and untreated controls, respectively. Although in our present study the oocyte recovery rate of 31.5% observed in the 7-day interval group is close the 31.9% reported by Boni et al. for their control group, variable differences (season, breed, days open and hormonal treatment) prevent direct comparison between the two studies. In Ding et al. (2008) 2008, Ding et al. collected oocytes at 3, 7, 10 and 14-day intervals for somatic cell nuclear transfer. After reconstruction, the highest blastocyst yield was observed from oocytes collected every 14 days; authors proposed oocytes from early atretic follicles in the second follicular wave had superior developmental competence.

Ferraz et al. (2007,2015) evaluated differences between follicular aspirations conducted every 7 or 14 days. Results revealed that increasing the OPU interval from 7 to 14 days resulted in more follicles available for puncture (15.6 vs. 12.8; $p < 0.0001$) and higher oocyte recovery rate (10 vs. 8.5; $p < 0.004$), indicating a less intensive OPU interval could enhance follicular recruitment. In addition, authors reported an interaction between bST treatment and the 14-day OPU interval, which resulted in reduced oocyte recovery rates for this treatment group. In our present study, the higher proportion of COCs recovered in the 14-day OPU interval is in agreement with these previous reports. Noteworthy, the 14-day OPU interval in our study resulted in a significantly higher number of medium-sized follicles available for puncture. It is possible that the higher proportion of recovered COCs could be attributable to the reduced proportion of large size follicles present at the time of aspiration, which in turn has been shown by other authors (Seneda et al., 2001) to result in lower COC recovery rates.

Our findings indicate that, although the 14-day interval improved the overall number of follicles available for puncture and oocyte recovery, this corresponded to an increase in the number of lower quality grade oocytes (grades III and IV). In buffalo, as in other domestic species, the relationship between oocyte quality and embryo development has been established (Chauhan et al., 1998; Nandi et al., 1998; Abdoon et al., 2001; Yousaf and Chohan, 2003). Noteworthy, most reports in buffalo have been conducted using abattoir-derived oocytes. Results from our present study using OPU-derived oocytes confirm that in vitro developmental competence from higher quality oocytes (grades I and II) is superior to the development from lower quality oocytes (grades III and V), strengthening the evidence that oocyte competence is a key limiting factor in the OPU efficiency for in vitro embryo production in buffalo.

In 1996, Boni et al. conducted a study in which the effect of twice-weekly OPU procedures was evaluated over a 2-month period of time. In that study, the average number of follicles available for puncture was 5.48, with an average of 2.71 oocytes recovered per donor per session. The study also indicated that only 53% of the recovered oocytes were suitable for in vitro embryo production due to poor oocyte quality. In our study, 100% of the higher quality oocytes recovered in both 14 and 7-day interval groups were considered suitable for IVF based on integrity of zona pellucida, ooplasm homogeneity and overall absence of degeneration. On the contrary, suitability of lower quality oocytes for IVF differed between 14 and 7-day treatment groups; whereas all grade III and IV oocytes recovered from the 7-day interval group were suitable for IVF, only 65% (75/115) from the 14-day interval group could be used for IVF due to evidence of oocyte degeneration. The reason for this difference is unclear. The definition of oocyte quality is quite complex; it involves morphologic, meiotic, cytoplasmic and molecular aspects with yet to be understood interactions (Lonergan et al., 1994; Sirard et al., 2006). Unfortunately, because current IVP systems require an intact oocyte with surrounding cumulus cells in order to maximize embryo development, our current method of oocyte quality assessment could be inadequate because it only addresses the morphology variable. It is possible that, although oocytes were classified under the same "low quality" category based on morphological assessment of cumulus investments and cytoplasmic aspect, other differences (cytoplasmic, meiotic, molecular) undetectable by morphological evaluation could be introduced by OPU interval and follicular origin (Raghu et al., 2002; Krisher, 2004). Because of the clear limitation oocyte quality currently plays in buffalo IVP systems, this issue should be further addressed in future studies.

Murrah buffaloes have been shown to have on average 26-day estrous cycles, 3 waves of follicular growth and an active functional CL until day 17 of the cycle (Carvalho et al., 2016). Although in the current study ultrasound evaluation and description of follicular wave emergence was not feasible between OPU procedures due to logistical issues regarding the access to donors, CL-like structures were observed at the time of aspiration both at 7- and 14-day OPU intervals, even with initial double dominant follicle ablation and the complete aspiration of all ultrasound-detectable follicles in all the procedures. This observation interested us, because other authors (Petyim et al., 2003; Boni, 2012) had previously reported these types of structures immediately after OPU.

Petyim et al. (2003) compared twice-weekly OPU procedures in either continuous or discontinuous regimes. Authors suggested continuity was an important aspect when analyzing the effect of OPU and reported CL-like structures between days 3 and 4 post-OPU in both continuous and discontinuous treatments. They proposed these CL-like structures fitted a 'subfunctional' classification (Petyim et al., 2001) due to their smaller size, shorter lifespan and aberrant sub-luteal progesterone production patterns when compared to normal, ovulation-derived CLs.

In cattle, low, sub-luteal progesterone concentrations (1–2 ng/ml) are associated with increased LH pulse frequency (Adams et al., 1992; Ginther et al., 1996). In turn, LH pulse frequency determines whether or not a dominant follicle ovulates or undergoes atresia (Savio et al., 1993; Lonergan, 2011). Although Petyim et al. (2003) reported that the presence of CL-like structures did not have an effect on the quantity and quality of oocytes recovered, others have reported that sub-luteal progesterone levels may affect follicular recruitment, growth and subsequent oocyte competence (Boni et al., 1996). Because it was not possible to analyze follicular dynamics or blood P_4 levels in our current study, no speculation regarding CL functionality, effect on follicular population or oocyte quality can be made. However, this is an interesting aspect to follow up on future studies, particularly in light of the increased oocyte degeneration and reduced suitability for IVP observed in the lower oocyte quality group.

In conclusion, results from this study indicate that it is possible to perform follicular aspirations every 14 days for oocyte collection in water buffalo without significantly compromising the proportion of good quality oocytes obtained; this approach could be considered as an alternative when access to animals is restricted. This study also confirmed that in buffalo, lower quality grade oocytes have compromised in vitro developmental after IVF and repeated, weekly oocyte collection may compromise subsequent developmental potential.

Conflict of interest statement

Authors declare no conflict of interest.

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Statement of animal rights

All animal procedures were reviewed and approved by the Animal Welfare Committee, (Universidad Nacional del Nordeste, Corrientes, Argentina), and conducted in accordance with ethical standards approved by the Institution.

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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