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The effect of parity on the efficacy of an ovulation synchronization (Ovsynch) protocol in buffalo (*Bubalus bubalis*)

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Abstract

The aim of the present study was to study the effect of parity on the efficacy of an Ovsynch protocol in buffalo. Buffalo heifers (HE; n = 8) and cows (BC; n = 9) were used to monitor ovarian follicular dynamics and evaluate serum progesterone profiles during this protocol. A total of 385 control buffalo heifers (CHE; n = 219) and cows (CBC; n = 166) were used to compare conception rates following the application of this protocol. The heifers and cows were cycling. All treated animals were injected with GnRH on day 0, PGF2α on day 7, GnRH on day 9 and artificially inseminated 16 h later. Ovarian changes were monitored daily using ultrasound and serum progesterone (P4) evaluated in the investigated animals. All heifers and 5 cows had follicles >8 mm at the first GnRH injection. The first GnRH injection resulted in ovulation in 7/8 HE (87.5%) and 5/9 BC (55.5%). Following the second GnRH injection, ovulation occurred in 100% of HE and 88.8% of BC. Ovulation occurred earlier in BC (10.4 ± 7.6 h) following the second GnRH injection than in HE (22.6 ± 5.4 h). Average P4 concentrations of HE were higher than those of BC on day 7 ($P < 0.04$). Conception rates were 62.5% (429/686) in HE, 59.8% (131/219) in CHE, 22.7% (62/273) in BC and 59.6% (99/166) in CBC. The present findings suggested that low conception rates in buffalo cows, compared to heifers, may be attributed to earlier ovulation and a less functional CL.

Keywords: buffalo parity, follicular dynamics, ovulation synchronization, progesterone, ultrasound.

Introduction

The Water buffalo is used in many countries, including Egypt, as a source of milk and meat. The population of buffalo in Asian and Mediterranean areas is about 150 Million, and 3.7 Million are bred in Egypt (Borghese, 2004). Silent heats and a long calving interval have been recognized as major causes of infertility and low productivity in buffalo. Seasonality of Egyptian buffalo is not clear. Productivity in domesticated buffalo is limited for reasons like inbreeding, feeding and health care, but the major problem seems to be infertility, which is much more prevalent than in cattle (Danell, 1987; Abol-Roos and Gaffar, 2000). Post-partum anestrus in buffalo is responsible for a long calving interval (Borghese *et al*., 1993; Campanile *et al*., 1993). Under typical management, upon reaching pubertal weight and age, buffalo heifers are housed with female adult buffalo for either natural mating or artificial insemination (AI). AI has made a significant contribution to genetic improvement in cattle and has the potential to improve genetic characteristics in buffalo. However, the widespread use of AI in buffalo is still limited due to a relatively low expression of estrus behavior (Seren *et al*., 1993; Ohashi, 1994). A highly variable duration of estrus (4-64 h) and difficulty in predicting the time of ovulation negatively influence the application of AI in buffalo (Baruselli, 2001). These considerations indicated a need for estrus synchronization using fixed-time insemination for the implementation of breeding programs in buffalo (Presicce *et al*., 2004; Ali and Fahmy, 2007). Estrus synchronization protocols, largely derived from cattle, have yielded variable results in buffalo (Singh *et al*., 1984; Barile *et al*., 1997; Zicarelli *et al*., 1997; Neglia *et al*., 2003; Presicce *et al*., 2004; Campanile *et al*., 2007a). Although failure of timed ovulation in synchronized buffalo has been suggested as an important cause of poor fertility (Hattab *et al*., 2000; Baruselli, 2001), it has not yet been fully studied. In addition, there is limited use of AI in buffaloes due to low conception rates following estrus synchronization (Zicarelli *et al*., 1997). There are some reports on ovarian follicular dynamics in buffalo (Manik *et al*., 1994; Taneja *et al*., 1995a,b, 1996; Baruselli *et al*., 1997), but a critical comparison of the effects of age and parity on ovarian follicular dynamics and hormonal profiles has not been widely studied (Presicce *et al*., 2004). The Ovsynch program has been applied to nulliparous and multiparous (Presicce *et al*., 2004) and cyclic and non-cyclic buffaloes (De Rensis *et al*., 2005; Ali and Fahmy, 2007). However, application of ovulation synchronization programs in Egyptian buffaloes has not been widely applied. Characterization of follicular turnover using ultrasonography and hormonal profiles in buffalo heifers and cows during an Ovsynch program under local conditions in Egypt has also not been critically studied. The aim of the present study was to monitor and compare ovarian follicular dynamics and serum progesterone profiles in Egyptian buffalo heifers and post-partum cows *(Bubalus bubalis*)

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during an ovulation synchronization protocol.

Materials and Methods

Animals and management

A total of 686 Egyptian buffalo heifers (HE; ages 19-27 months, average 24 ± 0.8 months and weighing 350-420 kg) and 273 parturient and lactating Egyptian buffalo cows (BC; 1-5 parities, weighing 415-530 kg and 45-65 days post-partum) were assigned for the present study. All animals were cycling and reproductively healthy. A total of 219 heifers (CHE) and 166 buffalo cows (CBC) inseminated during natural estrus using the same semen within the same season were used to compare conception rates between treatment and control groups. Experimental and control animals were housed in an open yard on the animal farm of Al-Azhar University, Assiut-Campus and Ard El-Khair farm, Misr El-Khair foundation, Assiut province, Egypt. The buffalo cows were milked twice daily, and kept on 40% dry matter forage (Egyptian clover) and 60% concentrate mixture. Wheat straw was also fed *ad libitum*. The ration provided 14% CP and 67% TDN. The experiment was conducted during Dec.-Feb. of 2009/2010 (57.3 ± 2.3% relative humidity and 13.4 ± 0.8ºC maximum atmospheric temperature). Body condition scoring (BCS) using a system from 1 = thin to 5 = fat was evaluated for each cow (Edmonson *et al*., 1989). Only cows between 2.5 and 3.5 BCS were included. Before starting the Ovsynch program, the reproductive tract of all HE and BC were examined rectally and ultrasonographically for the recording of ovarian and uterine findings for at least one cycle for each animal. The examination started on Day 25 post-parturition in BC. In control groups, routine rectal palpations were performed before insemination.

Ovsynch program

Experimental HE and BC were treated on day 0 (1st day of the program) with 100 µg GnRH im (Buserelin, Receptal®, Intervet International B.V., Boxmeer, Holland). Seven days later (day 7), 25 mg PGF2α (Dinoprost, Lutalyse, Pfizer, Pharmacia and Upjohn Company, NY, USA) was administered im. Forty-eight hours later (day 9), the animals received a second dose of 100 µg GnRH im. All animals were artificially inseminated 16-21 h following the second GnRH treatment with frozen-thawed semen from a superior-proven buffalo bull.

Ultrasound examination

Ovarian structures of only 8 heifers and 9 cows in each treated group were monitored ultrasonographically using a real-time, B-mode, diagnostic scanner equipped with a transrectal 5/7.5 MHz linear array transducer (Hitachi, EUB-405B, Japan). Ultrasound examinations were performed once daily from days 0 to 9 and every 12 h thereafter until ovulation or for a maximum of 48 h. All follicles >3 mm and CL were measured and mapped individually for each cow. Ovulation was identified when a tracked large, growing, antral follicle was no longer observed. Emergence of a follicular wave was defined as the day on which the retrospectively tracked dominant follicle (DF) was 4 mm in diameter (Ginther *et al*., 1997). Follicle luteinization was considered when a follicle did not ovulate; instead luteal tissue gradually developed and was detected as an echogenic ring that later increased in thickness and filled the whole follicular antrum. The CL was examined and an image of the largest cross-sectional area was evaluated. Luteal regression following PGF2α treatment was considered when P4 concentration was less than 1 ng/ml. The following ovarian characteristics were determined and compared between groups: (1) ovulation rates after the first and second GnRH treatments; (2) diameter of the ovulatory follicles; (3) interval from treatment to emergence of a new follicular wave after the first GnRH treatment; (4) number and diameter of the CL; and (5) luteal regression rate after PGF2α treatment. Pregnancy diagnosis was performed by ultrasonography 30 days after AI. The conception rate was determined and compared between groups.

Homonal analysis

Blood samples were collected from the jugular vein of 8 heifers and 9 cows (the same animals used to monitor ovarian changes) from the treated groups into plain tubes at days 0, 2, 4, 7, 9 and 10 of the Ovsynch program. The samples were transported to the laboratory in an ice box within 20-30 min, centrifuged at 1700 x g for 20 min and sera were stored at -20ºC until analyzed for progesterone (P4). P4 concentration was determined using a direct ELISA technique. Kits were provided by Diagnostic System Laboratory Co. (DSL, Catalogue No. 3900, USA). The coefficient of variance of intra- and inter-assays were 4.8 and 9.2%, respectively. The sensitivity of the assay was 0.12 ng.

Statistical analyses

The data are presented as mean ± SEM and statistical analysis was carried out using SPSS, version 10.0. Differences in ovulation rates after GnRH treatment, luteal regression rates and conception rates between HE and CHE heifers and BC and CBC cows were evaluated by χ^2-test. A t-test was used to compare groups for follicle and CL diameters within examination dates, the interval from treatment to ovulation and the interval to wave emergence. Differences among the HE, CHE, BC and CBC groups in serum P4 levels were evaluated using ANOVA. Level of significance was set at $P < 0.05$.

Results

Follicle turnover

At the time of the first GnRH injection, the mean number of small follicles (2-5 mm) in HE was 65 *vs*. 73 in BC. The number increased (P < 0.05) in HE after GnRH injection compared to BC. The mean number of medium sized follicles (5-8 mm) was similar in both groups at the time of the first GnRH injection (11 in HE vs. 10 in BC), then increased in both groups with a non-significantly larger number in HE than BC (P = 0.3). The number of the large follicles (>8mm) at the time of the first GnRH injection was higher (P < 0.05) in HE than BC (Fig. 1).

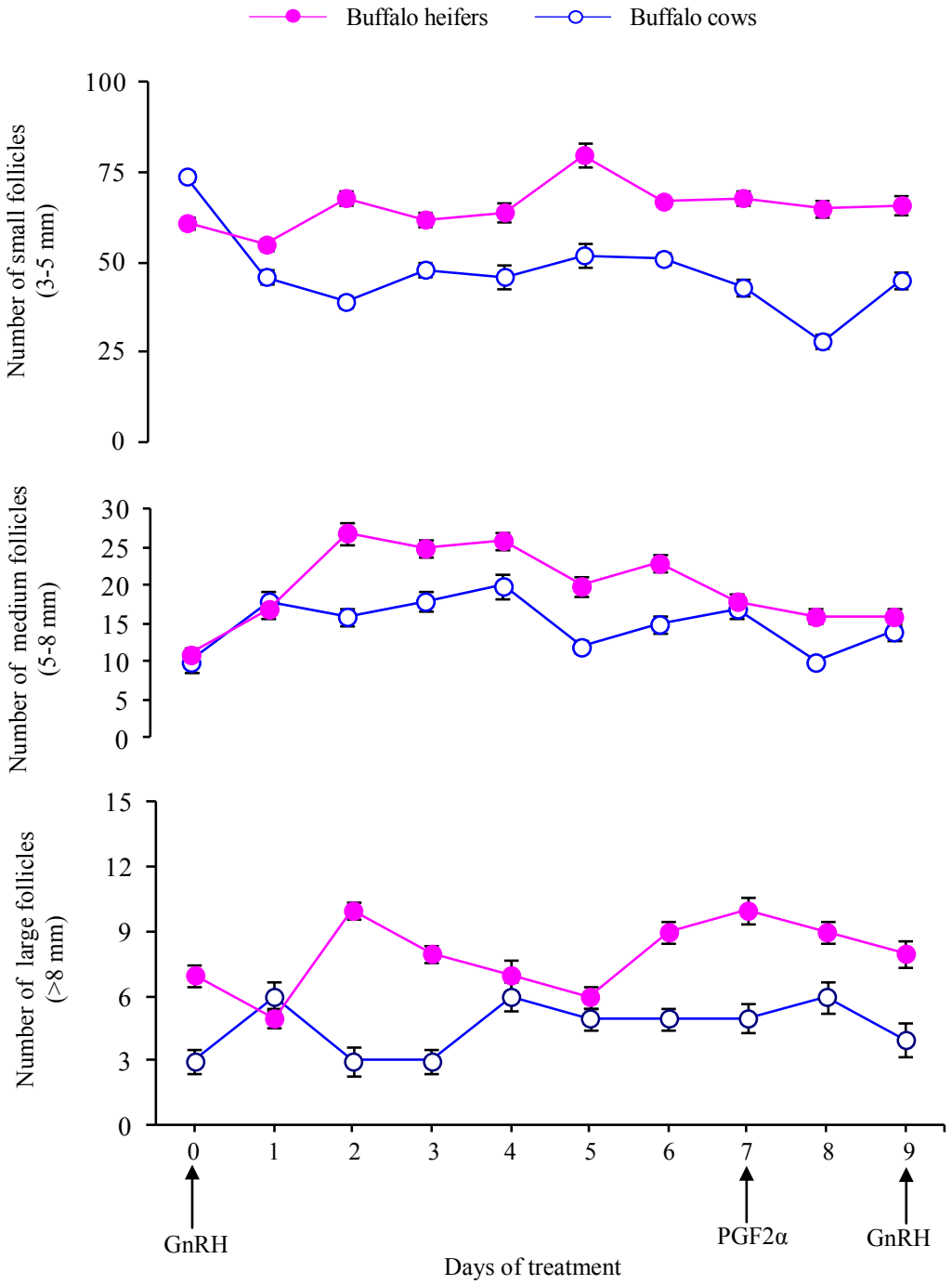

Figure 1. Follicular populations in Egyptian buffalo heifers (n = 8) and cows (n = 9) on both ovaries during the Ovsynch protocol.

Ovarian response to first GnRH injection

After the first GnRH injection, 87.5 and 100% of HE and BC ovulated, respectively (P = 0.88; Table 1). Follicle luteinization was observed in only one follicle (11.1%) in the HE group. A new follicular wave was recruited in all heifers after nearly two days (51.0 ± 3.4 h), and in 8/9 BC, a new dominant follicle developed within nearly the same period as in HE (52.6 ± 2.4 h).

Table 1. Ovarian response of buffalo heifers (HE) and buffalo cows (BC) to the first GnRH injection (day 0) during the Ovsynch protocol.

Ovarian findings	Buffalo heifers (HE, n = 8)	Buffalo cows (BC, n = 9)
Largest follicle	8/8[a]	5/9[b]
Number	8[a]	5[b]
Diameter (mm)	9.5 ± 0.1[a]	9.8 ± 0.3[a]
Medium follicle number	11[a]	10[a]
Small follicle number	65[a]	73[a]
CL number	4/8[a]	4/9[a]
CL size (mm)	15.5 ± 2.0[a]	18.9 ± 1.2[b]
Response to first GnRH		
Ovulation (n)	7/8[a]	5/5[a]
Time to ovulation (h)	48.7 ± 3.7[a]	64.2 ± 5.6[b]
Ovulatory follicle size (mm)	10.5 ± 1.0[a]	13.4 ± 4.3[a]
Follicle atresia	11[a]	10[a]
Follicle luteinization	1	0
Follicle wave number	8/8[a]	8/9[a]
Time to emergence of follicular wave (h)	51.0 ± 3.4[a]	52.6 ± 2.4[a]
Largest follicle growth rate (days 0-7; mm/day)	0.57 ± 0.04[a]	0.86 ± 0.06[b]

[a,b]Values with different letters differ significantly.

Ovarian findings on day 7

On the day of PGF2α injection (day 7), 3 HE had a single CL and 5 had double CL (Table 2); 2 of the double CL were already present at the time of the first GnRH injection and the others resulted from second ovulations. One buffalo cow had only double CL and the rest had a single CL. The mean diameter of the CL was larger (P < 0.003) in the BC group. A follicle >8 mm in diameter was detected in all HE (100%) and in 5/9 BC (55.5%) groups. The mean diameter of the second largest follicle was greater (P < 0.05) in BC than in HE (12.9 ± 0.2 *vs*. 10.1 ± 0.6 mm, respectively). The mean growth rate of the largest follicle between days 0 and 7 was higher (P < 0.03), and the maximum diameter was greater (P < 0.01) in BC.

Table 2. Ovarian response of buffalo heifers (HE) and buffalo cows (BC) to PGF2α injection on day 7 (day 0 = start of treatment) of the Ovsynch protocol.

Ovarian findings	Buffalo heifers (HE, n = 8)	Buffalo cows (BC, n = 9)
Corpus luteum (n)	3/8 had 1 CL	4/91 CL
	5/8 had 2 CL	1/9 had 2 CL
Diameter (mm)	14.8 ± 0.7[a]	16.2 ± 0.9[b]
Largest follicle		
Animals (n)	8/8[a]	5/9[b]
Number	10[a]	5[b]
Diameter (mm)	10.1 ± 0.6[a]	12.9 ± 0.2[b]
Growth rate (mm/day)	0.57 ± 0.04[a]	0.86 ± 0.06[b]
Response to PGF2α (Day 9)		
Luteolysis (n)	8/8[a]	1/5[b]
Largest follicle		
Animals (n)	8/8[a]	7/9[a]
Number	8[a]	7[a]
Diameter (mm)	10.7 ± 0.7[a]	11.7 ± 0.9[a]
Growth rate (mm/day)	0.59 ± 0.06[a]	0.58 ± 0.07[a]

[a,b]Values with different letters differ significantly.

Ovarian response to the PGF2α

Luteolytic responses to PGF2α treatment were 87.5 and 20.0% for HE and BC, respectively (Table 2). In HE, the CL regressed from an average diameter of 14.8 ± 0.7 mm on day 7 to 9.1 ± 0.7 mm on day 9. In BC, the CL decreased from 16.2 ± 1.2 mm on day 7 to 9.1 ± 0.6 mm on day 9. The diameter of the dominant follicle was 10.7 ± 0.7 mm and 11.7 ± 0.9 mm in HE and BC, respectively.

Ovarian response to the second GnRH injection

The size of the dominant follicle was 9.8 ± 1.2 and 12.0 ± 2.2 mm for HE and BC, respectively, at the time of second GnRH injection (Table 3). An ovulation rate of 100 and 88.8% was recorded for HE and BC, respectively. Time to ovulation averaged 22.6 h (range 16-36 h) and 10.4 h (range 6-24 h) in HE and BC, respectively ($P < 0.01$). The mean diameter of the CL on day 7 of the protocol was 15.4 ± 0.8 and 19.7 ± 1.3 mm in HE and BC ($P < 0.03$), respectively.

Table 3. Ovarian response and conception rates of buffalo heifers (HE) and buffalo cows (BC) after the second GnRH injection during the Ovsynch program.

Ovarian Findings	Buffalo heifers (HE, n = 8)	Buffalo cows (BC, n = 9)
Ovulation (n)	8/8[a]	8/9[a]
Interval to ovulation (h)	22.6 ± 5.4[a]	10.4 ± 7.6[b]
Ovulatory follicle diameter (mm)	10.9 ± 0.9[a]	13.6 ± 0.9[b]
Second CL diameter developed at day 7 (mm)	15.4 ± 0.8[a]	19.7 ± 1.3[b]
Conception rate	62.5% (429/686)[a]	22.7% (62/273)[b]

[a,b]Values with different letters differ significantly.

P4 concentrations

The average P4 concentrations were higher ($P < 0.04$; Fig. 2) in HE than in BC on day 7. The concentration of serum P4 correlated positively with the diameter of the CL ($r = 0.6$; $P < 0.005$).

Conception rate

In the treated groups, 429/686 HE (62.5%) and 62/273 BC (22.7%) conceived ($P < 0.01$). In the control groups, 131/219 CHE (59.8%) and 99/166 CBC (59.6%) conceived.

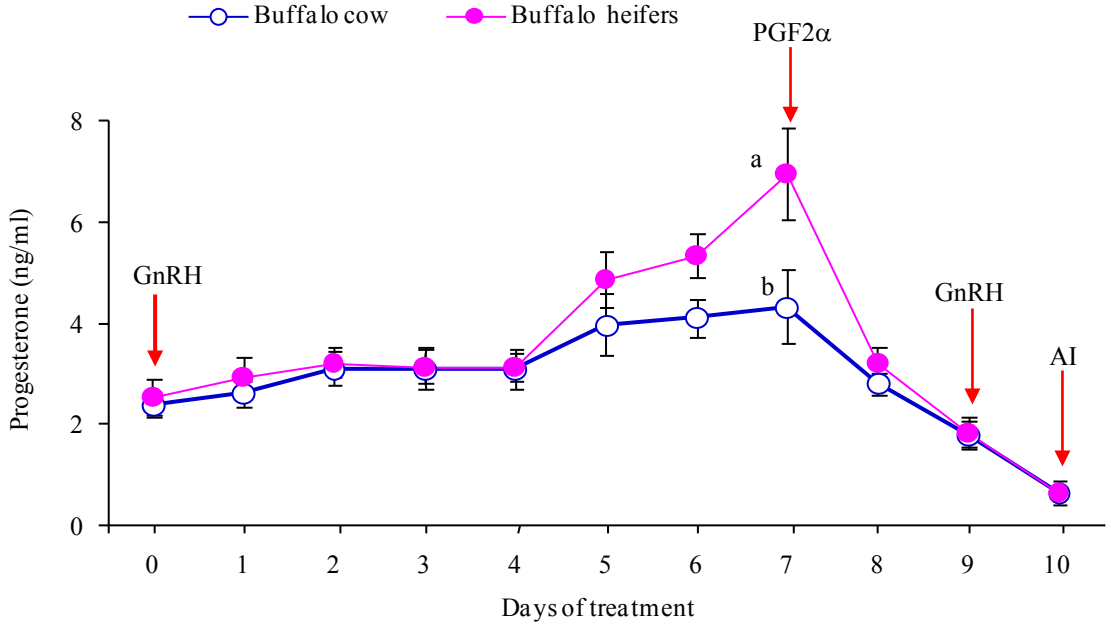

Figure 2. Progesterone concentrations in the serum of treated buffalo heifers (n = 8) and buffalo cows (n = 5) during the Ovsynch program. [a,b]Values with different letters differ significantly.

Discussion

This study aimed to describe the differences between heifers and buffalo cows in their response to different treatments in the Ovsynch program. The first GnRH injection was designed to enhance the ovulation of the largest functional follicle and to induce a new follicular wave. It is well accepted that an injection of a

GnRH agonist at any stage of the estrous cycle in buffalo 1) increases the number of medium-sized follicles within 3 days of treatment, 2) eliminates the large follicles by ovulation or atresia and 3) induces the emergence of a new follicular wave within 2 to 3 days of treatment (Jazayeri *et al*., 2010). The subsequent injection of PGF2α increases the percentage of synchronized animals by lysis of both the cyclic CL and the CL resulting from ovulation of the dominant follicle (Pursley *et al*., 1995). The second GnRH injection on day 9 of the protocol causes an induced LH surge responsible for ovulation of the dominant follicle and formation of a new CL (Senger, 2003).

The results of the present study showed that 87.5 and 100% of HE and BC, respectively, had ovulations after the first GnRH injection. In previous studies, an ovulation rate of approximately 86% was recorded in cyclic buffalo (Rao and Venkatramiah, 1991; De Araujo Berber *et al*., 2002), 90% in cyclic and 50% in non-cyclic buffalo (Neglia *et al*., 2003; Ali and Fahmy, 2007) and 82-90% in cyclic cattle (Pursley *et al*., 1995; Frike *et al*., 1998; Wiltbank, 1998) following the first GnRH administration. The present results coincide with the previous study of Neglia *et al*. (2003) in buffalo but disagree with previous reports in cattle (Pursley *et al*., 1995; Hussein, 2003). The discrepancy between the present findings and previous ones may be attributed to the lower number of dominant follicles in BC at the time of the first GnRH injection. The time to ovulation after GnRH injection depends mainly on the diameter of the largest follicle at the time of injection (Wiltbank, 1998; Hussein *et al*., 2002; Hussein, 2003). However, follicle diameter is not the only parameter that can affect ovulation rates. In a recent study, it was demonstrated that follicle size in buffalo that ovulated compared to those that did not ovulate is quite similar (Campanile *et al*., 2008). Moreover, the stage of follicular development (growth or regression phase) greatly affects its response to GnRH treatment (Dharani *et al*., 2010).

It has been noted that the first GnRH injection was successful in synchronizing a new follicular wave 1-3 days after treatment (Neglia *et al*., 2003; Ali and Fahmy, 2007). In cattle, the new wave started 1-2 days after GnRH treatment, regardless of the incidence of ovulation (Frike *et al*., 1998; Hussein, 2003; Hussein *et al*., 2004). In the present study, this wave resulted in the development of a new dominant follicle in all and 8/9 of HE and BC, respectively. The second dominant follicle developed faster from days 0 to 7 and reached a larger diameter in the BC cows, which may be attributed to the subnormal P4 concentrations. Sub-luteal circulating P4 levels have been reported to increase the frequency of LH pulses, and a prolonged growth phase of the dominant follicle (Bridge and Fortune, 2003). After the PGF2α injection, if the dominant follicle did not ovulate, a new wave of small follicles needs several days to grow and become able to produce estradiol-17β, leading to an induction of the preovulatory LH-surge (Bridge and Fortune, 2003). PGF2α was injected on day 7 to regress all CL. If a CL resulted from the initial injection of GnRH, the 7 day interval should have provided sufficient time for the CL to mature in order to respond to PGF2α (Wiltbank, 1998; Ali and Fahmy, 2007). In the present study, all treated HE and 5 BC showed at least one or double CL on the day of PGF2α treatment. Most HE (7/8) had a follicle >8 mm at that time. The high synchrony between animals (presence of functional CL and large active follicle) reported in this study is the result of the first GnRH treatment. A synchrony rate of 90% in Murrah buffalo (Paul and Prakash, 2005), 75% in Mediterranean Italian buffalo (De Rensis *et al*., 2005) and 84% in cattle (Frike *et al*., 1998) was previously reported. Regression of corpora lutea was recorded for all HE and BC, but a difference was only apparent in a low number of animals (8 HE *vs*. 5 BC) in the current study.

In order to increase synchrony of ovulation, a second dose of GnRH was injected to ovulate the preovulatory follicle at a precise time (Wiltbank, 1998). In the present study, the second dominant follicle ovulated in 100 and 89% of HE and BC, respectively. An ovulation rate of 90-93% in cyclic buffalo (Rao and Venkatramiah, 1991; De Araujo Berber *et al*., 2002; Paul and Prakash, 2005) and 86-100% in cyclic cattle (Frike *et al*., 1998; Wiltbank, 1998; Hussein, 2003) was reported. In the current study, the BC group started to ovulate earlier (6 h after the second GnRH injection) than the insemination time (16 h after second GnRH injection). Furthermore, those animals ovulated over a relatively longer time span (40 h). Early and asynchronous ovulation, as well as early application of this program in the post-partum BC, seemed to be problematic and might explain the low conception rate in this group (22.7%). Neglia *et al*. (2001) observed a pregnancy rate of 45% in buffalo cows synchronized with PGF2α alone and 48.8% when PGF2α was combined with GnRH injection at the time of AI. Similarly, 33.3, 43.7, 36.0 and a 15.0 *vs*. 51.4% pregnancy rate was recorded in Murrah buffalo (Paul and Prakash, 2005), Mediterranean Italian buffalo (De Rensis *et al*., 2005), Italian cyclic buffalo (Neglia *et al*., 2003) and Swamp buffalo heifers vs. cows (Chaikhun *et al*., 2010) after using the Ovsynch protocol and timed insemination, respectively.

In the current study, the circulating concentrations of P4 precisely indicated the presence or absence of a CL and reflected its size and activity. Concentration of P4 found here is in agreement with the levels recorded by others in skim milk (Qureshi *et al*., 2000) and plasma (Jazayeri *et al*., 2010) of buffalo. Serum progesterone levels in HE and BC subjected to the Ovsynch protocol compared to control groups were not significantly different until the day 5 of treatment.

On the day 7 of the program, serum progesterone values differed ($P < 0.05$) in treated HE and BC compared to the control groups. The present results indicated that the CL of the BC group was less active than that of the HE group, which might explain the low conception rate recorded in the BC group and suggests that a significant improvement in conception rate in the BC cows can be achieved with the supplementation of exogenous progesterone from days 0 to 7 post-insemination. It has been previously suggested that high P4 levels at the time of PGF2α application may be an important factor to improve conception rates for subsequent inseminations (Hussein, 2003, De Rensis *et al*., 2005). On the contrary, Pursley *et al*. (1997) reported that P4 supplementation on the day of PGF2α injection had no effect on the pregnancy rates. Attempts to replace the second GnRH injection with hCG failed to improve conception rates in buffalo after fixed time AI in Brazil (Carvalho *et al*., 2007). It was suggested that the presence of a large follicle at the beginning of the Ovsynch protocol is a determining factor for the successful synchronization of ovulation and high conception rates (De Rensis *et al*., 2005). It was concluded previously that buffaloes require exogenous hormone treatments that induce elevated P4 concentration throughout the period from initial development to embryonic attachment. The use of pharmacological treatments in order to increase P4 systemic levels between 25 and 40 days post-AI, characterized by a 45% embryo mortality rate in buffalo, play a determinant role in farms with a high incidence of embryonic mortality (Campanile *et al*., 2005, 2007b).

In conclusion, the application of the Ovsynch program in buffalo heifers could be better than in cows. Conception rates in buffalo heifers were acceptable and satisfactory, while in buffalo cows were very low. This difference may be attributed to earlier and long-lasting ovulation as well as a sub-functional CL in buffalo cows. In addition, early application of the program during the post-partum period may be another possible cause of lowering conception rates in buffalo cows. Further studies should focus on improving conception rates following application of the Ovsynch program.

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