Anim. Reprod., v.9, n.1, p.00-00, Jan./Mar.2012.

The effect of parity on the efficacy of 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 Ovsynch protocol in buffalo. Out of 686 buffalo heifers (HE) and 273 cows (BC), 8 heifers and 9 cows were used to monitor and evaluate ovarian follicular dynamics and serum progesterone profile during this protocol while the rest of animals were used to compare the conception rate following the application of this protocol, respectively. Total of 385 control buffalo heifers (CHE, n = 219) and cows (CBC, n = 166) were used as a reference for conception rate. The heifers and buffalo-cows were cyclic. All treated animals were injected with GnRH on Day 0, $PGF_2\alpha$ on day 7, GnRH on day 9 and inseminated artificially 16 h later. Ovarian changes were monitored daily using ultrasound and serum progesterone (P_4) in the investigated animals. All heifers (8) and 5 cows had F > 8 mm (LF) 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 second GnRH, ovulation occurred in 100% of HE and 88.8% of BC. Ovulation started earlier in BC (10.41 ± 7.6 h) following second GnRH and extended for longer (22.6 ± 5.4 h) in HE. The average P_4 concentrations of the HE were slightly greater than those of the BC on day 7 (P = 0.04). Conception rate in HE was 62.54 % (429/686) and was 59.82 % (131/219) in CHE, while it was only 22.71 % (62/273) in BC and 59.64 % (99/166) in CBC. It is suggested that the unsatisfactorily low conception rate in buffalo-cows -compared to heifers- may be attributed to the early ovulation and sub-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 production. The population of buffaloes in Asian and Mediterranean areas is about 150 Millions and 3.7 Millions are bred in Egypt (Borghese, 2004). Silent heats and long calving interval have been recognized as a major cause of infertility and low productivity in buffaloes. Seasonality of the Egyptian buffaloes is not clear. The productivity in domesticated buffaloes is limited for reasons like inbreeding, feeding and health care, but the major problem seems to be infertility that is much higher than that in cattle (Danell, 1987; Abol-Roos and Gaffar 2000). Postpartum anestrus in buffaloes is responsible for 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 buffaloes for either natural mating or artificial insemination (AI). AI has a significant contribution to the genetic improvement in cattle and has the potential to improve the genetic characters in buffaloes. However, widespread use of AI in buffalo is still limited due to relatively low expression of estrus behavior (Seren *et al*., 1993; Ohashi, 1994). Variable duration of estrus (4-64 h) and difficulty in predicting time of ovulation negatively influence the application of AI in buffaloes (Baruselli, 2001). This consideration indicated a need for estrus synchronization and fixed-time insemination for implementation of breeding programs in buffaloes (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 buffaloes has been suggested as an important cause of poor fertility (Hattab *et al*., 2000; Baruselli, 2001), yet it has not been fully studied. There is limited use of AI due to a low conception rate following estrus synchronization (Zicarelli *et al*., 1997). There are some reports of 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 largely studied (Presicce *et al*., 2004). The Ovsynch program has been applied in nulliparous and multiparous (Presicce *et al*., 2004), cyclic and non-cyclic buffaloes (De Rensis *et al*., 2005; Ali and Fahmy, 2007). However, application of ovulation synchronization program in Egyptian buffalo is not widely applied. Characterization of follicular turnover using ultrasonography and hormonal profile in heifers and buffaloes during ovsynch program under local condition in Egypt is not critically studied. The aim of the present study was to monitor and compare the ovarian follicular dynamics and serum progesterone profile in Egyptian buffalo heifers and post partum cows *(Bubalus bubalis*) during the ovulation synchronization protocol.

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Received: December 6, 2012
Accepted: March 16, 2012

Materials and Methods

Animals and management

Total of 686 heifers (HE, aging 19-27 average 24 ± 0.8 months and weighing 350-420 kg and 273 parturient and lactating cows (BC, 1- 5 parities, 415-530 kg body weight and 45-65 days post-partum) of cyclic and reproductively healthy Egyptian buffalo were assigned for the present study. 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 rate between treatment and control groups. Experimental and reference animals were housed in an open yard in 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 fed on 40% forage dry matter (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 the year 2009/2010 (57.29 ± 2.3% relative humidity and 13.45 ± 0.8ºC maximum atmospheric temperatures). Body condition scoring (BCS) system from 1 = very thin to 5 = very 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 was examined rectally and ultrasonographically for recording the ovarian and uterine findings for at least one cycle for each animal. The examination started after Day 25 from parturition in BC. In control groups, the routine rectal palpations were done before insemination.

Ovsynch program

The 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 intramuscularly (im). Forty-eight hours later (Day 9), the animals received a second dose of 100 µg GnRH im. All animals were inseminated artificially 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 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 each 12 h thereafter until ovulation or for a maximum of 48 h. All follicles >3 mm and CL were measured, and sketched individually for each cow. Ovulation was identified when a traced large growing antral follicle was no longer observed. Emergence of a follicular wave was defined as the day on which the retrospectively traced dominant follicle (DF) was 4 mm (Ginther *et al*., 1997). Follicle luteinization was considered when a follicle did not ovulate; instead lutein tissue gradually developed and detected as echogenic ring that increased in thickness later andfilled the whole follicular antrum. The CL was examined and an image of the largest cross-sectional area was estimated. Luteal regression following PGF2α treatment was considered when P4 concentration was less than 1 ng ml^{-1}. The following ovarian characteristics were determined and compared between groups: (1) ovulation rates after thefirst 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; (5) luteal regression rate after PGF2α treatment. Pregnancy diagnosis was performed by ultrasonography 30 days after AI. The CR was determined and compared between groups.

Serum hormonal analysis

Blood samples were collected from the jugular vein of 8 heifers and 9 cows (the same animals used to monitors the ovarian changes) of the treated groups into plain tubes at days 0, 2, 4, 7, 9 and 10 of the program. The samples were transported into ice box to the laboratory within 20-30 min, centrifuged at 1700 xg for 20 min and sera were stored at -20ºC until analyzed for P_4. The P_4 concentration was determined using direct ELISA technique. Kits were provided by Diagnostic System Laboratory Co. (DSL, Catalogue No. 3900, USA). The coefficient of variance of intra-and inter-assay were 4.8 and 9.2%, respectively. The sensitivity of the assay was 0.12 ng.

Statistical analysis

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

Results

Follicle turnover

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

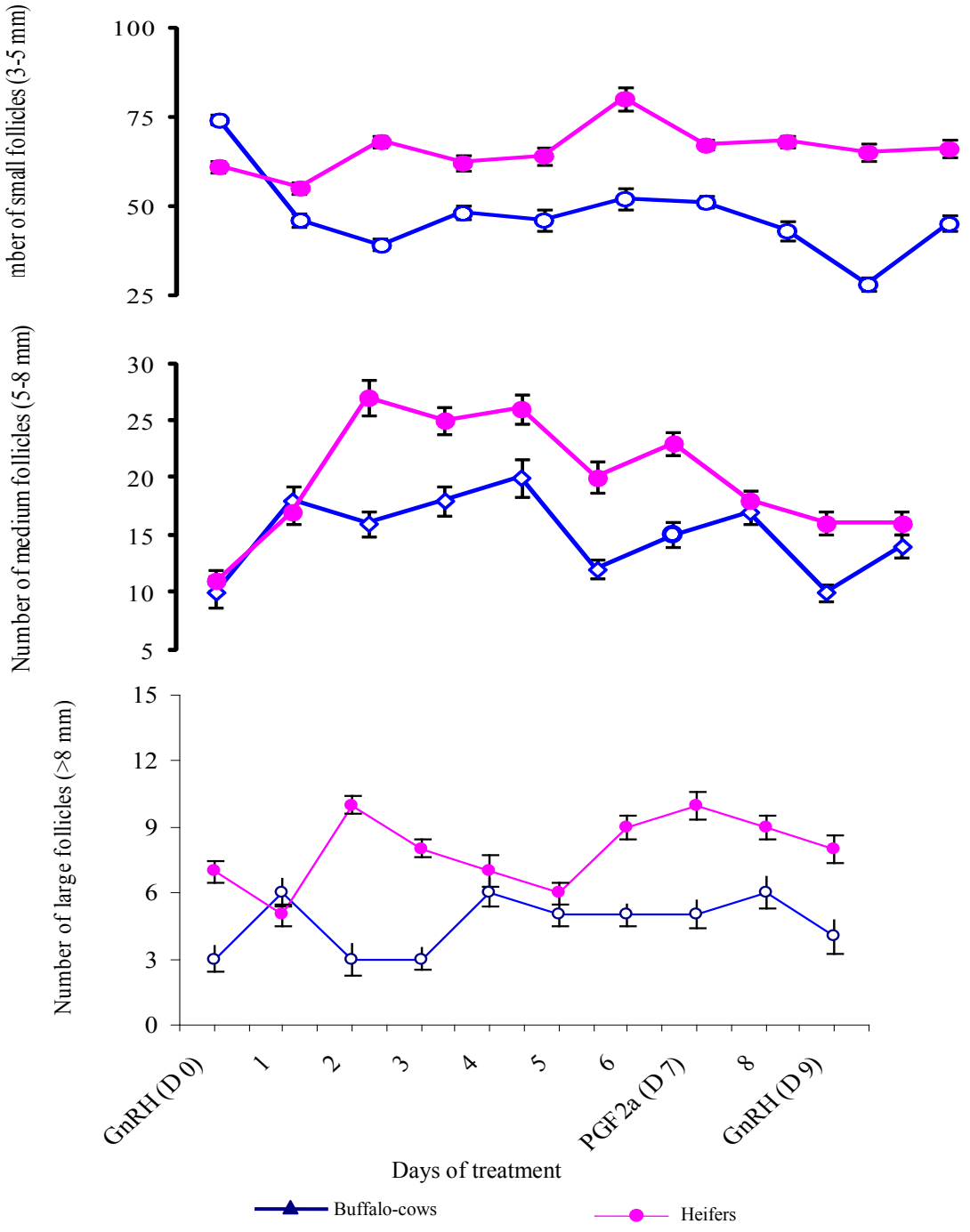

Figure1. Follicular population in Egyptian buffalo heifers (n = 8) and cows (n = 9) on both ovaries (Mean ± SEM, small follicle 2-5 mm, medium follicle 5-8 mm, large follicle >8 mm) during Ovsynch protocol.

Ovarian response to First GnRH (Table 1)

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

Ovarian Findings	Heifers (HE, n = 8)	Buffalo-Cows (BC, n = 9)
Animals had LF (n)	8/8[a]	5/9[b]
LF number (n)	8[a]	5[b]
LF diameter (mm)	9.5 ± 0.14[a]	9.8 ± 0.32[a]
MF number (n)	11[a]	10[a]
SF number (n)	65[a]	73[a]
CL (n)	4/8[a]	4/9[a]
CL size (mm)	15.48 ± 1.95[a]	18.90 ± 1.20[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]
Ov F size (mm)	10.5 ± 1.0[a]	13.4 ± 4.3[a]
F atresia	11[a]	10[a]
F lutenization	1	0
FW (n)	8/8[a]	8/9[a]
Time to FW (h)	51.0 ± 3.4[a]	52.6 ± 2.4[a]
FGR (days 0-7, mm day^{-1})	0.57 ± 0.04[a]	0.86 ± 0.06[b]

Values in means ± SEM. F: follicle; CL: corpus luteum; LF: large follicle; MF: medium follicle; SF: small follicle; FW: follicular wave; FGR: follicle growth rate; OvF: ovulatory follicle. Values with different letters ([a and b]) differ significantly.

After the first GnRH injection, 87.5 and 100% of HE and BC ovulated, respectively (P = 0.88). Follicle luteinization was observed only in 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 out of 9 BC, a new dominant follicle developed within nearly the same period as in HE (52.6 ± 2.4 h).

Ovarian findings on day 7 (Table 2)

Table 2. Ovarian response of Heifer (HE) and buffalo-cows (BC) to PGF2α injected on Day 7 (Day 0 = start of the protocol) of the Ovsynch protocol.

Ovarian Findings	Heifers (HE, n = 8)	Buffalo-Cows (BC, n = 9)
CL (n)	3/8 had 1 CL	4/91 CL
	5/8 had 2 CL	1/9 had 2 CL
CL size	14.85 ± 0.72[a]	16.20 ± 0.95[b]
Animals had LF	8/8[a]	5/9[b]
LF number (n)	10[a]	5[b]
LF diameter (mm)	10.11 ± 0.59[a]	12.90 ± 0.18[b]
FGR (days 0-7, mm day^{-1})	0.57 ± 0.04[a]	0.86 ± 0.06[b]
Response to PGF2α (Day 9)		
Luteolysis (n)	8/8[a]	1/5[b]
Animals had LF (n)	8/8[a]	7/9[a]
LF (n)	8[a]	7[a]
F size (mm)	10.68 ± 0.69[a]	11.66 ± 0.91[a]
FGR (days 7-9, mm day^{-1})	0.59 ± 0.06[a]	0.58 ± 0.07[a]

Values in means ± SEM. F: follicle; CL: corpus luteum; LF: large follicle; FGR: follicle growth rate. Values with different letters ([a and b]) differ significantly.

At day of PGF2a injection (Day 7), Three HE had single CL and 5 had double CL (2 of them were already present at the time of the first GnRH injection and the others resulted from second ovulations). One Buffalo-cow only had double CL and the rest had a single CL. The mean diameter of the CL was significantly larger in BC group (P = 0.003). A follicle larger than 8 mm in diameter was detected in all HE (100 %) and in 5/9 BC (55.5%) groups.

The mean diameter of the LF2 was significantly larger in BC than in HE (12.90 ± 0.18 *vs*. 10.11 ± 0.59 mm, respectively). The mean growth rate of the LF between days 0 and 7 was higher (P = 0.03), and the maximum diameter was larger (P = 0.01) in BC.

Ovarian response to the PGF2α (Table 2)

Luteolytic responses to PGF2α treatment were 87.5 and 20.0% for HE and BC, respectively. In HE, the CL was regressed from an average diameter of 14.85 ± 0.72 mm on Day 7 to 9.14 ± 0.7 mm on Day 9. In the BC, the CL was decreased from 16.20 ± 1.20 mm on Day 7 to 9.06 ± 0.6 mm on Day 9. The diameter of the DF was 10.68 ± 0.69 mm and 11.66 ± 0.91 mm in HE and BC, respectively.

Ovarian response to the second GnRH (Table 3)

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

Ovarian Findings	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]	10.41 ± 7.6 [b]
Ov F size (mm)	10.94 ± 0.91[a]	13.64 ± 0.89[b]
Second CL size (mm, developed at D7)	15.45 ± 0.8[a]	19.7 ± 1.3[b]
CR (n = 686 HE and 273 BC)	429/686[a]	62/273[b]
CR %	62.54%[a]	22.71%[b]

Values in means ± SEM. F: follicle; CL: corpus luteum; OvF: ovulatory follicle; CR: conception rate; Values with different letters ([a and b]) differ significantly.

The size of the dominant follicle was 9.82 ± 1.23 and 11.96 ± 2.15 mm for HE and BC at the time of second GnRH injection, respectively. An ovulation rate of 100 and 88.8% was recorded for HE and BC, respectively. Ovulation time 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 developed at Day 7 of the protocol was 15.45 ± 0.8 and 19.7 ± 1.3 mm in HE and BC, respectively (P = 0.03).

P4 concentrations (Fig. 2)

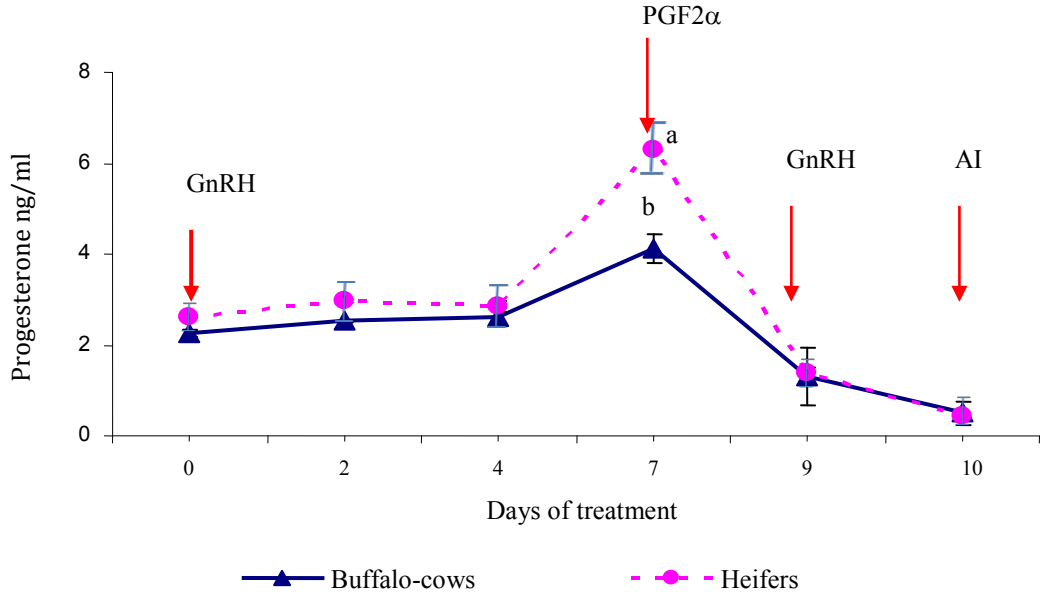

Figure 2. Progesterone levels in serum of heifers (n = 8) and buffalo-cows (n = 5) treated with the Ovsynch program. Values with different letters ([a,b]) differ significantly (P < 0.05).

The average P_4 concentrations were higher in HE than in BC on day 7 (P = 0.04). The concentration of serum P4 correlated positively correlation with the diameter of the CL (r = 0.6, P = 0.005).

Conception rate

In the treated groups, 429 out of 686 HE (62.54 %) and 62 out of 273 BC (22.71 %) had conceived (P = 0.01). In the control groups, 131 out of 219 CHE (59.82 %) and 99 out of 166 CBC (59.64 %) had conceived.

Discussion

This study aimed to describe the differences between heifers and buffalo-cows in their response to different treatments of the Ovsynch program. Thefirst GnRH was designed to enhance the ovulation of the large functional follicle and to induce a new follicular wave. It is well accepted that injection of a GnRH agonist at any stage of the estrous cycle in cattle 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 second injection of PGF2α increases the percentage of animals synchronized by lysis of both the cyclic CL and that resulted from ovulation of DF1 (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 buffaloes (Rao and Venkatramiah, 1991; De Araujo Berber *et al*., 2002), 90% in cyclic and 50% in non-cyclic buffaloes (Neglia *et al*., 2003; Ali and Fahmy, 2007) and 82-90% in cyclic cattle (Pursley *et al*., 1995; Wiltbank, 1998; Frike *et al*., 1998) following thefirst GnRH administration. The present results are coincident with the previous study of Neglia *et al*. (2003) in buffaloes and 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 fewer dominant follicles in BC at the time of first GnRH injection. The time of ovulation after GnRH injection depends mainly on the size of DF1 at the time of injection (Wiltbank, 1998; Hussein *et al*., 2002; Hussein, 2003). However, follicle size is not the only parameter that can affect the ovulation rate. In a recent study, it was demonstrated that follicle size in buffaloes that ovulate compared to those that did not ovulate is quite similar (Campanile *et al*., 2008). Moreover, stage of the follicular development (growth or regression phase) greatly affects the response to GnRH treatment (Dharani *et al*., 2010).

It is recorded that the first GnRH 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 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 DF (DF2) in all and 8/9 of HE and BC groups, respectively. The DF2 developed faster from days 0 to 7 and reached a larger diameter in the BC cows, this may be attributed to the subnormal P4 level. The sub-luteal circulating P4 has been reported to increase the frequency of LH pulses, and 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 some days to grow and become able to produce estradiol-17β leading to induction of preovulatory LH-surge (Bridge and Fortune, 2003). PGF2α was injected on day 7 to regress all CLs. If a CL resulted from the initial injection of GnRH, the 7 days 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 in the day of PGF2α. Most of HE (7/8) had a follicle larger than 8 mm. The high synchrony between animals (presence of functional CL and large active follicle), reported in this study, is the result of thefirst GnRH treatment. A synchrony rate of 90 % in Murrah buffalo (Paul and Prakash, 2005), 74.7% in Mediterranean Italian buffaloes (De Rensis *et al*., 2005) and 84% in cattle (Frike *et al*., 1998) was previously recorded. Regression of corpora lutea was recorded for all HE and BC, but the difference was only in number of animals (8 HE *vs*. 5 BC) in the current study.

In order to increase synchrony of ovulation, a second GnRH was injected to ovulate the preovulatory follicle at a precise time (Wiltbank, 1998). In the present study, the DF2 ovulated in 100 and 88.8% of the HE and BC groups, respectively. An ovulation rate of 90-93% in cyclic buffaloes (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 recorded. In the current study, the BC group started to ovulate earlier (6 h after second GnRH) than the inseminating time (16 h after second GnRH). Furthermore, those animals ovulated over a relatively longer time (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 very low CR in this group (22.71%). Neglia *et al*. (2001) observed a pregnancy rate of 45% in buffalo-cows synchronized with $PGF_2\alpha$ alone and 48.8% when PGF2α was combined with GnRH injection at the time of AI. Similarly, 33.3, 43.7, 36 and 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 Ovsynch protocol and timed insemination, respectively.

In the current study, the level of P4 indicated precisely 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 on skim milk (Qureshi *et al*., 2000) and plasma (Jazayeri *et al*., 2010) of buffaloes. Serum progesterone levels in HE and BC subjected to Ovsynch protocol, compared to control groups were not significantly different till the fifth day of treatment. At the seventh days of the program, serum progesterone values differed significantly in HE and BC compared to control groups ($P < 0.05$). 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 CR recorded in the BC group and suggested that a significant improvement in CR 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 P_4 level at the time of $PGF_2\alpha$ application may be an important factor to improve conception on subsequent insemination (Hussein, 2003, De Rensis *et al*., 2005). On the contrary, Pursley *et al*. (1997) reported that P_4 supplementation at the day of PGF2α injection had no effect on the probability of pregnancy. Attempts to replace the second GnRH injection by hCG failed to improve conception rate in buffalo after fixed time AI in Brasil (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 a 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 throughout the period from initial development to embryonic attachment and the use of pharmacological treatments in order to increase P4 blood levels between 25 and 40 days post AI, period characterized by 45% of embryo mortality in buffalo, play a determinant role in farms with high incidence of embryonic mortality (Campanile *et al*., 2005, 2007b).

In Conclusion, the application of Ovsynch program in buffalo heifers could be better than in cows. Conception rate in buffalo heifers was acceptable and satisfactory, while in buffalo cows was very low. The difference may be attributed to earlier and long-lasting ovulation as well as 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 the conception rate in buffalo-cows. Further studies should focus on improving the conception rate following application of Ovsynch program.

Acknowledgments

The authors are deeply grateful to Dr. M. Abdel-Raouf, Professor Emeritus, for his guidance, critical reading and suggestions during the manuscript preparation.

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