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OVARIAN AND HORMONAL CHANGES DURING OVSYNCH PROGRAM IN BUFFALO-COWS (BUBALUS BUBALIS)

(With 2 Tables and 3 Figures)

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التغيرات المبيضية والهرمونية المصاحبة لتطبيق برنامج توافق التبويض في الجاموس

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أجري هذا البحث بهدف معرفة أهم التغيرات المبيضية والهرمونية المصاحبة لبرنامج توافق التبويض في الجاموس المصري. أجري البحث علي عدد ثمانية جاموسات ، تم حقن كلا منها ثلاث جرعات من الهرمونات. الجرعة الاولي عبارة عن المحفز لهرمون الحاثة المنسلية ، والجرعة الثانية بعد الاولي بسبعة أيام وهي عبارة عن هرمون البروستاجلاندين ، اما الجرعة الثالثة فكانت عبارة عن المحفز لهرمون الحاثة المنسلية للمرة الثانية وذلك بعد الحقنة الثانية بيومين. تم تلقيح جميع الحيوانات اصطناعيا بغض النظر عن ظهور او عدم ظهور علامات الشبق وذلك بعد الحقنة الثالثة بـ ٩ ، ٢٤ ساعة. تم فحص الحيوانات يوميا باستخدام الموجات فوق الصوتية لمتابعة التغيرات التي تحدث في المبيض ، كما تم اخذ عينات لبن في الايام ٠ ، ٤ ، ٧ ، ٨ ، ٩ ، ١٠ لتحديد مستوي هرمون البروجستيرون. اوضحت النتائج انه بعد الحقنة الاولي للمحفز لهرمون الحاثة المنسلية تم التبويض في عدد ٨/٧ من الحيوانات وكانت الفترة من الحقن الي التبويض ٢,١٣ ±٠,٢ يوم. بعد حقن هرمون البروستاجلاندين لوحظ اضمحلال الجسم الاصفر في عدد ٨/٦ من الجاموس. أما بعد الحقنة الثانية من المحفز لهرمون الحاثة المنسلية حدث تبويض في عدد ٨/٦ من الحيوانات ما بين ٢٤ - ٤٨ ساعة من الحقن وبفحص الحيوانات بعد شهر من التلقيح بواسطة الاشعة فوق الصوتية لوحظ حدوث حمل في ٨/٥ من الحيوانات. يستخلص من هذا البحث ان تطبيق هذا البرنامج من الاهمية بمكان حيث يمكن بواسطته التغلب علي مشكلة الشيوع الصامت في الجاموس.

SUMMARY

The objective of the current study was to monitor ovarian and hormonal changes during the ovsynch program in eight Egyptian buffalo-cows. At random stages of estrous cycle, each cow received GnRH on Day 0, $PGF_2\alpha$ on Day 7, and GnRH on Day 9 with AI 9 and 24h later. Ovarian structures were monitored by daily ultrasound examination. Milk samples were collected on Days 0, 4, 7, 8, 9 and 10 for progesterone (P_4) analysis. The 1st GnRH injection resulted in development and subsequent ovulation of a large follicle (DF1) in 7/8 cases. This injection was also successful in synchronizing a new follicular wave. The mean P_4 level increased significantly from Day 0 through Day 7. By the day of $PGF_2\alpha$ injection, 4/8 cows showed double CL, 4/8 had single one and 7/8 revealed large follicle (DF2). After $PGF_2\alpha$ injection, the CL regressed in 6/8 animals. After the 2nd GnRH injection, the DF2 ovulated in 6/8 cases. All ovulation occurred between 24 and 48h after injection. Five of the eight buffalo-cows conceived. In conclusion, this study provided some basic knowledge about the ovsynch program in buffaloes, which might be used to solve the problem of silent heat.

Key words: *Estrus synchronization, GnRH, $PGF_2\alpha$, milk progesterone, buffalo.*

INTRODUCTION

Silent estrus has been recognized as a major cause of infertility in buffalo (Hafez, 1954; Schmidt, *et al.*, 1963; El-Sheikh and Abdel-Hadi, 1971; Kanai and Shimizu, 1983; Shah, *et al.*, 1990; Barkawi, *et al.*, 1993). Other than the acceptance of the male, signs such as swollen vulva, mucus discharge, increased frequency of urination are not reliable indicators of estrus (Hafez, 1954; Schmidt, *et al.* 1963; Danell, 1987). Moreover, the estrus in buffalo commences towards late evening, with peak sexual activity occurring during the hours of darkness (Jainudeen, 1986). This difficulty is usually overcomed by the traditional practice of running a bull with the females (Gill, *et al.*, 1973). Unfortunately, the service rate is likely to increase by using a bull because the bull can be more persistent and adapt at checking for estrus (Alonso, *et al.*, 1992). However, the use of natural service bulls rather than proven AI sires will decrease genetic gain and will bring possible disease problems. Some aids such as pedometers, heat mount detectors and exposure to a vasectomized bull have been used to improve the efficiency of heat detection in buffaloes (Gill, *et al.*, 1973; Williams, *et al.*, 1986; Alonso, *et al.*, 1992; Palo, *et al.*, 2000). The efficiency of such estrus detection aids may, however, be reduced because of the wallowing habits of the animals as well as due to the rarely occurrence of homosexual mounting behavior.

These considerations indicated a need for estrus synchronization with fixed-time insemination for implementing breeding programs in the buffalo. To date, the most common synchronization schemes in buffaloes are limited either to induce regression of the corpus luteum (CL) by injection of $PGF_2\alpha$ or its synthetics analogs (Perera, *et al.*, 1977; Pathiraja, *et al.*, 1979; Rao and Venkateswara, 1979; Kamonpatana, *et al.*, 1987; Singh and Dabas, 1998; Abol-Roos and Gaffar, 2000; Brito, *et al.*, 2002) or by prolonging the life-span of the CL by progesterone or progestagen preparations (Rao, 1981; Subramaniam and Devarajan, 1991; Luthra, *et al.*, 1994; Barile, *et al.*, 2001). The difficulty with these approaches is the variability in time from AI to ovulation. Moreover, the conception rate was low and needed improvement if fixed–time insemination hase to replace insemination at detected estrus.

The effect of GnRH on the ovarian activity in buffaloes had been investigated in some previous studies. GnRH injection resulted in earlier resumption of ovarian activity and enhanced conception rate (Shah, *et al.*, 1990). It also induced ovulatory estrus in anestrus animals (Mohammed, *et al.*, 1999). Rao and Venkatramiah (1991) investigated a combination of GnRH- $PGF_2\alpha$ treatment. This combination resulted in a conception rate of 37% in anestrus buffaloes. The ovysynch program, developed by Pursley *et al.* (1995), is a new technique in bovine estrus control designed to allow AI without estrus detection. In this program, each cow gets three injections for each breeding: the first is GnRH, the second is $PGF_2\alpha$ 7 days later, and the third is another GnRH 2 days later with AI at 0 to 24 hours after the second GnRH. This regime was also investigated in few reports in buffaloes (Zain, *et al.*, 2001; Berber, *et al.*, 2002; Neglia, *et al.*, 2003). However, ovarian and hormonal changes associated with this program have not been intensively studied as in cattle.

The aim of the current study was to apply ovarian ultrasonography and hormonal analysis to study ovarian and hormonal changes associated with the ovsynch program in the buffalo-cows.

MATERIALS and METHODS

Eight Egyptian river buffalo-cows (Bubalus bubalis), between 3.5 and 6 years old, weighing 558-657 kg and 52-217 days after parturition were used. Two cows were primipara (No. 3, 7) and six were multipara (No. 1, 2, 4, 5, 6, 8). The animals were housed in an open yard in the animal farm of Al-Azhar University, Assiut branch. They were milked twice daily, and fed on Egyptian clover (Trifolium alexandrium), concentrated mixture and wheat straw. All the cows were cyclic before starting the program.

The animals were treated at a random stage of the estrous cycle (D 0) with an intramuscular injection of GnRH (20 µg buserelin, Receptal, Intervet International B.V., Boxmeer, Holland). Seven days later (D 7), $PGF_2\alpha$ analogue (0.750 mg tiaprost, Iliren, Intervet International B.V., Boxmeer, Holland) was injected intramuscularly. Forty-eight hours later (D 9) the animals received a second injection of 20 µg GnRH. All cows were artificially inseminated with fresh extended semen 9 and 24 hours after the second injection of GnRH. The semen was collected from a buffalo bull kept at the farm, examined and extended with egg yolk-citrate on the same day of insemination.

Ovarian structures were monitored ultrasonographically using a real-time, B-mode, diagnostic scanner equipped with a transrectal 6/8 MHz linear array transducer (Pie Medical, 100 LC, Holland). Ultrasound examination was performed once daily from the day of the 1st GnRH injection, until two days after the 2nd GnRH injection. All follicles $\geq$ 3 mm and CL were measured, counted, and mapped individually for each cow. Follicular populations were classified into small (< 5 mm in diameter), medium (5-8 mm in diameter) and large (> 8 mm diameter) follicles. The animals were examined for pregnancy by ultrasonography 30 d after AI.

Milk samples were obtained from each cow on the morning on days 0, 4, 7, 8, 9 and 10. The samples were centrifuged at 1200 x g. The supernatant containing milk-fat was discarded, while the fat-free part (skim milk) was collected and kept at −20 °C until analyzed for progesterone hormone (P_4). The P_4 concentration was determined in each sample by using ELISA technique. Kits and assay procedure was provided by Oima (Gesellschaft fuer Diagnostika mbH, Cat. No. EHPGN96q, Rev. 04.2002.03, Germany). Coefficient of variance of intra- and interassay were 6.5 and 8.3%, respectively. The sensitivity of the assay was 0.02 ng P_4. A P_4 level > 1 ng/mL skim milk was taken as indicator for the presence of a functional CL (Qureshi, *et al.*, 2000).

The data were presented in mean ± SEM. They were statistically analyzed by SPSS program, version 10.0 (1999). Changes in P_4 levels, diameters of the corpora lutea, diameters of the dominant follicle (DF) and follicular population through the days of treatment were compared by GLM-repeated measures. Differences between mean diameters of DF developed after first GnRH injection and those developed after second GnRH treatment, as well as between mean P_4 in animals with single and those with double CL were compared by t-test. The degree of association between size of CL and level of P_4 in skim milk were tested by the correlation coefficients.

RESULTS

Ovarian structures on the first day of the ovsynch program (D 0) are presented in Table 1, and the ovarian changes during the program are illustrated in Fig. 1. On the second Day of the 1st GnRH injection (D 1), one of the medium follicle in cows bearing small and medium follicles (No. 1 - 6) increased in size to reach a diameter between 9 and 11 mm. While, the largest follicle in cows having small, medium and large follicles (No. 7, 8) showed a minimum change in size. Consequently, all the treated animals showed a large follicle (DF1) one day after GnRH injection. On the third day of GnRH injection (D 2), this large follicle disappeared (ovulated) in 6 cows, while it ovulated in the fourth day in one cow (No. 6), and became atretic in another one (No. 1). The mean time between GnRH injection and ovulation was 2.13 ± 0.2 days. The average diameter of the ovulated DF1 was 11.63 ± 0.4 mm (range 10 - 13 mm, n = 7). A new follicular wave started in all cows between Days 1 and 3 of the GnRH injection (average 2.22 ± 0.3 days). This wave resulted in a development of a new dominant follicle (DF2) in all but one cow (No. 1). By Day 4, six of the eight cows showed a double CL, one of which was present already at the time of GnRH injection (old CL) and the other one originated as a result of ovulation of DF1 (new CL). The old CL was non significantly larger than the newly formed one (15 ± 7.3 vs 12.2 ± 2.9 mm in diameter). Two cows showed only one CL (No. 1, 7). One of the two CL (old one) regressed spontaneously between Days 6 and 8 in cows No. 3 and 6.

Table 1: Ovarian structures on the first day of the Ovsynch program

Animal No	Ovarian Structures			
	Follicles			Corpus luteum
	Small (< 5 mm)	Medium (5 – 8 mm)	Large (> 8 mm)	(13 – 15 mm)
1	+	+	-	+
2	+	+	-	-
3	+	+	-	+
4	+	+	-	+
5	+	-	-	+
6	+	+	-	+
7	+	-	+	-
8	+	+	+	+

On the Day of prostaglandin injection all the included animals, except one (No. 1), showed a large follicle (DF2) between 8 and 13 mm in diameter (average 9.71 ± 0.6 mm). Parallel, 4 cows still presented double CL (No. 2, 4, 5, and 8) with a diameter for each between 10 and 21 mm (average 16.13 ± 1.7 mm). There was no significance difference between the mean diameter of the old and the new CL (17.25 ± 1.7 vs 15.00 ± 1.9). The other 4 cows (No. 1, 3, 6 and 7) showed only one CL with a diameter between 15 and 18 mm (average 15.75 ± 0.7 mm).

After prostaglandin injection, the CL regressed significantly in size in 6/8 cows. They reduced from an average diameter of 16.0 ± 0.6 mm at Day 7 to 11.33 ± 1.7 mm at Day 8 and 8.13 ± 2.2 mm at Day 9 (P = 0.0001). In two cows (No. 1 and 6) the CL showed little changes in size. On the other hand, the DF2 increased significantly in size in 7/8 animals. They increased from 9.71 ± 0.6 mm at Day 7 to 11.29 ± 0.8 mm at Day 8 and 12.71 ± 0.3 mm at Day 9 (P = 0.006). In one cow (No. 1), the largest follicle (7 mm in diameter) showed little changes in size after prostaglandin injection.

After injection of the 2nd GnRH on Day 9, the DF2 increased non-significantly in size, from 12.71 ± 0.3 mm at Day 9 to 13.86 ± 0.6 mm at Day 10. These DF2 ovulated in 6/8 cows between 24 and 48 hours after the second GnRH injection. In two cows (No. 1 and 7) no ovulation was observed up to 72h after GnRH injection. The DF2 ovulated by an average diameter of 14.30 ± 0.4 mm (range 13-15 mm, n = 6). The average diameter of DF2 was significantly larger than that of the DF1 which ovulate after the first GnRH injection (P = 0.04).

Changes in follicular population during ovsynch program are illustrated in Fig. 2. The 1st GnRH injection caused nonsignificant changes in the number of small sized follicles (P = 0.07). While it caused a significant decrease (P = 0.006), followed by a significant increase (P = 0.04) in the number of the medium sized follicles. The maximum number reached on Day 4. Then, the number gradually decreased to reach a minimum on Day 7. The same pattern was nearly repeated after the 2nd GnRH injection.

Changes in P4 levels during this program are presented in Table 2 and Fig. 3. On the Day of the 1st GnRH injection, 6/8 cows showed P_4 level higher than 1.5 ng/mL skim milk. The other two cows (No. 2 and 7) showed P_4 level lower than 0.5 ng/mL. On Day 4, all the included cows showed P_4 levels higher than 1.0 ng/mL. Difference between the mean value of P_4 on Day 0 and that of Day 4 was significant (P = 0.007). Then, the mean P_4 level increased slightly on Day 7. By this day, animals with double CL showed significantly higher mean P_4 level than those with single CL (P = 0.003). One day after prostaglandin injection (D 8), the mean P_4 levels reduced (P = 0.009). Through Days 9 and 10, the mean P_4 level progressively decreased in 6/8 cows, while, in two animals (No. 1 and 6) the P_4 level remained higher than 1.0 ng/mL. In general, there was a positive correlation (r = 0.7, P = 0.0005) between the diameter of the CL and the level of P_4 in the skim milk.

Table 2: Changes in the progesterone concentrations in skim milk during ovsynch program in buffalo cows

Days	Treatments	Mean Progesterone concentration (ng/mL) ± SEM in cows developed:		
		SCL n=4	DCL n=4	All cows n=8
0	GnRH	1.18 ± 0.4^{ab1}	1.68 ± 0.5^{a1}	1.43 ± 0.3^{ac}
4	-	2.0 ± 0.5^{ab1}	3.0 ± 0.05^{ab1}	2.48 ± 0.3^{b}
7	$PGF_2a\alpha$	2.0 ± 0.05^{a1}	4.0 ± 0.05^{b2}	2.68 ± 0.4^{b}
8	-	1.45 ± 0.3^{ab1}	1.03 ± 0.3^{ac1}	1.24 ± 0.2^{c}
9	GnRH AI	1.1 ± 0.3^{ab1}	0.3 ± 0.1^{c2}	$0.71 \pm 0.2c^{d}$
10	AI	0.78 ± 0.4^{b1}	0.28 ± 0.1^{c1}	0.53 ± 0.2^{d}

SCL: single corpus luteum, DCL: double corpora lutea, AI: artificial insemination
a,b,c,d Values with different superscript letters in the same column differ significantly (p<0.05).
1,2 Values with different superscript number in the same row differ significantly (p<0.05).

Five of the eight cows conceived according to ultrasonographic pregnancy diagnosis one month after insemination. Three animals did not conceive, two of which (No. 1 and 7) did not ovulate after the 2nd

GnRH injection, while the third one (No. 6) showed high P_4 level at the time of insemination. Interestingly, all the animals (4/4) with double CL by the day of $PGF_2\alpha$ injection became pregnant in comparison to only one (1/4) from those with single CL.

DISCUSSION

The Ovsynch program has created considerable interest among dairy producers seeking to improve herd reproductive performance and / or elimination of heat detection (Frike, *et al.*, 1998). In the ovsynch program, each buffalo-cow received three injections for each breeding. The first injection of GnRH was designed to ovulate large functional follicle and to induce a new follicular wave (Wiltbank, 1998). The second reason for the first GnRH injection was to increase the percentage of animals synchronized to a single injection of $PGF_2\alpha$. In the current study, when there was no large follicle on the ovary, the first GnRH resulted firstly in the development of a follicle to reach the ovulatory size. The ovulation of DF1 occurred when the follicle reached a diameter of 11.6 mm. In cattle, only follicle larger than 10 mm (Frike, *et al.*, 1998) or larger than 15 mm (Pursley, *et al.*, 1995) responded to the exogenous GnRH. Ovulation of the DF1 occurred in 7/8 buffalo-cows, which is comparable to 86% ovulation rate recorded in buffaloes (Rao and Venkatramiah, 1991) and 82 – 90% in cattle (Pursley, *et al.*, 1995; Frike, *et al.*, 1998; Wiltbank, 1998). In addition, the injection of GnRH was successful in synchronizing a new follicular wave 1-3 days later. In cattle the new wave started 1-2 after GnRH injection (Pursley, *et al.*, 1995; Frike, *et al.*, 1998; Wiltbank, 1998). This wave resulted in the development of a new DF in 7/8 cases.

$PGF_2\alpha$ was injected on day 7 to regress all CL. 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 response to $PGF_2\alpha$ (Wiltbank, 1998). In the present study, by the day of $PGF_2\alpha$ injection, all the animals showed at least one CL and 7/8 had large DF2. This high synchrony between animals is the beneficial of the first injection of GnRH. A synchrony rate of 84% was recorded by Frike *et al.* (1998) in cattle. After $PGF_2\alpha$ injection, the CL regressed ideally in 6/8 animals, indicating the maturity of the CL by this time. In two cases, the CL regressed slowly. Wiltbank (1998) recorded 95% regression rate of CL in cattle. Parallel, the DF2 increased significantly in size up to

Day 10. However, Hussein (2003) reported in cattle that the DF2 showed little changes in size after $PGF_2\alpha$ injection.

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 work, the DF2 ovulated in 6/8 cows. An ovulation rate of 93% was recorded in buffaloes (Rao and Venkatramiah, 1991) and 89-100% was observed in cattle (Frike, *et al.*, 1998; Wiltbank, 1998). Size of DF2 at the time of ovulation was larger than that of DF1. This difference may be due to that DF2 took sufficient time for development than DF1. Ovulation of DF2 occurred between 24 and 48h after the 2nd GnRH injection. This is comparable to 26h recorded in buffaloes (Rao and Venkatramiah, 1991), a study used ultrasonography every 6 or 12 hours, and 24-32h registered in cattle (Frike, *et al.*, 1998; Wiltbank, 1998).

Hussein (2003) registered the changes in follicular population during ovsynch in cattle. Except the slightly reduction in the number of follicles one day after the GnRH injection, the pattern of follicular changes observed in the present study was similar to that reported in cattle.

Milk P_4 monitoring offers an objective and accurate method for assessing reproductive status in dairy buffaloes (Qureshi, *et al.*, 2000). Milk is preferred over serum or plasma for P_4 assay especially under field condition, because collection of blood from dairy animals is more invasive and disliked by farmers. The level of P_4 in the present work is in agreement with the levels recorded by other works on skim milk in buffaloes (Vale, *et al.*, 1993; Qureshi, *et al.*, 2000). In the current study, the P_4 level in the skim milk indicating precisely the presence or absence of a CL as well as it reflects its size and activity. All animals with higher P_4 (double CL) at the time of $PGF_2\alpha$ application conceived on the subsequent insemination. Hussein (2003) recorded nearly the same phenomenon in cattle. In contrast, Pursley *et al.* (1997) reported that P4 on day of $PGF_2\alpha$ injection had no effect on the probability of pregnancy.

Although the present protocol was not focused mainly on the conception rate (CR) as compared with other management strategies. However, CR of 5/8 is encouraging, since the normal CR in buffaloes with AI ranged from 40-60% using fresh semen after estrus detection (Jainudeen, 1986, Tailor, *et al.*, 1990). In previous studies in buffalo utilizing ovsynch regime a CR between 36 and 57% was recorded (Berber, *et al.*, 2002; Neglia, *et al.*, 2003). However, in those studies part of the animals were in anestrous, and / or received one AI.

In summary, ultrasonic ovarian examination and hormonal analysis indicating that buffalo-cows responded efficiently to the ovsynch program. Consequently, this protocol could have a major impact on managing reproduction in buffalo-cows, because it allows for AI to occur at an expected time of ovulation and eliminates the need for detection of estrus, which is a major problem in buffaloes.

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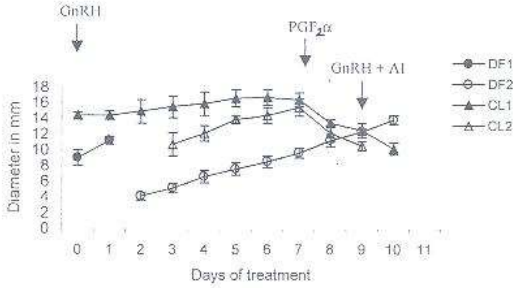

Fig. 1: Ovarian changes during ovsynch program in 8 buffalo cows (data in means ± SEM). The 1st GnRH injection resulted in development and ovulation of a large follicle (DF1) in 7/8 cases, and recruited a new follicular wave in all cows. By the Day of $PGF_2\alpha$ injection, 4/8 cows showed double corpora lutea (CL1, CL2), 4/8 had single one and 7/8 revealed large follicle (DF2). After $PGF_2\alpha$ injection, the CL regressed in 6/8 animals. After the 2nd GnRH injection, the DF2 ovulated in 6/8 cases.

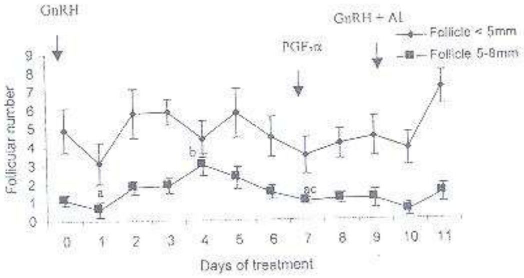

Fig. 2: Changes in Follicular Population during during Ovsynch program in 8 Buffalo-cows. The 1st GnRH injection caused nonsignificant changes in the number of small sized follicles. While it caused a significant decrease, followed by a significant increase in the number of the medium sized follicles. The maximum number reached on Day 4. Then, the number gradually decreased to reach a minimum on Day 7. The same pattern was nearly repeated after the 2nd GnRH injection. Values with different superscript letters differ significantly.

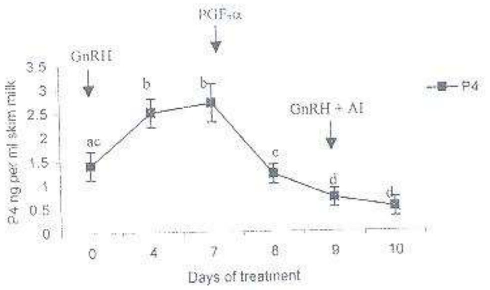

Fig 3: Changes in Progesterone (P4) Concentration during Ovsynch program in 8 Buffalo-cows. The mean P_4 level increased significantly from Day 0 through Day 7. After $PGF_2\alpha$ injection on Day 7, the P_4 level sharply decreased. Values with different superscript letters differ significantly.