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Original Article

Preovulatory follicular dynamics and ovulatory events following the use of GnRH 84 h after medroxyprogesterone acetate sponge removal in postpartum buffaloes

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Abstract. Herein, we evaluated the effects of gonadotropin hormone-releasing hormone (GnRH) administration 84 h after medroxyprogesterone acetate (MAP) sponge removal on follicular growth, ovulation timing, and pregnancy per artificial insemination (AI) in cosynchronized postpartum Nili Ravi buffaloes. In this study, 58 Nili Ravi postpartum buffaloes (DIM = 103 ± 1.64) were randomly divided into two treatment groups (n = 29/treatment): GnRH-TAI-84 and TAI-84. All buffaloes were administered a MAP sponge for seven days. Upon MAP sponge removal, all the subjects received prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and Timed AI (TAI) was performed 84 h after sponge removal. In the GnRH-TAI-84 group, the buffaloes received GnRH alongside insemination, whereas in the TAI-84 group, the buffaloes were inseminated without GnRH administration. Follicle diameter and blood estradiol levels were measured every 6 h from 72–108 h after MAP sponge removal. The animals were checked for pregnancy using ultrasonography 40 days after AI. Animals subjected to the GnRH-TAI-84 protocol had a higher follicular growth rate and preovulatory follicle size than those in the TAI-84 group. The follicular diameter was also larger in animals that received GnRH-TAI-84 than in those that received TAI-84 90 and 96 h after MAP sponge removal. Buffaloes in the GnRH-TAI-84 group had lower estradiol concentrations at 90, 96, 102, and 108 h than those in the TAI-84 group. Ovulation in GnRH-TAI-84 buffaloes occurred 11 h earlier than that in buffaloes from the TAI-84 group. A shorter interval between AI and ovulation in GnRH-TAI-84 buffaloes (14 h *vs*. 25 h) led to greater pregnancies per AI (62% *vs*. 17%) compared to buffaloes from the TAI-84 group.

Key words: Buffalo, Gonadotropin hormone-releasing hormone (GnRH), MAP sponge, Timed artificial insemination

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In the field of reproductive management, the implementation of reproductive protocols, such as Ovsynch, G6G (Incorporation of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) followed by an injection of gonadotropin hormone-releasing hormone (GnRH) two days later, with the Ovsynch protocol being administered six days afterward), and Cosynch, has revolutionized the practices of large-scale cattle farming [1]. Over the course of several decades, estrus synchronization techniques adapted from the cattle industry have also been employed to enhance the reproductive efficiency of buffaloes, achieving variable success rates [2–5]. Because of the weak estrus expression in buffaloes, it is imperative to adopt an approach to enhance fertility, which involves implementing Ovsynch, or using progesterone in conjunction with GnRH and estradiol benzoate, thus eliminating the need for estrus detection, and instead employing timed artificial insemination (TAI) for both dairy cows [6–8] and buffaloes [9–11]. To successfully apply hormonal protocols and TAI in buffaloes [12–14], a comprehensive understanding of ovulation timing is crucial. There was variation in ovulation time following the removal of Controlled Internal Drug Release (CIDR) devices in buffaloes [15, 16]. As such, further research focusing on tightening ovulation synchrony following the removal of progesterone devices is required to optimize TAI in buffaloes. GnRH and estradiol are used in the TAI protocol to synchronize ovulation near the TAI in buffaloes [17–19] and bovine [20–22]. In this context, several studies have previously reported that increasing time from $PGF_{2\alpha}$ to final GnRH administration was associated with a higher pregnancy per artificial insemination (P/AI) rate due to reduced progesterone at TAI, optimized proestrus hormonal environment, and improved endogenous estradiol concentrations, leading to synchronized ovulation in bovines. [23–25].

More recently, Haider *et al.* [26] evaluated the efficacy of the CIDR Cosynch protocol, originally developed for cows, in buffaloes, identifying a 25% P/AI. However, when a modification involving a 12-h delay in administering GnRH after CIDR removal was introduced, the P/AI significantly increased to 65%. The potential reason for this rise in P/AI in buffaloes in the 84-h CIDR-Cosynch protocol (administering GnRH and TAI concomitantly at 84 h after CIDR removal) was unclear, with the researchers being uncertain as to whether this was due to delayed AI or the administration of GnRH

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to larger follicles leading to higher P/AI, as described by Perry *et al*. [27]. It is also important to delineate the effect of the GnRH treatment based on the delay in AI timing, as both were implemented simultaneously.

Hence, the primary objective of this study was to assess the effect of the GnRH-TAI-84 (Administering GnRH and TAI concomitantly 84 h after sponge removal) and TAI-84 (timed artificial insemination performed 84 h after sponge removal) protocols on follicular size, follicle growth rate, ovulation interval, estrus intensity score, ovulation induction, and P/AI using a 7-day medroxyprogesterone acetate (MAP) sponge Cosynch in postpartum Nili Ravi buffaloes. We hypothesized that GnRH administration 84 h after sponge removal in a 7-day MAP sponge Cosynch would lead to better ovulation synchronization and, subsequently, a higher P/AI in postpartum buffaloes, as GnRH is an effective ovulation inducer and remains a reliable option for TAI.

Materials and Methods

Prior to the experiments, approval was granted by the Animal Use and Research Committee of the Buffalo Research Institute, Pattoki, Pakistan (Approval # 340118-3).

Power and sample size calculations

The 2-tailed sample size was calculated using the POWER procedure in SAS version 9.4 (SAS/STAT, SAS Institute Inc.) using data regarding the chance of pregnancy from previous studies [26, 28]. Data from these studies suggest that the P/AI ratio remains between 59% and 65% when the interval from TAI to ovulation is 8–20 h. However, the P/AI was reduced by 10–18% when the interval from TAI to ovulation was too early (4 h) or too late (28 h). In addition, Haider *et al.* [26] suggested that the pregnancy rate would be 65% when the interval from TAI to ovulation was 15 h after the administration of GnRH-TAI-84 h post CIDR removal. In this background, we anticipated a P/AI of ~60% in buffaloes administered GnRH-TAI-84 h post CIDR removal. However, the P/AI was 20% when buffaloes were bred for TAI-84 h after CIDR removal without receiving GnRH. Therefore, we calculated the sample size based on a 40%-point difference in the P/AI between treatments ($\alpha = 0.05$; $\beta = 0.20$). We further conducted the power analyses for the timing of ovulation based on the previous data [26], and calculated the sample size to anticipate the mean difference of 8 h with a standard deviation of 7.5 between treatments ($\alpha = 0.05$; $\beta = 0.20$). The sample size for P/AI was 23 experimental units per treatment, whereas it was 14 experimental units for the timing of ovulation. We opted for P/AI sample size calculations and enrolled additional buffaloes to accommodate potential attrition during the experiment.

Animals and management

This study was carried out at the Buffalo Research Institute, Pattoki, Punjab, Pakistan (31°1'0"N, 73°51"E), during the breeding season (September to November 2022), with average temperature ranges from 20–35ºC and a humidity level of 45–65% [29]. A total of fifty-eight adult Nili Ravi buffaloes, aged six–eight years, with a body condition score (BCS) rated on a 1–5 scale [30], weighing 540–570 kg, and with days in milk (DIM) ranging from to 90–110, were selected for this investigation, as shown in Table 1. All animals were housed in a free-stall system with unrestricted access to water and were fed a regular diet containing 30–40 Kg of green fodder and 1–2 kg of a concentrate mixture with 15% crude protein and 65% total digestible nutrients per head per day. Prior to initiating the synchronization technique, all animals were scanned to determine whether they possessed a normal reproductive tract with a small uterus and no uterine contents after parturition. Ultrasonography was performed on day –7 before the start of the protocol, and on day 0 at the initiation of the protocol to confirm the acyclic status, absence of the corpus luteum, and the absence of signs of postpartum estrus.

Experimental design

In a randomized controlled trial, randomly selected buffaloes were assigned to two treatment groups: administration of GnRH 84 h after MAP sponge removal (GnRH-TAI-84, n = 29) or untreated (TAI-84), and timed insemination 84 h after MAP sponge removal (Fig. 1). Briefly, for seven days, all animals were administered an intravaginal MAP sponge (NIAB Heat Sponge^{TM}, containing 250

Table 1. Body performance and production parameters at protocol initiation, ovulation intervals, ovulation rate, estrus intensity score, and pregnancy per artificial insemination (P/AI)

Characteristics	GnRH-TAI-84 (n = 29)	TAI-84 (n = 29)	SEM	P-value
Body performance and production parameters				
Body weight (Kg)	544.2	543.9	1.5	0.88
BCS (1–5)	3.56	3.49	0.03	0.10
Age (years)	6.53	6.68	0.17	0.83
DIM (days)	103.5	103.7	1.64	0.94
Parity (n)	2.51	2.62	0.17	0.68
Reproductive parameters				
Interval from $PGF_{2\alpha}$ [2]/MAP [1] sponge removal to ovulation (h)	98.06	109	0.63	0.01
Interval from TAI to ovulation (h)	14.06	25	0.63	0.01
Progesterone at TAI (ng/ml)	0.31	0.33	0.01	0.46
Ovulation [3]; (%) n/n	29/29 (100)	25/29 (86)	6.0	0.97
Estrus intensity score [4]	3.82	3.68	0.14	0.51
P/AI [5]; (%) n/n	18/29 (62)	5/29 (17)	9.0	0.002

Values shows as the least square means and associated standard error of means. [1] MAP medroxyprogesterone acetate (250 mg); [2] $PGF_{2\alpha}$, Prostaglandin $F_{2\alpha}$. [3] Total ovulation represents the number of animals ovulated divided by the total number of buffalo treated × 100. [4] Estrus intensity Score: 1 = poor sign, 2 = satisfactory sign, 3 = good sign, 4 = Very Good sign, 5 = excellent sign. [5] Pregnancy per AI; P/AI = [(number of pregnant buffaloes divided by the number of treated buffaloes) × 100].

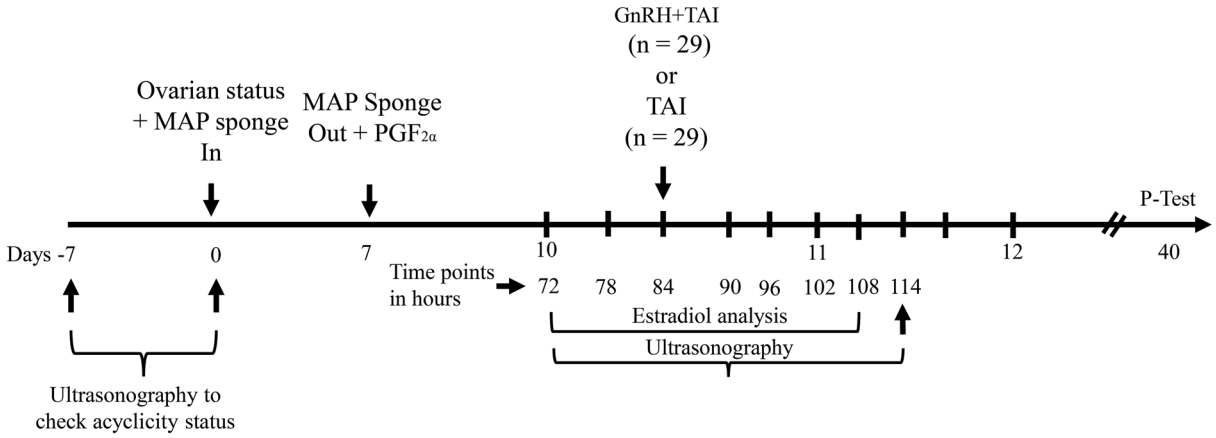

Fig. 1. Schematic illustration of the experimental design. MAP sponge, medroxyprogesterone acetate (250 mg) sponge; P-test, pregnancy test; TAI, timed-artificial insemination; $PGF_2\alpha$, prostaglandin $F_2\alpha$; GnRH, gonadotropin-releasing hormone.

mg of medroxyprogesterone acetate/sponge, Faisalabad, Pakistan). Intramuscular administration of $PGF_{2\alpha}$ (Trometamol 5 mg; DPROST, Selmore®, Lahore, Pakistan) was performed upon removal of the MAP sponge simultaneously. The buffaloes in GnRH-TAI-84 group (n = 29) were randomly assigned to receive an intramuscular GnRH injection (GnRH; 10 µg of buserelin acetate, Bosol, Selmore®, Lahore, Pakistan) concomitantly with AI at 84 h following sponge removal/$PGF_{2\alpha}$ administration. Conversely, buffaloes in the TAI-84 (n = 29) group were inseminated following the TAI schedule at 84 h after sponge removal/$PGF_{2\alpha}$ administration without receiving any GnRH injection.

Ultrasonography, ovulation, and pregnancy diagnosis

At the beginning of the synchronization protocol, the ovaries and uteruses of each buffalo were scanned using a B-mode ultrasound console (HS-1500V; Honda Electronics Ltd., Tokyo, Japan) with a 7.5 MHz linear probe to ascertain the presence or absence of a fetus, corpus luteum, follicle, or any structural abnormality. During the synchronization protocol, ultrasonic tracking was conducted at regular intervals of six hours, commencing at 84 and continuing until 114 h following sponge removal/$PGF_{2\alpha}$ injection, including all the animals in experimental groups. Ovulation was defined as the disappearance of a preovulatory follicle observed during the last ultrasound scan [15]. A subset from each buffalo group (GnRH-TAI-84; n = 12 and TAI-84; n = 9) underwent a comprehensive study of follicle diameter, preovulatory follicle size, and follicle growth subsequent to ovulation using ultrasound at regular intervals of six hours, starting from 72 to 114 h, leading to ovulation [31]. Subsequently, ultrasonography was performed 7 days following AI to confirm ovulation by observing the corpus luteum (CL) in buffaloes. Ultrasonography was performed to confirm pregnancy on days 35–40 after AI, revealing the amniotic membrane, embryonic fluid, and heartbeat.

Measurement of estrus intensity and AI

Using the aforementioned 1–5 scale, all animals were assessed for estrus intensity. The estrus intensity score was determined based on factors such as the tone in the uterine horns, mucous secretion, edematous vulva, bellowing, restlessness, and other signs [18]. A skilled technician performed TAI following standard procedures.

Blood sampling

To determine estradiol concentration (pg/ml), six buffaloes from each group (GnRH-TAI-84 *vs.* TAI-84) were subjected to blood sample collection through the jugular venipuncture using disposable syringes at various time points (72 to 108 h) following sponge removal/$PGF_{2\alpha}$ administration. Conversely, for the progesterone (ng/ml) assay, blood samples were collected through jugular venipuncture in the two groups at TAI (days 10.5) after MAP sponge removal/$PGF_{2\alpha}$ (n = 29/treatment). The samples were maintained at room temperature for coagulation and serum harvesting. The serum was carefully collected, transferred into Eppendorf tubes after separation, and centrifuged at 2000 rpm for ten min at room temperature to remove any impurities or blood cells. The transparent supernatant serum was transferred into separate tubes and stored at –20ºC till further analysis. Radioimmunoassay (RIA) was employed to assess estradiol and progesterone levels in the serum samples. Commercially available solid-phase RIA kits (Beckman Coulter, IMMUNOTECH, Prague, Czech Republic; estradiol; Ref A21854, progesterone; Ref IM1188) were used for this assay [32]. The intra- and inter-assay coefficients of variation were 10%, 16.4%, 9.48%, and 16.8% for estradiol and progesterone levels, respectively.

Statistical analysis

Data normality was determined using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Significance was set at $P < 0.05$. The MIXED and GLIMMIX procedures in SAS (SAS ver. 9.4 Institute, Inc., Cary, NC, USA) was used to examine factors including body weight, BCS, age, days in milk, parity, follicular size, follicular growth rate, estradiol concentrations, progesterone concentrations, ovulatory intervals, estrus intensity score, ovulation rate, and P/AI. The model included the fixed effects of treatment, time, and their interactions. Repeated measures were used to analyze estradiol levels and follicular diameter over equal time intervals across all treatments. The choice of the autoregressive (1) covariance structure was based on the AICC values, while the experimental unit was the buffalo. Binary responses, such as ovulation and P/AI, were subjected to logistic regression analysis using the GLIMMIX procedure in SAS.

Results

At the beginning of the protocol, the physical and production performance parameters of the treatment groups showed no significant differences (Table 1). Follicle diameter measured after sponge removal at six-hour intervals from 72 to 114 h until ovulation showed an

interaction (P < 0.001) between time and treatment (Fig. 2). At 72, 78, and 84 h after sponge removal, the follicle diameter between the treatments remained the same, but significantly differed at 90 and 96 h after sponge removal. Specifically, at the 90 and 96-h time points, the follicle diameter was 1.3 and 1.5 mm larger in animals that received GnRH-TAI-84 compared with the TAI-84 group (Fig. 2). Moreover, the preovulatory follicle size was significantly larger (P < 0.002) in the GnRH-TAI-84 group than that in the TAI-84 group (Fig. 3A). The follicular growth rate, between 84 h after $PGF_{2\alpha}$/MAP sponge removal to the preovulatory follicle, was also significantly higher (P < 0.001) in GnRH-TAI-84 compared with the TAI-84 group (Fig. 3B).

Furthermore, maximum ovulation occurred in buffaloes treated with GnRH-TAI-84 (69%) at 96 h, and in the TAI-84 group (66%) at 108 h after sponge removal (Fig. 4). Four animals in the TAI-84 group did not ovulate until the last scan, performed at 114 h after sponge removal. These animals remained anovulatory and no corpus luteum was observed on day 7 after TAI or pregnancy on the scheduled day of diagnosis.

We observed a significant interaction (P < 0.001) between time and treatment for estradiol concentration in subjected animals (Fig. 5). Estradiol concentrations did not differ across treatments at 72, 78, or 84 h after sponge removal. Later, estradiol concentrations were 1.7, 2.7, 1.2, and 0.8 pg/ml lower in GnRH-TAI-84 than in the TAI-84 group at time points 90, 96, 102, and 108 h after sponge removal, respectively. Progesterone concentrations remained the same at TAI between treatments.

Table 1 shows the ovulatory intervals, ovulation rates, estrus intensity scores, and P/AI results in both groups. Similarly, the interval from $PGF_{2\alpha}$/MAP sponge removal to ovulation (in hours) was reduced, a 12-h duration, (P < 0.001) in the GnRH-TAI-84 group compared with TAI-84 treated animals. The ovulation rate and estrus intensity score did not differ significantly between treatments. The P/AI was significantly higher (P < 0.001) in the GnRH-TAI-84 group than that in the TAI-84 group.

Discussion

This study is one of the first reports on the success of GnRH treatment using the TAI protocol for buffalo conception. The rationale for the present experiment was based on a preliminary study conducted by our group on cyclic buffalo heifers, which revealed an increased follicular growth rate 84 h following CIDR removal and a shortened interval from TAI to ovulation [26]. Furthermore, the study by Haider *et al.* previously postulated whether this effect was due to the shorter ovulation time or better corpus luteum development due to larger preovulatory follicle size. The current study therefore aimed to delineate the effect of GnRH treatment in inducing ovulation 84

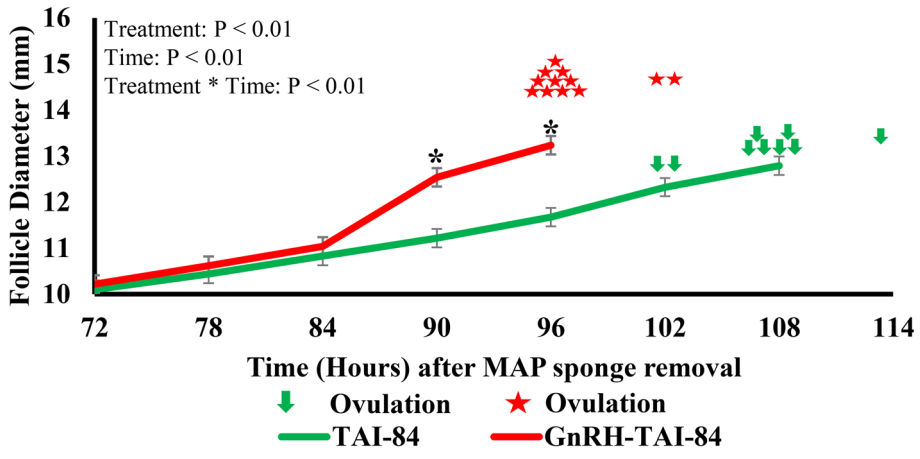

Fig. 2. Follicle diameters and ovulation times in the GnRH-TAI-84 (n = 12) and TAI-84 (n = 9) groups. * indicates a significant difference between treatments.

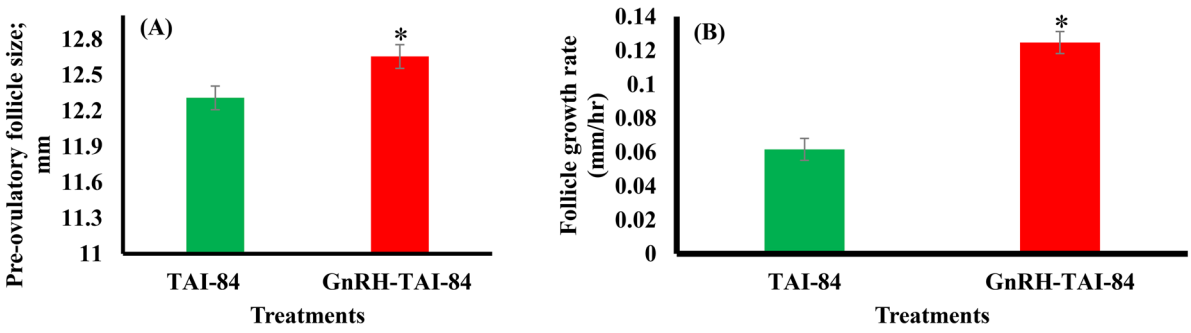

Fig. 3. (A) preovulatory follicle size and (B) follicle growth rate in the GnRH-TAI-84 (n = 12) and TAI-84 (n = 9) groups. The follicular growth rate between 84 h after $PGF_{2\alpha}$ /MAP sponge removal to preovulatory follicle = (Follicle size at the time of appearance of preovulatory follicle – Follicle size at 84 h after $PGF_{2\alpha}$/MAP sponge removal) / (Hours after the $PGF_{2\alpha}$ /MAP sponge removal to the appearance of preovulatory follicle – (minus) 84). * indicates a significant difference between treatments.

h after sponge removal. These findings could have an impact on the reduction of postpartum calving intervals in adult buffaloes.

In the current study, GnRH treatment improved P/AI by reducing the interval from TAI to ovulation, increasing follicular growth rate, and inducing ovulation, as indicated by a decrease in estradiol levels. Most follicles acquire LH receptors on the granulosa cells when the follicle diameter reaches approximately 8.5 mm in buffaloes [33] and 10 mm in cows [34]. Intriguingly, the follicle was medium in size and mature at the time of GnRH administration, which initiated a preovulatory LH surge and increased fertility. Moreover, follicular diameters remained similar across groups at different time points until 84 h. However, we observed an increase in follicular diameter at time point 90 in animals subjected to the GnRH-TAI-84 protocol compared to the TAI-84 protocol, indicating a sharp increase in the follicular growth rate following GnRH injection, which subsequently induced maximum ovulation at 96 h. The data on ovulation in buffaloes at 96 h in the GnRH-TAI-84 group suggests a role of GnRH in initiating a preovulatory gonadotropin surge before the dominant follicle attains physiological maturity; hence, GnRH-induced ovulation of immature follicles exerts a negative impact on P/AI [27, 35]. Thus, the success of ovulation depends on the optimal size and maturity of the dominant follicle at the time of GnRH injection [36–38]. The size of the preovulatory follicles in this species was consistent with previous reports [39, 40], which described that ovulation of appropriately sized follicles led to a larger CL and increased progesterone production for successful fertility.

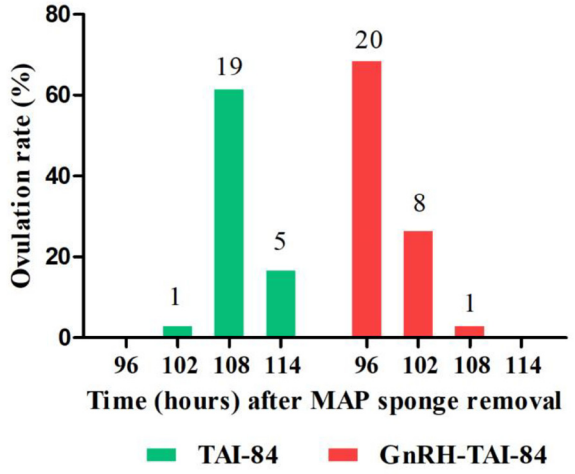

Fig. 4. Ovulations (%) in the GnRH-TAI-84 (n = 29) and TAI-84 (n = 25) groups. The numbers above the bars represent the number of ovulations in each treatment interval. Four animals in the TAI-84 group remained anovulatory.

In our study, the plasma estradiol concentration remained consistent across different time points until 84 h after sponge removal, and administering GnRH at 84 h at the peak estradiol concentration may have resulted in a higher release of LH to initiate the preovulatory LH surge during the periovulatory period. Subsequently, we observed a sharp decrease in estradiol concentration by 108 h in the GnRH-TAI-84 group compared to the TAI group. It is imperative to mention that an increase in serum estradiol concentration at TAI is positively correlated with improved P/AI owing to the increased length of proestrus, which leads to an improvement in the follicular and uterine steroid environments, as seen in bovines [41–43]. Consistent with our findings, previous reports have similarly reported that the administration of GnRH agonists before or during the LH surge can increase P/AI by enhancing the spontaneous preovulatory LH surge [37, 44]. Furthermore, it has been observed that administering $PGF_{2\alpha}$ before GnRH can enhance the pituitary release of LH, leading to an additive effect that may further enhance the preovulatory LH surge following GnRH administration [45, 46]. Similar to the findings of Mirmahmoudi and Prakash [47], ovulation was detected in both treatments when estradiol was at basal level (1 pg/ml).

Notably, the primary concern in the Cosynch protocol utilizing progesterone (CIDR, progesterone-releasing intravaginal device (PRID), MAP sponge) is the interval to ovulation [48]. Indeed, prior research has demonstrated that application of the GnRH-TAI-84 procedure following CIDR removal shortened the ovulation interval in buffalo heifers [26]. Our study observed a similar trend of shortened interval to ovulation after sponge removal in the GnRH-TAI-84 group compared to those not receiving GnRH. Based on this information, it can be inferred that the administration of GnRH 84 h following sponge removal better synchronizes ovulation at 12 h, and TAI performed– 8–20 h before ovulation results in a higher P/AI in

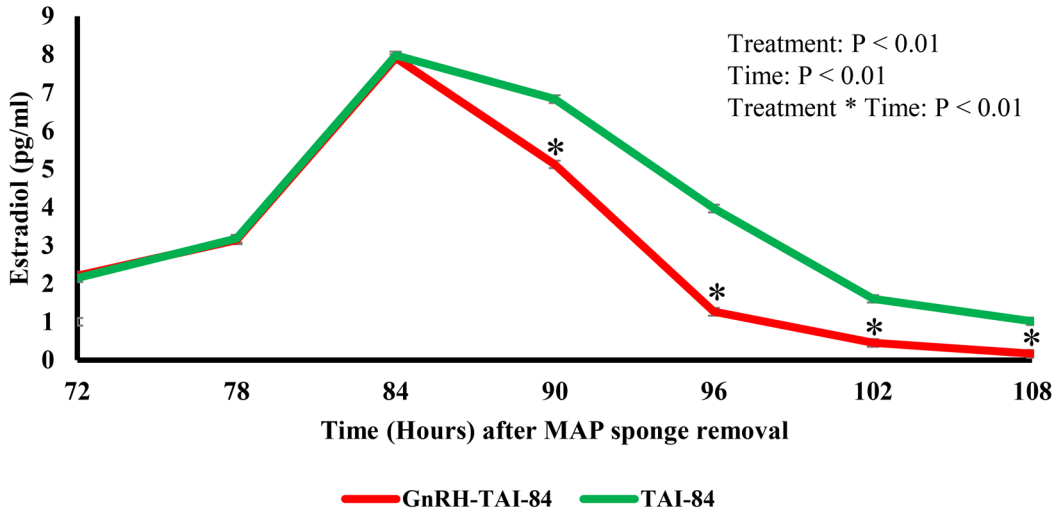

Fig. 5. Serum concentrations of estradiol in the GnRH-TAI-84 (n = 6) and TAI-84 (n = 6) groups. * indicates a significant difference between treatments.

buffaloes, as the average time frame for sperm to reach the uterus after introduction is typically 10–14 h before ovulation [26, 49]. The estimated time of ova viability is 6–12 h after ovulation [50]. The presence of capacitated sperm at the site of fertilization within 6 to 8 h after mating in cows [51] suggests that performing AI too early or late in relation to ovulation reduces fertilization and P/AI because of the presence of premature [51] or aging oocytes at the time of fertilization [52]. Hence, ovulation time and oocyte age at fertilization are critical events for conception.

In postpartum buffaloes, exposure to subluteal progesterone stimulates the growth and maturation of the dominant follicle by increasing LH release and inducing LH receptors, resulting in increased estradiol concentration and ovulation rate [53, 54]. Moreover, progesterone pre-exposure can predict the duration and amplitude of the LH surge by GnRH injection after the progesterone device, which may, in turn, augment CL growth, endogenous progesterone production, and pregnancy maintenance [55, 56]. Initially, the MAP sponge helped to fine-tune the hypothalamus to improve gonadotropin control, folliculogenesis, and estrus expression in buffaloes [57, 58]. It further has the potential to induce ovulation of the follicle earlier due to the increased frequency of LH pulses following the removal of the MAP sponge or $PGF_2\alpha$ administration.

The utilization of progesterone in conventional farming systems has been shown to enhance estrus intensity by increasing the sensitivity of the hypothalamus to estrogen, resulting in more pronounced estrus in buffaloes [18, 49]. The current investigation revealed that estrus intensity was uniformly high across all treatment groups, indicating the application of MAP sponges in pronounced estrus expression, as was previously observed in beef cattle [59]. In the present study, MAP sponge insertion for seven days, with GnRH administration at the time of AI (84 h after MAP sponge removal) may have enhanced the P/AI in buffaloes.

In the present study, the P/AI was significantly higher in animals in the GnRH-TAI-84 group than in those in the TAI-84 group. Indeed, similar observations of P/AI have been made in cows when the CIDR Cosynch protocol was applied [45, 60]. The GnRH-induced LH surge, which recruits more granulosa cells to become luteal cells for progesterone production, or an increase in progesterone production by existing cells [61], may also have enhanced pregnancy/AI in the current experiment. In light of our research, we confirmed that ovulation synchronized 12 h after the administration the GnRH-TAI-84 protocol. Overall, our findings provide crucial information regarding the timing of ovulation and insemination, the lifespan of sperm, and the likelihood of P/AI in this species. As such, these findings have significant implications for improving buffalo TAI protocols.

In conclusion, the administration of GnRH 84 h following sponge removal improves follicular growth rate, shortens the interval from AI to ovulation, reduces preovulatory follicle size, and sharply decreases estradiol levels, leading to ovulation, which may enhance P/AI in buffaloes. This protocol could have a major impact on the management of reproduction in postpartum buffaloes as it allows insemination at a known time of ovulation by eliminating estrus detection. In addition, it is a cost-effective and less labor-intensive approach. Future studies should be designed to test this protocol in larger buffalo herds to synchronize ovulation, which could enhance P/AI.

Conflict of interests: None of the authors have any conflicts of interest to disclose in regards to this manuscript.

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