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Effect of anti-stressor nutritional supplement in combination with progesterone implant on estrus induction and conception rate in summer anestrus Murrah buffaloes

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The present study was carried out to determine the effect of an anti-stressor nutritional supplement in combination with a progesterone implant on estrus induction and conception rates in summer anestrus Murrah buffaloes. In this study, 33 anovular lactating buffaloes (> 90 days postpartum) were monitored for one month and adjudged anovular through transrectal ultrasonography at 10-day intervals. Subsequently, buffaloes were divided into treatment (n=16) and control groups (n=17), and the treatment group buffaloes were given an anti-stressor feed supplement (a preparation consisting of sodium bicarbonate and additional minerals and vitamins ~ 100g daily) for 40 days, while the control group animals received no feed supplement and were fed the normal diet as per the farm schedule. A controlled internal drug-releasing device (CIDR, 1.38g progesterone, Pfizer Pharmaceuticals Ltd.) was administered intravaginally 15 days prior to the start of the feeding trial and was left in place. After 7 days, implants were removed, and 400 IU PMSG (Folligon, MSD Animal Health, i.m) and 500 ug Cloprostenol (Injection Vetmate, 2 ml, i.m) were administered at the time of implant removal. Fixed-time insemination using frozen-thawed semen was carried out at 48 and 60 h after implant removal. The feeding trial was continued until 15 days post-artificial insemination (AI), and CIDR was inserted intravaginally simultaneously in the control group and kept in place for 7 days, similar to the treatment group. Within 15 days of feed supplementation, 37.5% (6/16) buffaloes in the treatment group resumed cyclicity as compared to 11.8% (2/17) in the control group. In response to hormonal treatment, the overall estrus induction rate was 100% for both the treatment and control groups. No difference in estrus intensity was observed between the groups. Following fixed-time AI, the conception rate was lower in the treatment group (37.5%) as compared to the control group (64.7%). However, approximately 70% of buffaloes became pregnant within two AIs in both groups. Furthermore, no significant difference (P = 0.60) in overall conception rates was found between the treatment (93.7%) and control groups (87.4%) within 90 days of CIDR removal. However, a significant difference

(P = 0.03) in conception rates was noticed between 30–90 days of CIDR removal between the two groups (56.2% *vs* 17.7%). In conclusion, the inclusion of anti-stress supplements along with CIDR supplementation may enhance estrus induction in postpartum anestrus buffaloes during the summer season.

KEYWORDS

buffalo, conception rate, estrus, progesterone, season

1 Introduction

In buffaloes, heat stress is the major cause of infertility and reproductive inefficiency, resulting in profound economic losses. It affects fertility and reproductive performance by compromising the functions of the reproductive tract, disrupting hormonal balance, decreasing oocyte quality, and thereby reducing embryo development and survival (Jasinski et al., 2023; Gendelman and Roth, 2012). Lower circulating concentrations of gonadotrophins and progesterone, accompanied with prolonged postpartum estrus and longer inter-calving intervals, have been detected during summer along with higher prolactin levels in buffaloes (Barile, 2005). Mineral deficiency greatly influences ovarian activity in ruminants, as trace minerals such as sodium, potassium, copper, cobalt, zinc, and selenium are involved in the synthesis of hormones essential for reproduction (Van Emon et al., 2020). Owing to their role in follicular dynamics, ovarian activity, and fertility, minerals are essential components of the animal's diet; thus, deficiencies of single or combined minerals or their imbalances result in reproductive failure (Dantas et al., 2019). Anestrus animals were found to be deficient in copper and zinc, which have a significant correlation with reproductive hormones, mainly progesterone and estradiol (Krishnamoorthy et al., 2017). Reports on anestrus Murrah buffaloes, Sahiwal and crossbred cows, and heifers showed that deficiency of copper could be responsible for the anestrus condition in these animals (Mudgal et al., 2012). Moreover, during summer, copper deficiency in cows and buffaloes has been associated with retained placenta, embryonic death, decreased conception rates, and anestrus (Mudgal et al., 2014). Kumar et al. (2011) used a combination of ascorbic acid, sodium bicarbonate, and potassium carbonate and found it very effective in reducing heat stress in buffaloes.

Thus, to overcome the deficiency, strategic dietary supplementation of copper with better bioavailability could be a suitable approach. Sejian et al. (2014) reported that the use of antioxidants such as vitamin E, vitamin A, and selenium helps reduce the impact of heat stress by improving oxidant balance. Resum et al. (2017) and Wani et al. (2018) found positive outcomes following supplementation of vitamin E and selenium in postpartum anestrus buffaloes along with a controlled internal drug-releasing device (CIDR). Thus, supplementation of anti-stress nutrients to buffaloes during summer months may help improve the effectiveness of the progesterone device for estrus induction and pregnancy rates. Considering this, the present study was conducted to test the hypothesis of whether an anti-stressor nutritional supplement in combination with a progesterone implant can enhance estrus induction and conception rates in summer anestrus Murrah buffaloes.

2 Materials and methods

2.1 Study location and animals

The present study was carried out in the organized herd of the ICAR-Central Institute for Research on Buffaloes (ICAR-CIRB). All the buffaloes were maintained under a semi-intensive management system with free access to clean drinking water. The study was conducted during the summer months (May to August), and the environmental temperature varied between 20 and 46 °C during the study period. Prior to the start of treatment, as well as after the end of treatment, all lactating buffaloes were maintained on a uniform pattern of feeding standard (ICAR, 2014) as followed at the farm. Thirty-three anovular lactating pluriparous buffaloes (> 90 days postpartum; average body weight: 400 ± 25 kg; BCS >3) were screened using ultrasound from a herd of 140 lactating buffaloes. All lactating buffaloes (n=33) selected for this experiment were kept under observation for a period of one month and scanned by ultrasound at 10-day intervals to confirm anovular status. Subsequently, buffaloes were randomly divided into two groups: treatment (n=16) and control (n=17). The treatment group buffaloes were given an additional anti-stressor feed supplement under test for a period of 40 days, while the control group animals were not given this feed supplement. All procedures were approved by the Institute Animal Ethical Committee (IAEC).

2.2 Group 1 (Treatment group)

Animals in this group (n=16) were fed an anti-stress feed supplement (a preparation consisting of sodium bicarbonate and additional minerals and vitamins ~ 100g daily) for a period of 40 days. The composition of the anti-stress feed supplement is shown in Supplementary file S1. A controlled internal drug-releasing device (CIDR, 1.38g progesterone, Pfizer Pharmaceuticals Ltd.) was inserted intravaginally 15 days after the start of the feeding trial. After 7 days, implants were removed and the animals were injected with 400 IU PMSG (Folligon, MSD Animal Health) and 500 ug cloprostenol (Injection Vetmate, 2 ml) at the time of implant removal. Fixed-time insemination using frozen-thawed semen from buffalo bulls was performed at 48 and 60 h after implant removal. All the semen doses were obtained from the semen freezing lab of ICAR-CIRB and had post-thaw motility of more than 50%. The feeding trial was continued until 15 days post-A.I (Figure 1).

2.3 Group 2 (Control group)

In this group, buffaloes were fed the normal diet as per the farm schedule without extra supplementation. The controlled internal drug-

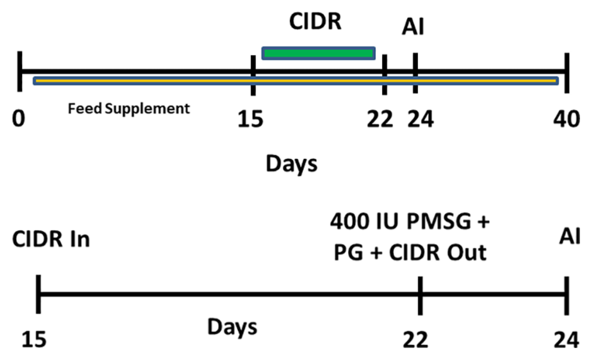

FIGURE 1
Schematic representation of experiment schedule in treatment group. Progesterone implant (CIDR).

releasing device (CIDR) was inserted intravaginally along with the treatment group and kept in place for 7 days, similar to the treatment group. Fixed-time insemination using frozen-thawed semen was performed at 48 and 60 h after implant removal (Figure 2). All the semen doses were obtained from the semen freezing lab of ICAR-CIRB and had post-thaw motility of more than 50%.

2.4 Ultrasonography

Transrectal ultrasonography examinations were performed by a single operator using a Hitachi Aloka Color Doppler Prosound F31 ultrasound scanner equipped with an intraoperative electronic convex sector probe (IPX7). Transrectal ultrasonography examination was performed on more than 90-day postpartum buffaloes that were not inseminated or were found non-pregnant for identification of acyclic buffaloes for this experiment. A cyclicity was confirmed by the absence of corpus luteum (CL) in either of the ovaries. Ultrasound examination was repeated on day 15 of the feeding trial and, simultaneously, CIDR was inserted intravaginally and kept in place for 7 days in both groups. Ultrasound was performed on the day of CIDR removal (day 7) and before first AI (day 9) to check ovarian activity. Finally, pregnancy diagnosis was performed by ultrasound on day 30 post-AI. The animals that were found non-pregnant were inseminated again and, subsequently, pregnancy was checked further with ultrasound for overall conception rate.

2.5 Estrus induction rate and induction of cyclicity

Estrus signs expressed by individual buffaloes were observed during the experiment period in both treatment and control groups.

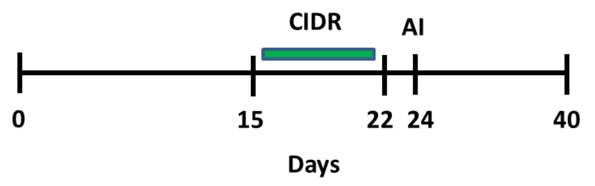

FIGURE 2
Schematic representation of experiment schedule in the control group.

The most common behavioral estrus signs observed included bellowing, micturition, vaginal mucus discharge, and excitement. At the time of AI, uterine tone and vaginal discharge were also considered to evaluate estrus intensity. Based on these collective estrus signs, uterine tone, and vaginal discharge, the estrus induction rate was evaluated and expressed as a percentage. Estrus induction rate was estimated as the percentage of animals that exhibited signs of heat in the treatment and control buffaloes. Induction of cyclicity was adjudged by the presence of CL on either ovary with the help of ultrasound on day 15 from the start of the feeding trial in both groups. Animals having CL in either of the ovaries were considered cyclic, and others were considered acyclic.

2.6 Statistical analysis

Categorical data as binary response variables (induction of ovarian cyclicity, estrus induction rate, estrus intensity, and conception rate) were classified into two groups (treatment and control). The association of these variables with groups was determined using Fisher's exact test due to sample size < 50. The confidence level (95%) and the criterion for statistical significance ($P < 0.05$) are now clearly mentioned. The study had approximately 80% statistical power to detect biologically meaningful differences (25–30%) in estrus induction and conception rates between groups at $\alpha = 0.05$. R 4.5.1 software was used for statistical analysis to determine the association between binary response variables and treatment groups.

3 Results

3.1 Estrus induction rate following using anti-stress feed supplement

During the observation period of one month without any treatment, none of the buffaloes were found in heat on visual observation or by ultrasound examination for the absence of corpus luteum. Buffaloes were divided into treatment (n=16) and control (n=17) groups. The treatment group buffaloes were given additional anti-stressor feed supplement under test for a period of 40 days, while the control group animals were not given this feed supplement. The progesterone implant was inserted 15 days after the start of anti-stress feed supplementation in both control and treatment groups. Within 15 days of feed supplementation, 37.5% (6/16) of buffaloes in the treatment group resumed cyclicity, while in the control group only 11.8% (2/17) of buffaloes developed CL in one of the ovaries, indicating ovulation of the dominant follicle ($P = 0.11$; Table 1).

3.2 Estrus response following CIDR supplementation

Behavioral signs of estrus were carefully observed in the morning and evening in the treatment and control groups 36 h after CIDR removal. Estrus response was classified into three categories. Animals were considered to have intense estrus when

TABLE 1 Induction of ovarian cyclicity (%) in treatment and control groups using anti-stress feed supplement alone within 15 days of feeding trial.

Group	N	Buffaloes induced to cyclicity (n)	Induction of cyclicity (%)	P- value
Treatment	16	6	37.50%	0.11
Control	17	2	11.80%	

they exhibited prominent bellowing during estrus period, vaginal mucus discharge, excitement along with edematous vulva, frequent micturition, and a good uterine tone on rectal palpation. Moderately responsive animals exhibited less prominent bellowing, excitement, a lesser degree of vulvar swelling, mucus discharge, and uterine tone. Poor estrus induction was recorded when buffaloes showed absence or minimal mucus discharge only on back-racking and mildly toned uterus with no evidence of bellowing. In response to hormonal treatment, the overall estrus induction rate was 100% in both treatment and control groups. No difference (P = 0.94) in estrus intensity was observed between the groups (Table 2).

3.3 Conception rate and fertility status

Following fixed-time AI, the conception rate was lower in the treatment group (37.5%) compared to the control group (64.7%). However, nearly 70% of buffaloes became pregnant within two AIs in both groups. Furthermore, no significant difference (P = 0.60) in overall conception rates was found between the treatment (93.7%) and control group (87.4%) within 90 days of CIDR removal (Tables 3, 4). However, a significant difference (P = 0.03) in conception rates was noticed between 30–90 days after CIDR removal between the two groups (56.2% *vs* 17.7%). At day 90, one buffalo in the treatment group and three in the control group remained acyclic.

4 Discussion

Buffaloes are sensitive to environmental temperature and radiation due to their inherent thick black skin and very few sweat glands (Jasinski et al., 2023). Heat stress is the major cause of poor reproduction, resulting in great economic losses to stakeholders. Although buffalo is a polyestrous animal, there is distinct seasonal variation in display of estrus, conception rate, and calving interval due to environmental factors (Singh et al., 2000). In India, the incidence of anestrus during the summer season has been

TABLE 2 Estrus response (%) and its intensity following progesterone implant.

Group	n	Estrus response (%)	Intensity of estrus (%)			P- value
			Intense	Moderate	Poor	
Treatment	16	100%	68.75% (11)	18.75% (3)	12.50% (2)	0.94
Control	17	100%	64.70% (11)	23.53% (4)	11.76% (2)	

TABLE 3 Conception rate at different AI attempts and overall conception rate (OCR) within 90 days of first AI in treatment and control groups during the summer season.

Groups	n	Conception rate at different AI attempts				Overall CR %	P-value
		FTAI	AI 2	AI 3	AI 4		
Treatment	16	37.50% (6)	31.20% (5)	6.30% (1)	18.70% (3)	93.70% (15)	0.60
Control	17	64.70% (11)	5.90% (1)	5.90% (1)	5.90% (1)	82.40% (14)	

FTAI, Fixed-time artificial insemination; AI, Artificial insemination; CR, Conception rate.

TABLE 4 Post-treatment conception rate at different time periods in treatment and control groups during the summer season.

Group	n	Pregnant within 30 days of CIDR removal	P- value	Pregnant between 30−90 days of CIDR removal	P- value	Cumulative pregnancy within 90 days of CIDR removal	P- value	Acyclic status at Day 90
Treatment	16	37.50% (6)	0.16	56.20%[a] (9)	0.03	93.70% (15)	0.60	6.30% (1)
Control	17	64.70% (11)		17.70%[b] (3)		82.40% (14)		17.60% (3)

CIDR, Controlled internal drug-release; AI, Artificial insemination.
Different superscript (a, b) differs significantly (P < 0.05) between treatment and control.

reported to be between 9.18 to 82.50% (Thakor and Patel, 2013), and approximately 31–40% of buffaloes did not exhibit estrus signs for more than 150 days postpartum (El-Wishy, 2007). In our study, it was observed that 40% of buffaloes did not resume cyclicity until 90 days postpartum during the summer season and were classified as anovular/anestrus. Several strategies, *viz*., extra nutritional supplementation, hormonal therapy, and heat amelioration measures, have been used to improve the reproductive efficiency in anestrus buffaloes with varying degree of success (Rathore et al., 2017; Ahmad and Arshad, 2020; Kumar et al., 2025a, b). Anovular condition primarily arises due to failure of the dominant follicle to ovulate, as 10–12 mm follicles are present in the ovary. Due to anovulation, animals are not primed for progesterone and remain acyclic. In contrast, in cyclic animals under the influence of progesterone priming, peripheral estrogen increases during the pre-ovulatory period due to progressive growth of the pre-ovulatory follicle during the peri-estrus period. This ascertains the importance of progesterone priming for estrus expression, induction, and ovulation. CIDR is a progesterone-impregnated device used effectively for induction and synchronization of estrus in acyclic buffaloes. However, during the summer season, hormonal therapy alone may not be as effective as during the breeding season.

Several investigators have suggested that dietary supplements of vitamins, trace minerals, and ruminal buffer are helpful in heat stress amelioration (Lundqvist et al., 2008; Krishnamoorthy et al., 2017). Supplementation of trace minerals and vitamins along with CIDR in acyclic buffaloes improved estrus induction rates ranging from 66 to 100% (Resum et al., 2017; Wani et al., 2018). In our experiment, higher numbers of buffaloes (37.5%) were found in heat after 15 days of anti-stress supplementation in the treatment group compared to the control group (11.8%) without hormonal therapy. However, during the observation period, none of the buffaloes were detected in heat either by visual observation or by transrectal ultrasonography. CIDR induced estrus response in 100% of buffaloes in both groups, and these findings were similar to earlier reports (Resum et al., 2017; Wani et al., 2018; Singh et al., 2023). Progesterone implant along with PMSG acts synergistically in inducing ovarian cyclicity (Rathore et al., 2017; Sharma et al., 2017) by increasing luteinizing hormone (LH) pulse frequency. The higher circulating estrogen level induces behavioral estrus (Sharma et al., 2017), ascertaining the necessity of P_4 priming of the hypothalamus for behavioral estrus, with the stimulatory effect of PMSG in inducing follicle turnover (Wiltbank et al., 2011), with non-significant alterations in the follicular hormonal milieu. In our study, the conception rate on FTAI was lower in the treatment group; however, nearly 70% of buffaloes conceived within two inseminations in both treatment and control groups. Furthermore, no significant difference in overall conception rate was found between the treatment (93.7%) and the control group (87.4%) within 90 days of CIDR removal. This could be due to similar health and nutritional status of buffaloes selected for the investigation, as well as the study design involving organized herd buffaloes maintained under uniform management conditions, as compared to field-based studies. The feeding strategy followed in our organized herd corroborates the non-significant effect of anti-stress supplement feeding with respect to overall conception rates in both study groups. However, supplementation induced estrus in buffaloes at the initial stage of feeding. Interestingly, 6.3% of buffaloes in the treatment group and 17.6% of buffaloes in the control group returned to an ovulatory/anestrus condition. This further suggests the beneficial effect of the anti-stress feed supplement used in our study.

It is noteworthy that anti-stress supplementation appeared to increase early induction of cyclicity between 30 to 90 days postpartum, but overall pregnancy outcomes after CIDR-based hormonal treatment were similar between groups. This may be explained by changes in several post-estrus phenomena, *viz*., uterine hormonal milieu and receptivity, contributing to conception rates. Moreover, interactions of the constituents of the anti-stress formulation with reproductive processes and hormones might impact fertilization as well as early embryonic growth, thereby affecting conception rates, which needs further investigation. Moreover, overall pregnancy rate within 90 days of feeding this supplement was 93.7% in the treatment group and 82.4% in the control group. Similar conception rates have been reported by Zanella et al. (2010); Izquierdo et al., 2010) in cows following anti-stress supplementation. In contrast, Scales (1976) and Spears et al. (1986) found no beneficial effect of anti-stress supplements (vitamin E and selenium (Se)) on fertility in cows. This study has limitations, including a small sample size, the fact that the study was conducted in an organized herd, and paucity of information regarding antioxidant status of the study animals before and after supplementation, which needs further investigation. Thus, anti-stress supplementation can augment estrus induction response in buffaloes through its antioxidant properties. In conclusion, the inclusion of anti-stress supplements along with progesterone supplementation can improve overall health and fertility of buffaloes for better productivity.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by the ICAR-Central Institute for Research on Buffaloes IAEC. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

RK: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. JP: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. RS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. VM: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. SP: Conceptualization, Data curation, Formal analysis, Investigation, Resources, Validation, Writing – original draft, Writing – review & editing. YB: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Writing – original draft, Writing – review & editing. JA: Data curation, Formal Analysis, Investigation, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fanim.2026.1745664/full#supplementary-material

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