# Evaluation of the Effects of Bio-Stimulation and Gonadotropin-Releasing Hormone on First-Service Conception Rate in Buffalo Cows

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**Abstract:** This study investigated the efficacy of combining bio-stimulation and hormonal interventions to enhance the first AI conception rate (CR-FAI) in buffaloes. Under field conditions, 224 adult female buffaloes were randomly assigned (lottery method) to one of two treatment protocols. In group A, AI was conducted using a modified penis-like device (mPLD) and the application of clitoral massage (CM) for 30 seconds (AI + mPLD + CM). In group B, AI included mPLD and CM supplemented with an intramuscular injection of 500 µg of gonadotrophin-releasing hormone (GnRH) immediately post-insemination (AI + mPLD + CM + GnRH). Confounding variables, including age, parity, body condition score (BCS), and breed of the buffaloes, were recorded at the time of AI. The results demonstrated that the CR-FAI in group B (77.4%) was significantly higher than in group A (63.6%; $p < 0.05$). While age categories exhibited significant variation in CR-FAI ($p < 0.05$; $\chi2 = 8.80$), no significant variation was found for parity, BCS, or breed. Logistic regression revealed that buffaloes at parity 1 and 2 possessed 3.6-times and 2.7-times higher odds of conception, respectively, [Wald=1.02, OR =3.61, 95% CI (0.30-43.99) and Wald =1.81, OR =2.65, 95% CI (0.64-11.0)], relative to other parity groups. Furthermore, age was highly significant and positively correlated with parity and BCS ($p < 0.01$). In conclusion, AI integrating bio-stimulation with concurrent GnRH administration substantially enhances the conception rates in buffaloes.

**Keywords:** Artificial insemination, GnRH, mPLD, Pregnancy, River-type buffalo.

## INTRODUCTION

The water buffalo (*Bubalus bubalis*) is an important species in the global livestock economy, particularly in developing countries such as Bangladesh. It serves as a principal source of quality milk, meat, and draught power. In coastal Bangladesh, buffaloes are reared under ‘semi-bathan’ (a seasonal system in which animals are housed during rice cultivation and graze on islands for the remainder of the year) practices [1]. This species is also known for its resistance to several infectious diseases, its tolerance to salinity, and its adaptability to adverse climatic conditions. Despite its economic importance, the species exhibits inherent reproductive inefficiencies that limit its genetic progress and profitability. Challenges such as late maturity, a high incidence of ‘silent’ estrus, seasonal anestrus, and lower first-service conception rates (CR) compared to cattle remain significant barriers for buffalo producers [2]. In many coastal and tropical regions, the first-service CR in buffaloes often ranges from 30% to 40%, necessitating the development of robust management interventions to optimize fertility. The CR is significantly higher in natural service due to bull exposure, the natural method of estrus detection, a high volume of semen, and the sex-driven release of ovulation-stimulatory luteinizing hormone (LH) and oxytocin hormones [3]. The mechanism involves olfactory and/or tactile signals relayed to the hypothalamus, leading to increased GnRH secretion and subsequent LH secretion [4]. The CR for buffaloes after natural service typically ranges from 61.28% to 75%, which is significantly higher than that achieved through artificial insemination (AI) under field conditions [5]. Although AI is a keystone of livestock genetic improvement programs globally, success largely depends on accurately detecting estrus and timing insemination, resulting in a low overall CR in buffaloes [6]. To address these reproductive hurdles, hormonal synchronization and bio-stimulation have emerged as two of the most promising strategies. Among hormonal treatments, Gonadotropin-Releasing Hormone (GnRH)

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is widely applied to manage the timing of ovulation by triggering a pre-ovulatory surge of LH [2, 7]. GnRH administration at the time of artificial insemination (AI) or during the early luteal phase ensures precise synchronization between gametes and enhances corpus luteum development [8]. Parallel to hormonal interventions, bio-stimulation offers a non-invasive or minimally invasive approach to enhancing fertility. This technique leverages the 'male effect' or mechanical stimuli to activate the hypothalamic-pituitary-gonadal axis. Although traditional bio-stimulation involved the physical presence of a bull, recent innovations have introduced mechanical devices, such as the modified Penis-Like Device (mPLD), to simulate natural coitus during AI [3]. Research by Sarker *et al*. [9] indicates that such mechanical stimuli can increase pregnancy rates by over 10%, potentially by facilitating oxytocin release and improving intrauterine sperm transport. Furthermore, the combination of mechanical bio-stimulation with clitoral massage has shown synergistic effects, significantly outperforming conventional AI protocols in coastal buffalo populations [3]

With intra-vaginal bio-stimulation with a penis-like device (PLD) following AI, the pregnancy rate increased by 15.5% in cattle [10] and 12% in buffaloes [9], compared to the standard AI procedure. Paul *et al*. [3] observed that combining the modified PLD (mPLD) with clitoral massage (CM) significantly boosted buffalo pregnancy rates (52.5%) across selected coastal areas of Bangladesh. Because these bio-stimulation (mPLD and CM) protocols significantly increased the conception rate, this protocol served as the base category for this study. The combined use of these strategies (bio-stimulation and hormonal) holds promise for further improving reproductive outcomes. The central hypothesis is that integrating bio-stimulation into established GnRH-based AI protocols will create a synergistic effect. It will optimize both the endocrine environment for conception and the physiological conditions by simulating a natural service. Evaluating the efficacy of these combined approaches is essential to developing robust, efficient reproductive management systems. It can sustainably enhance conception rates in buffalo populations. However, to my knowledge, the injection of GnRH along with bio-stimulation has not been explored in buffalo cows. Therefore, this study aimed to enhance conception rate at first AI (CR-FAI) through the synergistic action of mechanical bio-stimulation (mPLD, clitoral massage) and a hormonal (GnRH) protocol in buffalo cows.

## MATERIALS AND METHODS

### Anthelmintics and Vitamin-Minerals

Anthelmintics, consisting of levamisole (1200 mg) and triclabendazole (1800 mg) (Navadex® DS Vet 2 g bolus at a dose of 1 bolus per 150 kg body weight and repeated with the same dose after a 7-day interval), were purchased from NAVANA Pharmaceutical Ltd, Dhaka, Bangladesh. VitaminAD3E (Injection Renasol AD3E ® Vet 30 ml vial at a dose of 10 ml intramuscularly (IM) at 7-day intervals for a total of three injections) was purchased from Renata PLC, Dhaka, Bangladesh. Hormones (Gonavet® 5 ml vial) were purchased from Chemist Laboratories Limited, Barishal, Bangladesh. The Gonavet® injection contained gonadorelin acetate BP (105 µg equivalent to 100 µg of gonadorelin). Each animal received a total dose of 500 µg via IM injection.

### Making of Modified Penis-like Device (mPLD)

The mPLD was constructed following the procedure established by Biswas *et al*. [10] and Paul *et al*. [3], as illustrated in Figure **1**. The device measured 20 cm in length, tapering from 12 cm to 5 cm, and was fitted with a 12 cm handle. It incorporated two apertures for the AI

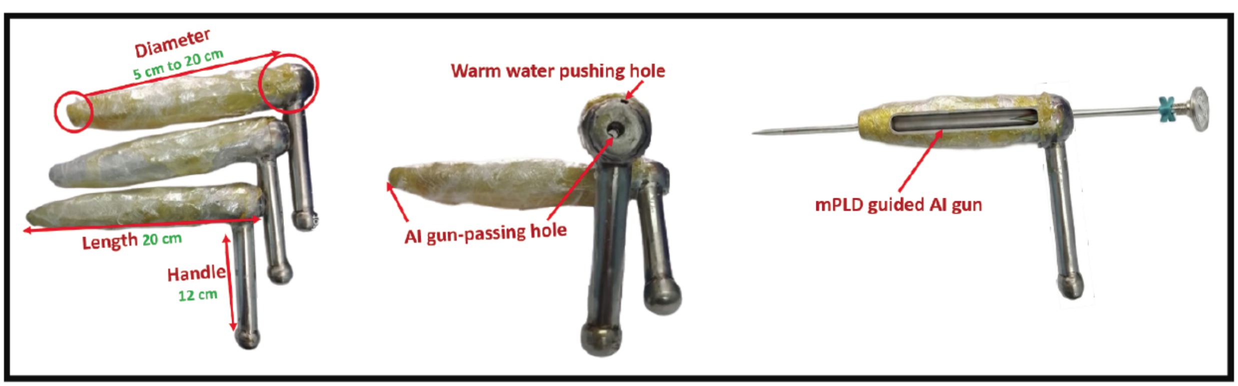


**Figure 1:** Illustration of Modified Penis-like Device (mPLD).

gun and an injection port situated above the handle to facilitate the introduction of warm water. This design was intended to stimulate the thermal and physical characteristics of an erect buffalo bull's penis.

**Semen Straws**

Frozen semen straws were sourced from the Buffalo Breeding Station at the Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka, Bangladesh. The straws were maintained in liquid nitrogen (-196 °C) according to standard cryopreservation protocols.

**Research Area and Period**

The study was conducted from July 2024 to June 2025 across two selected coastal regions in Bangladesh: Charfasson Upazila in the Bhola district (22° 11' 4.92" N, 90° 45' 45.00" E) and Bauphal Upazila in the Patuakhali district (22° 25' 45.12" N, 90° 30' 50.04" E). These locations were selected based on the strategic research scope of the Barishal division.

**Animal Selection and Management**

A total of 224 adult female river-type buffaloes were selected by simple random sampling from the study area. The selection criteria prioritized effective communication and farmer compliance. Ethical clearance and informed consent were obtained from all participating farmers after a thorough briefing on the experimental protocols. Buffaloes were maintained under a standardized nutritional regimen, fed 7 to 10 kg per animal daily of the concentrate admixture consisting of 30% rice polis, 30% wheat bran, 20% broken rice, and 20% oil cake. It was supplemented with ad libitum water prior to grazing. Prophylactic measures included the administration of anthelmintics and Vitamin AD3E consistent with veterinary standards. All treatments were executed during the winter season (November - February) to ensure uniform environmental conditions. Phenotypic data, age, parity, body condition score (BCS), and breed were recorded at the time of insemination.

**Grouping of Animals**

Animals were classified into discrete variables based on the research hypothesis. The cohort included indigenous (n=192) and crossbred (n=32) buffaloes. Age was estimated via dental eruption patterns and birth records. They were categorized as 3-4 years (n=82), 6-8 years (n=120), and ≥9 years (n=22) of age. The population comprised nulliparous (n=7) and parous (n=117) buffaloes. Further, stratified by parity: 1 (n=55), 2 (n=77), 3 (n=56), and ≥4 (n=29). BCS was grouped as 2.0-<2.5 (n=8), 2.5-3.0 (n=184), and >3.0-4.0 (n=32) on a 1-5 scale.

**Application of mPLD**

The mPLD was deployed according to the methodology described by Paul *et al*. [3]. The device was disinfected by spraying with 70% ethanol, lubricated with coconut oil, and primed with warm water (45-48 ºC) to mimic physiological conditions. A loaded AI gun was integrated into the mPLD, inserted intravaginally, and advanced. Following intrauterine semen deposition, the device was manipulated in a slow cranial-caudal (push-pull) motion for 30 seconds. To ensure animal welfare and prevent mucosal injury, all procedures were performed by a certified technician. Observation of any deviations was recorded.

**Estrus Detection, Artificial Insemination, and Pregnancy Diagnosis**

Estrus was initially identified by farmers observing behavioral signs (bellowing, mucous discharge from the vagina, and standing to be mounted). Clinical confirmation of cyclicity was performed via transrectal palpation to assess uterine tonicity and the coiling of the uterine horns. Insemination followed the standard 'AM-PM' rule (12 to 18 hours post-onset of heat) as described by Praveen *et al*. [11]. Primary pregnancy assessment was based on non-return to estrus 20-25 days post-AI. Confirmatory diagnosis was performed via transrectal palpation on days 60 and 90, identifying physiological markers such as asymmetry of the uterine horns, membrane slip, and a persistent corpus luteum. To eliminate observational bias, pregnancy was confirmed by two independent, trained personnel, neither of whom performed the insemination on the buffalo in this study.

**Experimental Design**

This study was conducted under field conditions. A total of 224 buffaloes were assigned to one of two experimental groups (Group A or Group B) according to specific treatment protocols (Figure **2**). Upon clinical confirmation of natural estrus, animals were randomly allocated to a treatment regimen using a simple randomization method (lottery). The efficacy of each protocol was evaluated based on the conception rate at first AI (CR-FAI). The experimental groups were defined as follows:

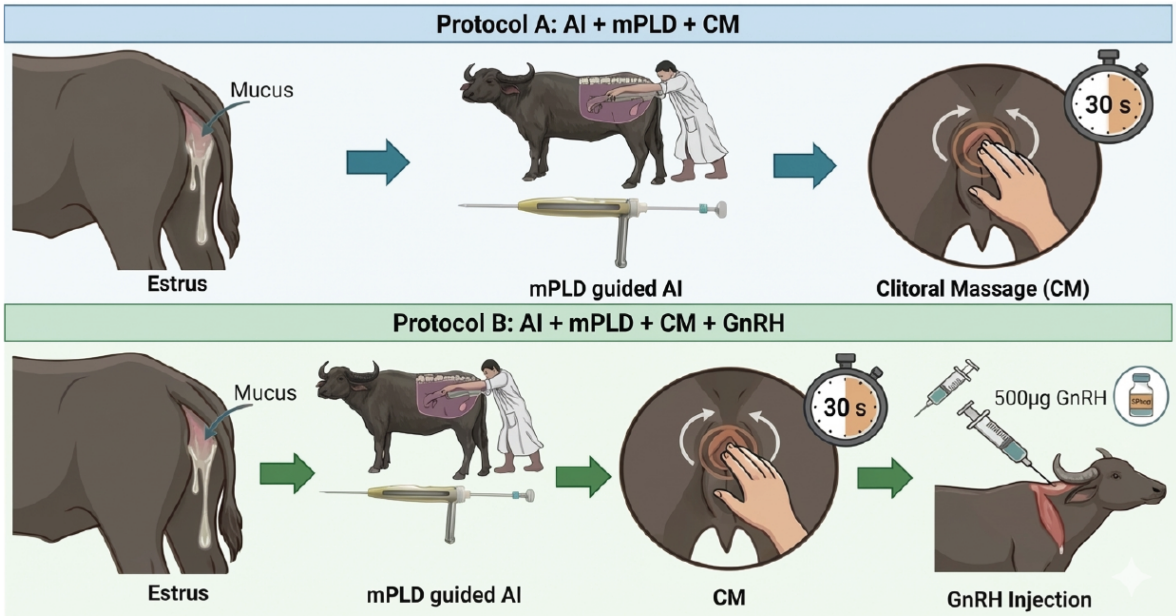


**Figure 2:** Experimental Protocols (AI: artificial insemination; mPLD: modified penis-like device; CM: clitoral massage; GnRH: gonadotropin-releasing hormone).

**Group A (n =118; Control):** AI was performed in conjunction with the modified penis-like device (mPLD) and manual clitoral massage (CM) for 30 seconds (Protocol A: AI + mPLD + CM).

**Group B (n=106):** AI was conducted using the mPLD and CM (30 seconds), supplemented with a concurrent intramuscular injection of 500 µg GnRH immediately post-insemination (Protocol B: AI + mPLD + CM + GnRH).

**Statistical Analysis**

Data were tabulated and coded in Microsoft Excel to ensure data integrity, including the identification of duplicates and missing values. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, Version 26.0; IBM Corp., Armonk, NY). Data normality was assessed via visual inspection of histograms. Conception rates at first AI were expressed as percentages (%). The degree of association or variation in conception rate between two experimental groups and across different categorical variables (age, parity, BCS, and breed) was evaluated using the Chi-square (χ2) test. Binary logistic regression was used to estimate odds ratios and predict the probability of conception based on the aforementioned independent predictors. Furthermore, partial correlation coefficients were used to assess the strength and direction of the linear relationship between continuous variables and conception outcomes. For all analyses, statistical significance was set at $p < 0.05$.

$$\text{Conception rate at first AI (\%)} = \frac{\text{Number of buffalo conceived on first AI}}{\text{Number of buffalo conduct first AI}} \times 100$$

Chi-square (χ2) formula:

$$\chi^2 = \sum \frac{(O_i - E_i)^2}{E_i}$$

Where, $O_i$ = observed frequency; $E_i$ = Expected frequency

The logistic regression statistical model:

$$Logit\ P(Y = 1) = In\left\{\frac{P(Y=1)}{1 - P(Y=1)}\right\} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots.. + \beta_n X_n$$

Where,

P (Y = 1) = Probability of a successful event (e.g., conception or pregnancy).

P (Y = 0) = Probability of a failure event (e.g., no conception or pregnancy).

$\beta_0$ = Intercept (constant).

$\beta_1$, $\beta_2$, …, $\beta_n$ = Coefficients of the predictors.

$X_1$, $X_2$, …, $X_n$ = independent variables

Partial correlation coefficient formula:

$$Pxy.z\ (r) = \frac{r_{xy} - r_{xz}r_{yz}}{\sqrt{1-r^2xy}\ \sqrt{1-r^2yz}}$$

Where, x, y denoted continuous variables (age, parity, BCS, breed) and z denoted conception

Pxy.z: Partial correlation coefficient

rxy: Pearson correlation between x and y

rxz: Pearson correlation between x and z

ryz: Pearson correlation between y and z

## RESULTS

### Comparison of the Efficacy of Experimental Protocols on CR-FAI

The conception rate at first AI (CR-FAI) was 63.6% for group A (AI + mPLD + CM) and 77.4% for group B (AI + mPLD + CM + GnRH). (Figure **3**). Statistical analysis revealed that group B achieved a significantly higher CR-FAI than group A ($p < 0.05$; $\chi2 = 5.07$). Notably, no adverse effects, post-procedural discomfort, or welfare-related complications were observed following the application of the mPLD.

### Factors Influencing Conception Outcomes

The CR-FAI stratified by categorical factors is presented in Table **1**. Results from the binary logistic regression and partial correlation analyses are summarized in Tables **2** and **3**, respectively.

### Age

The overall CR-FAI for aged 3-5, 6-8, and ≥ 9 years was 79.3%, 61.7%, and 81.8%, respectively (Table **1**). A significant variation was observed among the categorical variables ($p < 0.05$, $\chi2 = 8.80$). The CR-FAI for the ≥9-year category was numerically higher than that for the 6-8-year category and comparable to that for the 3-5-year cohort. Across all age strata, protocol B consistently yielded higher conception rates than protocol A. Logistic regression identified age as a significant predictor of CR-FAI, particularly in the 3-5- and ≥9-year groups ($p < 0.05$; Wald = 5.23 and 3.34, respectively; Table **2**). Age exhibited highly significant positive correlations with parity ($r = 0.70$, $p < 0.01$) and BCS ($r = 0.27$, $p < 0.01$), while displaying a negligible negative correlation with breed ($r = -0.01$, $p > 0.05$; Table **3**).

### Parity

The CR-FAI of nulliparous heifers, and animals of parity 1, 2, 3, and ≥4 was 85.7%, 78.2%, 70.1%, 60.7%, and 69.0%, respectively. No significant variation was observed among groups ($p > 0.05$, $\chi2 = 4.90$). The CR-FAI across all categories was also higher in response to protocol B than in protocol A (Table **1**). Although parity did not exert a significant independent effect ($p > 0.05$), buffaloes at parity 1 and 2 demonstrated 3.6- and 2.7-fold higher odds of

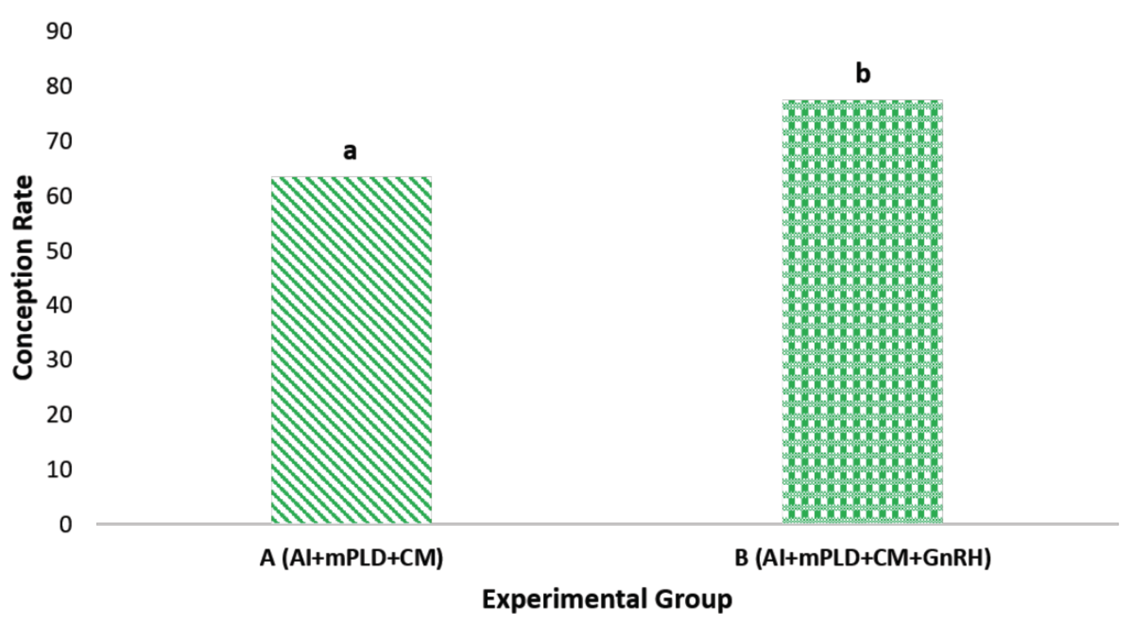


**Figure 3:** Comparisons of Two Protocols on CR-FAI in Buffaloes. Different letters (**a**, **b**) indicate a significant difference ($p < 0.05$).

**Table 1: First AI Conception Rates of Different Factors**

| Factors | Category | Frequency (n) | Overall CR-FAI % (n) | χ2 & p value | CR-FAI % (n) | |
|---|---|---|---|---|---|---|
| | | | | | Protocol A | Protocol B |
| Age | 3 to 5 | 82 | 79.3 (65) | 8.80<br>0.01 | 76.1 (35/46) | 83.3 (30/36) |
| | 6 to 8 | 120 | 61.7 (74) | | 55.9 (38/68) | 69.2 (36/52) |
| | ≥9 | 22 | 81.8 (18) | | 50 (2/4) | 88.9 (16/18) |
| Parity | Nulliparous | 7 | 85.7 (6) | 4.90<br>0.30 | 0 (0/1) | 100 (6/6) |
| | 1 | 55 | 78.2 (43) | | 76.7 (23/30) | 80 (20/25) |
| | 2 | 77 | 70.1 (54) | | 62.5 (30/48) | 82.8 (24/29) |
| | 3 | 56 | 60.7 (34) | | 59.3 (16/27) | 62.1 (18/29) |
| | ≥4 | 29 | 69 (20) | | 50 (6/12) | 82.4 (14/17) |
| BCS | 2 to <2.5 | 8 | 75 (6) | 0.56<br>0.76 | 66.7 (2/3) | 80 (4/5) |
| | 2.5 to 3 | 184 | 69 (127) | | 63.8 (67/105) | 75.9 (60/79) |
| | >3 to 4 | 32 | 75 (24) | | 60 (6/10) | 81.8 (18/22) |
| Breed | Indigenous | 192 | 70.3 (135) | 0.03<br>0.86 | 63.8 (67/105) | 78.2 (68/87) |
| | Crossbred | 32 | 68.8 (22) | | 61.5 (8/13) | 73.7 (14/19) |

CR-FAI: conception rate at first insemination; n: number; P: pregnant; A: AI + mPLD + CM; B: AI + mPLD + CM + GnRH.

conception, respectively [Wald=1.02, OR =3.61, 95% CI of OR (0.30-43.99) and Wald =1.81, OR =2.65, 95% CI of OR (0.64-11.0)] than the other categories (Table **2**). Parity was strongly and positively correlated with age and BCS ($p < 0.01$) and weakly correlated with breed ($p > 0.05$).

### Body Condition Score (BCS)

CR-FAI BCS categories 2-2.5, 2.5-3, and >3-4 were recorded at 75%, 69%, and 75%, respectively. Congruent with other factors, protocol B elicited a superior response across all BCS cohorts (Table **1**). However, no significant variation or predictive effect of BCS on CR-FAI was detected using chi-square or logistic regression ($p > 0.05$; Table **2**). BCS showed a highly significant positive correlation with parity ($r = 0.27$, $p < 0.01$), but a significant negative correlation with breed ($r = -0.14$, $p < 0.05$; Table **3**).

### Breed

Indigenous and crossbred buffaloes exhibited CR-FAI of 70.3% and 68.8%, respectively, representing no significant inter-breed variation ($p >0.05$; $\chi^2 = 0.03$). Both breeds responded more favorably to protocol B (78.2% and 73.7%, respectively) than to protocol A (63.8% and 61.5%, respectively). Logistic regression indicated that crossbred buffaloes had 1.1-fold higher, though non-significant, odds of pregnancy ($p > 0.05$, Wald = 0.07, OR = 1.12, 95% CI of OR (0.47-2.69). Breed was negatively correlated with age and BCS, and positively associated with parity.

## DISCUSSION

This study investigated the comparative efficacy of artificial insemination (AI) augmented by bio-stimulation alone (protocol A) versus a synergistic approach combining bio-stimulation with exogenous gonadotropin-releasing hormone (GnRH) injection (protocol B). Our objective was to elucidate the impact of these protocols, alongside intrinsic factors such as age, parity, BCS, and breed, on the conception rate at first AI (CR-FAI) in river-type buffaloes in the coastal region. The CR-FAI observed in group B (77.4%) was significantly higher than that in group A (63.6%; $p <0.05$). The findings of this study were comparatively higher than those reported by Paul *et al*. [3], who observed a 52.5% conception rate using mPLD and CM, and Sarker *et al*. [9], who reported 44% using a standard PLD. The enhanced outcomes observed here may be attributed to the synergistic interaction between mechanical bio-stimulation and hormonal intervention. In contrast, Sarker *et al*. [9] also reported that the conception rate of buffaloes after natural insemination was higher (64%) than that with only AI (32%) due to the bull effect of mating. On the other hand, Maurya *et al*. [12] reported that the massage of the clitoris immediately after AI hastens the release of oxytocin from the posterior pituitary gland. Oxytocin increases

**Table 2: Logistic Regression Analysis of Factors associated with the First AI Conception Rate**

| Factors | Category | Wald | D.F. | p value | O.R. | 95% C.I. of O.R. | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower | Upper |
| Age | 3 to 5 | 5.23 | 2 | 0.07 | - | - | - |
| | 6 to 8 | 0.89 | 1 | 0.35 | 0.45 | 0.08 | 2.39 |
| | ≥9* | 3.34 | 1 | 0.07 | 0.25 | 0.06 | 1.11 |
| Parity | Nulliparous | 2.51 | 4 | 0.64 | - | - | - |
| | 1 | 1.02 | 1 | 0.31 | 3.61 | 0.30 | 43.99 |
| | 2 | 1.81 | 1 | 0.18 | 2.65 | 0.64 | 11.00 |
| | 3 | 1.17 | 1 | 0.28 | 1.10 | 0.57 | 6.99 |
| | ≥4* | 0.31 | 1 | 0.58 | 1.41 | 0.42 | 4.78 |
| BCS | 2 to <2.5 | 0.79 | 2 | 0.67 | - | - | - |
| | 2.5 to 3 | 0.08 | 1 | 0.78 | 0.76 | 0.11 | 5.11 |
| | >3 to 4* | 0.78 | 1 | 0.38 | 0.66 | 0.26 | 1.67 |
| Breed | Indigenous | - | 1 | - | - | - | - |
| | Crossbred* | 0.07 | 0 | 0.80 | 1.12 | 0.47 | 2.69 |

*Indicated reference category; D.F.: degree of freedom; O.R.: odd ratio; C.I.: confidence interval.

**Table 3: Correlation-Coefficients between Variables in relation to the Conception**

| Variables | Age | Parity | BCS | Breed |
|---|---|---|---|---|
| Age | 1 | 0.70** | 0.27** | -0.01 |
| Parity | 0.70** | 1 | 0.27** | 0.01 |
| BCS | 0.27** | 0.27** | 1 | -0.14* |
| Breed | -0.01 | 0.01 | -0.14* | 1 |

** indicated p <0.01; * p <0.05 (2-tailed).

uterine tone and facilitates the rapid transport of sperm toward the oviduct. Furthermore, Sarker *et al*. [9] also assumed that the physio-mechanical intravaginal bio-stimulation by the PLD during AI triggers the surge release of luteinizing hormone (LH), which is responsible for ovulation. Therefore, the use of mPLD with CM during AI significantly increased the conception rate, as achieved with natural service. In this study, the bio-stimulation functioned as a proxy for the ‘bull effect’ observed during natural mating, while GnRH exerted a stimulatory effect on the pituitary gland, increasing the rate. Although the underlying endocrine pathways were not quantified in this study. The observed conception rate was also considerably higher than that reported by Binyameen *et al*. [13], who reported a 60% pregnancy rate following exogenous GnRH injection with the traditional AI technique. The exogenous administration of GnRH at the time of AI acts directly on the anterior pituitary gland to induce an LH surge [7]. However, GnRH injection at AI significantly improves the probability of ovulation [14] and with a fixed-timed AI synchronization protocol [15]. Furthermore, Berean *et al*. [16] demonstrated that administering GnRH at the time of, or shortly after, AI can enhance corpus luteum function and increase progesterone levels, thereby reducing early embryonic loss. Consequently, it is hypothesized that integrating GnRH injection with a bio-stimulation-assisted AI protocol may mitigate conception failure due to slow sperm transportation and delayed ovulation. This is particularly relevant for buffalo populations in coastal Bangladesh, where high ambient temperatures and humidity often suppress endocrine function, thereby reducing reproductive efficiency [17]. Therefore, protocol B offers a promising strategy to mitigate heat-stress-related infertility.

In this study, various influencing factors were measured by their effect on conception rate. While a significant association between CR-FAI and age was observed across categories ($p < 0.05$; $\chi 2 = 8.80$), no such association was found for parity, BCS, or breed. Buffaloes in the 3-5 and ≥ 9-year categories exhibited higher CR-FAI (79.3% and 81.8%, respectively), with a significantly greater probability of conception (Wald = 5.23 and 3.34, $p < 0.05$) than those in the 6-8-year-old group (61.7%). Regarding the determinants of conception, age demonstrated a highly significant positive correlation with parity and BCS. These findings align with Paul *et al*. [3] and Sarker *et al*. [9], who reported a higher conception rate in buffaloes aged 4 to 5.5. Although Pehan *et al*. [18] and Calanni *et al*. [19] reported that conception rates declined with increasing age. Interestingly, the older buffaloes (≥9 years) showed a superior response to the treatment protocol. It is postulated that GnRH-mediated physio-mechanical bio-stimulation enhances endocrine function in older buffaloes, thereby improving conception outcomes.

Regarding the parity, buffaloes with first and second parities exhibited non-significantly 3.6-fold (Wald =1.02, OR =3.61, 95% CI =0.30-43.99) and 2.7-fold (Wald =1.81, OR =2.65, 95% CI =0.64-11), respectively, higher odds of pregnancy compared to other parity categories. These findings are consistent with Sarker *et al*. [9], who reported a significantly higher pregnancy rate in parity 2 (68.2%; $p < 0.05$), and with Paul *et al*. [3], who noted a higher probability of conception in primiparous buffaloes. Primiparous buffaloes possess better uterine health and greater hormonal sensitivity compared to multiparous animals [20]. Nevertheless, this study observed consistently higher conception rates across both primiparous and multiparous buffaloes.

Regarding BCS, the majority of buffaloes were in BCS 2.5-3.0. The CR-FAI for this range was recorded at 69% (127/184), which did not differ significantly from other categories. Furthermore, BCS exhibited a significantly negative correlation with breed. These results parallel those of Sarker *et al*. [3], who observed a non-significantly higher pregnancy rate at a BCS 2.5. A BCS range of 2.5 to 3.0 is considered optimal for the reproductive performance of buffalo cows [18].

In terms of breed variation, this study demonstrated no significant effect on conception rates. The CR-FAI was 70.3% (135/192) in indigenous buffaloes and 68.8% (22/32) in crossbred individuals. In contrast, Sarker *et al*. [9] reported a significantly higher pregnancy rate in indigenous buffaloes (51.8%; $p < 0.05$) relative to other breed categories. The comparable performance observed between indigenous and crossbred buffaloes suggests that these reproductive interventions are broadly efficacious across diverse genotypes.

## LIMITATIONS

Although this study explored a novel protocol to enhance the conception rate of buffaloes, several limitations still remain. The study was conducted at the field level, not in a controlled management system. The primary estrus detection was dependent on farmers' observations. Additionally, there was no true control group (AI without bio-stimulation) in this study, and the hormonal mechanisms were not studied.

## CONCLUSION

In conclusion, the combined application of intravaginal bio-stimulation via a modified penis-like device, clitoral massage, and GnRH therapy at the time of artificial insemination effectively improves the first-service conception rate in buffaloes, independent of various confounding factors. Indigenous buffaloes with a BCS 2.5-3.0 exhibited particularly high responsiveness to this GnRH-based AI protocol. Nevertheless, Further research incorporating endocrine analysis under controlled management conditions is warranted to elucidate the precise physiological efficacy of this treatment.

## LIST OF ABBREVIATIONS

AI = Artificial Insemination

CR = Conception Rate

CR-FAI = Conception Rate at First Artificial Insemination

CM = Clitoral Massage

mPLD = Modified Penis-like Device

FSH = Follicular Stimulating Hormone

LH = Luteinizing Hormone

## AVAILABILITY OF DATA AND MATERIALS

Data and materials used for this research are available upon reasonable request to the corresponding author.

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## RESEARCH ETHICS AND POLICIES

The study protocol and policies were reviewed and approved by the Institutional Ethical Committee of Patuakhali Science and Technology University (Approval ID:PSTU/IEC/2025/09).

## DECLARATION OF INTEREST

The authors declare no conflicts of interest that could be perceived as prejudicing the impartiality of the submitted study.

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## AUTHORS CONTRIBUTION

Ashit Kumar Paul conceptualized and conducted the experiment, analyzed the data, and wrote the draft manuscript. Md. Fakruzzaman and Md. Ashadul Alam formulated the methodology, validated the data, and revised the manuscript. Gautam Kumar Deb and M. A. M. Yahia Khandoker contributed to the finalization, review, and proofreading for publication. All authors contributed to fund acquisition (Grant number for this experiment: 12-ES-BRDP).

## REFERENCES

[1] Sagor SI, Marma U, Chowdhury S, Ape TS, Rumi HJ, Hossain MH, *et al.* Buffalo farming in Bangladesh: a guide for buffalo farmers, veterinarians and veterinary students. Chattogram (BD): Udder Health Bangladesh 2024; pp. 22-23.

[2] Coman S, Berean DI, Cimpean R, Ciupe S, Coman I, Bogdan LM. Clinical modalities for enhancing reproductive efficiency in buffaloes: a review and practical aspects for veterinary practitioners. Animals (Basel) 2024; 14: 2642. https://doi.org/10.3390/ani14182642

[3] Paul AK, Fakruzzaman M, Alam MA, Deb GK, Khandoker MAMY. Intra-vaginal bio-stimulation and clitoral massage to enhance pregnancy rates in water buffalo in coastal Bangladesh. Animals (Basel) 2025; 15: 597. https://doi.org/10.3390/ani15040597

[4] Fiol C, Ungerfeld R. Biostimulation in cattle: stimulation pathways and mechanisms of response. Trop Subtrop Agroecosyst 2012; 15: S29-S45. https://doi.org/10.56369/tsaes.1342

[5] Rahman MK, Sarkar M, Rahim A, Nandi R, Hasan MM, Debnath RC. Effectiveness of artificial insemination in buffalo at coastal region of Bangladesh. Int J Curr Res Life Sci 2020; 9: 3385-8.

[6] Baruselli PS, de Carvalho NAT, Gasparrini B, Campanile G, D'Occhio MJ. Development, adoption, and impact of assisted reproduction in domestic buffaloes. Animal 2023; 17(1): 100764. https://doi.org/10.1016/j.animal.2023.100764

[7] Uddin AHMM, Petrovski KR, Song Y, Garg S, Kirkwood RN. Application of exogenous GnRH in food animal production. Animals (Basel) 2023; 13: 1891. https://doi.org/10.3390/ani13121891

[8] Pasha MH, Ali GA, Rahman SU. Estrus synchronization of native buffaloes using PGF2α and GnRH-based protocols in Bangladesh. Bangladesh J Livest Res 2024; 28: 48-56.

[9] Sarker A, Biswas D, Fakruzzaman M, Deb GK, Hossain SMJ, Alam MA, *et al.* Enhancement of the pregnancy rate of buffalo cows through intra-vaginal bio-stimulation with penis-like device in the coastal area of Bangladesh. Adv Anim Vet Sci 2024; 12: 1301-8. https://doi.org/10.17582/journal.aavs/2024/12.7.1301.1308

[10] Biswas S, Swarna M, Paul AK. Improvement of bovine pregnancy rate through intra-vaginal bio-stimulation with penis-like device. Bangladesh J Sci Ind Res 2022; 57: 101-8. https://doi.org/10.3329/bjsir.v57i2.60406

[11] Praveen KS, Radhika V, Chandrika T, Srihari J, Manoranjan I, Srikanth NR, *et al.* Artificial insemination in cattle and buffalo: an update. Int J Vet Sci Anim Husb 2024; 9: 221-5. https://doi.org/10.22271/veterinary.2024.v9.i5d.1690

[12] Maurya V, Mehrotra S, Krishnaswamy N, Singh G. Effect of clitoral stimulation after artificial insemination on conception and hormonal profile in the Murrah buffalo. Indian J Anim Sci 2017; 87: 70-5. https://doi.org/10.56093/ijans.v87i1.66907

[13] Binyameen M, Imran M, Waseem M, Anwar S, Tahir H, Azam B, *et al.* Effect of a single injection of GnRH analog alone and at the time of AI on reproductive performance of Nili Ravi buffaloes. Farm Anim Health Nutr 2024; 3: 17-21. https://doi.org/10.58803/fahn.v3i1.38

[14] Kolachi HA, Zhang X, Rahimoon MM, Shahzad M, Oluwaseun AJ, Panhwar MI, *et al.* Fixed-time artificial insemination technology in buffaloes: a review. Front Vet Sci 2025; 12: 1586609. https://doi.org/10.3389/fvets.2025.1586609

[15] Hoque MN, Talukder AK, Akter M, Shamsuddin M. Evaluation of ovsynch protocols for timed artificial insemination in water buffaloes in Bangladesh. Turk J Vet Anim Sci 2014; 38: 418-24. https://doi.org/10.3906/vet-1302-35

[16] Berean D, Bogdan LM, Opris P, Cimpean R. Economical implications and the impact of gonadotropin-releasing hormone administration at the time of artificial insemination in cows raised in the extensive system in North Romania. Front Vet Sci 2023; 10: 1167387. https://doi.org/10.3389/fvets.2023.1167387

[17] Perera BMAO. Reproductive cycles of buffalo. Anim Reprod Sci 2011; 124: 194-9. https://doi.org/10.1016/j.anireprosci.2010.08.022

[18] Pehan EA, Miah M, Rahman MH, Shejuty SF, Haque MN, Huda MN, *et al.* A holistic review of buffalo productivity, reproductive efficiency, genetic improvement, and disease management in Bangladesh. Vet Anim Sci 2025; 29: 100496. https://doi.org/10.1016/j.vas.2025.100496

[19] Calanni Macchio A, Santinello M, Bifulco G, Matera R, Biffani S, Gomez-Carpio M, *et al.* The role of age at first calving in shaping production and reproductive outcomes in Italian buffaloes. J Dairy Sci 2025; 108: 7235-47. https://doi.org/10.3168/jds.2025-26369

[20] Otava G, Squicciarini S, Marc S, Suici T, Onan GW, Hutu I, *et al.* Effects of age and season on conception rate of Mediterranean Italian dairy buffalo (*Bubalus bubalis*) following oestrus synchronization and fixed-time artificial insemination. Reprod Domest Anim 2021; 56: 1511-8. https://doi.org/10.1111/rda.14013

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