Effects of Calf Sex Altering Oral Preparation on Pregnancy Rate and Female Calf Production in the Buffalo

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Abstract: *Purpose of the Study*: The use of calf sex-altering oral preparations (CSAOP) to increase female offspring can offer advantages in field applicability and affordability compared to other sex-manipulation techniques. The current study evaluated the effect of CSAOP (FemEXX®) on pregnancy rate and calf sex ratio in buffaloes.

Methods: Parous buffaloes (N = 240) were selected and randomly divided into four groups (60 buffaloes per group). The groups received one of the following treatments: CSAOP alone, CSAOP combined with a special feed supplement, special feed supplement alone, or no treatment (control). At estrus, all buffaloes were bred naturally, and those in the CSAOP alone and CSAOP + special feed supplement groups were orally drenched with 300 mL of FemEXX®, 30 minutes after natural mating. Logistic regression was used to evaluate the effects of treatment and other factors on pregnancy and female calf birth rates, and linear regression was applied for gestation length. Variables significant in univariable analysis were included in multivariable models, with statistical significance set at $p< 0.05$.

Results: The overall pregnancy rate was 76.62%, and no significant difference in pregnancy rate was observed among treatment groups ($p = 0.76$). However, the proportion of female calves was significantly higher in the CSAOP (78.9%) and CSAOP with special feed (77.1%) groups than in controls (42.5%), with higher odds of female calf birth (OR = 4.64, 95% CI: 1.68-12.78 and OR = 4.22, 95% CI: 1.52-11.72, respectively). Pluriparous buffaloes had higher odds of producing female calves than primiparous animals (OR = 2.18; 95% CI: 1.01-4.70). Gestation length did not differ among treatment groups but was shorter in female-bearing buffaloes ($p < 0.01$).

Conclusion: In conclusion the use of CSAOP is effective in increasing the number of female calves without affecting pregnancy rate in buffaloes.

Keywords: Buffalo, FemEXX®, Sex-altering oral preparation, Calf sex ratio, Pregnancy rate, Gestation period.

INTRODUCTION

Buffaloes are an important livestock species that contribute to nutrition, livelihoods, and employment in many Asian countries, particularly in South Asia. The global buffalo population is estimated at 205 million, with about 98% located in Asia [1]. They are major dairy animals in countries such as India, Pakistan, China, Nepal and Egypt [2]. Buffaloes are well adapted to tropical and subtropical environments and produce milk with higher fat and solids than cow milk, while showing good feed efficiency and greater resistance to parasitic, reproductive, and mammary diseases [3]. These traits have supported the expansion of buffalo farming beyond Asia to the Mediterranean region, Latin America and Europe [4]. Given the trend of increasing buffalo rearing, it is important to improve the conception rate, which we addressed in our recent study [5]. Additionally, increasing the proportion of female calves is also essential for herd growth and replacement in dairy systems. However, calf sex ratios are often equal or male-biased. For example, Nili Ravi buffaloes show a male-biased ratio of 1.36:1 [6], while male calf production in Surti and Murrah breeds ranges from 49% to 55.64% [7-9], with similar trends reported in Iran and Brazil [10, 11].

Several approaches have been investigated to manipulate offspring sex ratio, including timing of insemination, dietary and mineral supplementation, modification of vaginal or uterine pH, use of sexed semen and chemical interventions [12-15]. However, except for sexed semen, most methods have shown inconclusive results. Although sexed semen can bias calf sex, it is often associated with reduced pregnancy rates [16]. Flow cytometry-based sperm sorting also

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has major limitations, including sperm damage, reduced fertility and high costs [17, 18]. In this context, calf sex-altering oral preparations (CSAOPs), containing natural or synthetic compounds, present a novel approach to influencing the offspring sex ratio. These supplements are administered before artificial insemination or after natural breeding and are hypothesized to create uterine or systemic conditions that favor X- or Y-bearing spermatozoa [19]. FemEXX® (AnshVetlife Pvt. Ltd., Vadodara, India) is a liquid CSAOP containing active constituents such as ethanoic acid, acetic acid and monosodium ethanoate (US patent no. 7351581). It is reported to promote Y-sperm binding ligand mimics in the female plasma before conception [20], thereby increasing the likelihood of female zygote formation [21].

The use of CSAOP presents several advantages over other calf sex manipulation techniques. As an oral supplement, it is practical, affordable, and more suitable for field use than methods that require specialized equipment and trained personnel. Furthermore, it can enhance pregnancy rates and has been reported to increase the proportion of female calves [20]. However, no study to date has provided a comprehensive understanding of the relationship between CSAOP, pregnancy rate, and the sex ratio in buffaloes. Therefore, this study was conducted to evaluate the effect of CSAOP, with and without special feed supplementation, on pregnancy rate, sex ratio, and gestation length, as well as the factors associated with these parameters in buffaloes.

MATERIALS AND METHODS

Research Site and Animal Selection

The research was conducted on buffaloes from the Fattepur area of Saptakoshi Municipality, Saptari district, Nepal, during 2023-24, as shown in Figure **1**. Ethical approval for the study (Approval No. AFU-FWA00031653) was granted by the Institutional Review Board of Agriculture and Forestry University, Nepal. The buffaloes were of the riverine type and were managed under a semi-intensive housing system, with free grazing during the daytime and stall feeding at night. A total of 240 postpartum non-pregnant parous buffaloes without any reproductive disorders were selected after examination through transrectal palpation and ultrasonography. A structured questionnaire survey was conducted to collect data on nutritional and management factors that could affect the efficacy of the experiment. Only buffaloes with a Body Condition Score (BCS) of 2.25 to 3.5 (scale 1-5, 0.25 increment) [22] were selected.

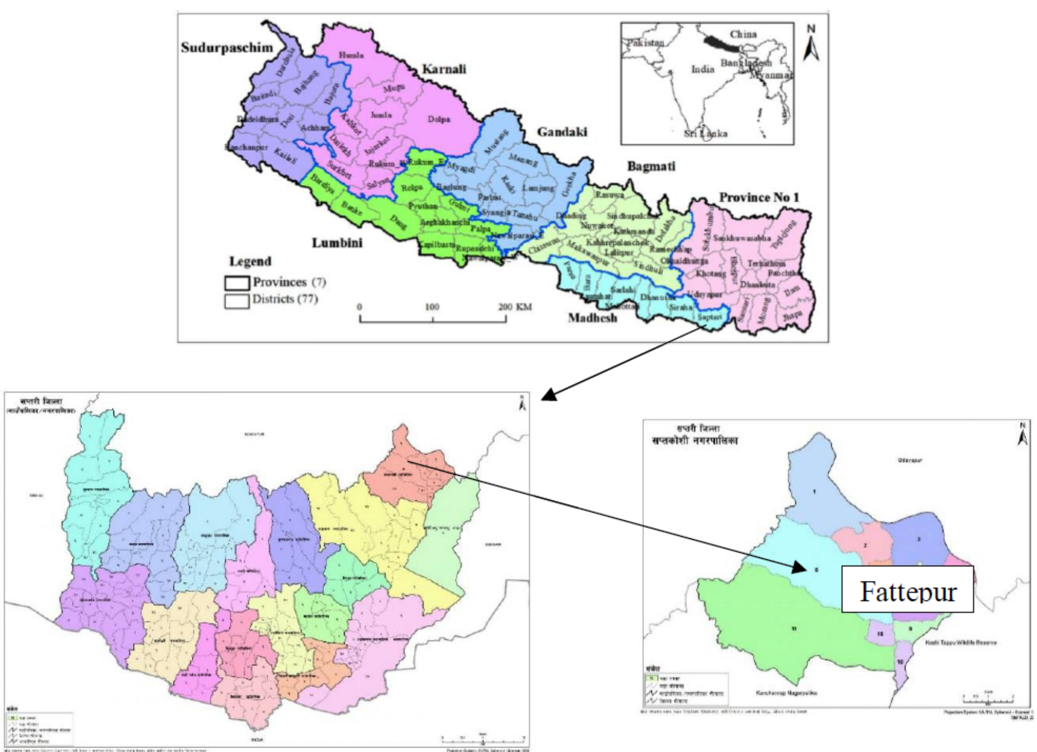

Figure 1: Map showing the location of the research site.

Experimental Design

The selected buffaloes were randomly divided into four treatment groups, with 60 buffaloes in each group, as shown in Table **1**. Randomization was performed at the individual-animal level. During the course of the study, some buffaloes were removed due to farmer-initiated culling, sale, or relocation, which was unrelated to treatment assignment. As a result, 201 buffaloes remained available for evaluation at calving and were included in the final analysis. Animals were excluded from analysis only if calving records were unavailable. In the CSAOP group, buffaloes in estrus were administered FemEXX® 300 ml orally, 30 minutes after natural breeding. It contains derivatives of polyhydric alcohols, a combination of amino acids, a buffer, and curcumin, along with edible-grade pharmaceutical aids.

In the CSAOP + special feed group, buffaloes received a daily ration of 1.5 kg commercial cattle feed (Fine Feeds Industries Pvt. Ltd., Narayangadh, Chitwan) that contains 20% protein and 2800 Kcal energy. 0.5 kg maize flour (9.5% protein, 365 Kcal/100 g), and 40 g mineral-vitamin mixture for 30 days. Likewise, the mineral-vitamin mixture comprised chelated Calcium (255g), Phosphorus (127.5g), Magnesium (6.0g), Manganese (1.5g), Iron (1.5q), Iodine (325mg), Copper (4.2g), Zinc (9.6g), Cobalt (150mg), Sulfur (7.2g), Potassium (100mg), and Sodium (6mg), Selenium (10 mg), Vitamin A (700000IU), Vitamin D3 (70000 IU), Vitamin E (250mg), Nicotinamide (1000mg), and Chromium (78mg) every 1.2 kg. Thereafter, when the buffaloes were in estrus, FemEXX® 300 ml was administered orally 30 minutes after natural breeding. In the special feed group, buffaloes received only the nutritional supplementation as described above, without FemEXX® administration following natural breeding. In the control group, buffaloes were naturally mated during estrus without any supplementation or intervention.

Estrus was detected through visual observation twice daily (morning and evening) for signs including restlessness, mounting activity, bellowing, mucous discharge, and vulvar swelling in buffaloes. Natural mating was performed within 12-24 hours of estrus detection. Similarly, before breeding, fecal samples were collected directly from the rectum and analyzed by sedimentation to detect gastrointestinal parasites, with results recorded as present or absent.

Pregnancy Diagnosis and Sex Determination of Calves

Pregnancy was confirmed within three months of natural breeding using transrectal palpation and/or ultrasonography. Buffaloes that did not conceive at the initial estrus and returned to estrus later, including those in the CSAOP, supplement-only, and control groups, were not re-treated and were considered pregnancy-negative. The sex of the calves was determined at birth through visual inspection of the external reproductive organs.

Statistical Analysis

Data were entered in Microsoft Excel 2016 (version 2307; Microsoft Corp., Redmond, WA, USA) and analysed using SPSS version 26 (IBM Corp., Armonk, NY, USA). Pregnancy and female calf birth rates were analysed using binary logistic regression. Each variable (treatment group, parity, BCS, and endoparasitic infestation) was first assessed using univariable analysis, and those that were significant were included in the multivariable model. If there was no significant factor or only one, multivariable analysis was not performed. Interaction effects among significant variables were tested and included in the final model only if statistically significant. Multicollinearity among predictors was examined using the variance inflation factor (VIF), with VIF > 5 indicating collinearity, and the final model was refined accordingly. Associations were expressed as odds ratios (OR) with 95% confidence intervals (CI). Significance was set at $p < 0.05$.

Table 1: Experimental Design of the Study

Treatment groups	No. of buffaloes selected initially	No. of buffaloes presented for pregnancy diagnosis
CSAOP	60	53
CSAOP and Special feed	60	47
Special feed	60	53
Control	60	48
Total	240	201

Gestation length was analyzed using linear regression, including treatment group, parity, BCS, endoparasitic infestation, and calf sex as covariates. Results are presented as mean ± standard error (SE) for continuous data and percentages for categorical data.

RESULTS

Effect of CSAOP and Other Factors on Pregnancy Rate

The effects of CSAOP and other factors on pregnancy rate are shown in Table **2**. Out of the total 240 buffaloes selected initially, only 201 buffaloes were presented for pregnancy diagnosis, and they remained until calving. The remaining 39 were either culled or sold by farmers. The overall pregnancy rate was 76.6% (154/201). Pregnancy rates were 71.7% for CSAOP, 74.5% for CSAOP with special feed, 77.4% for special feed alone, and 83.3% in the control group. Logistic regression analysis showed that CSAOP supplementation, either alone or in combination with special feed, did not significantly influence pregnancy rate in buffaloes. Similarly, none of the other factors were significantly associated with pregnancy outcomes.

Effect of CSAOP and Other Factors on Female Calf Birth Rate

Univariable logistic regression indicated that both treatment group and parity significantly influenced the proportion of female calf births (Table **3**). Compared with the control group (42.5%), CSAOP (78.9%; OR = 5.07, 95% CI: 1.87-13.80; p = 0.001) and CSAOP with special feed (77.1%; OR = 4.57, 95% CI: 1.68-12.51; p = 0.003) significantly increased the likelihood of female calf births. Special feed alone showed no effect (p = 0.925). Pluriparous buffaloes produced a higher proportion of female calves than primiparous (65.7% vs. 41.9%; OR = 2.67, 95% CI: 1.30-5.49; p = 0.008). Body condition score and endoparasitic infestation were not associated with calf sex ratio. In the multivariable logistic regression model (Table **4**), both the treatment group and parity remained significant predictors of female calf birth rate. The buffaloes supplemented with CSAOP (OR = 4.64, 95% CI: 1.68-12.78; p = 0.003) and CSAOP with special feed (OR = 4.22, 95% CI: 1.52-11.72; p = 0.006) had significantly increased odds of female calf births compared with controls. Pluriparous buffaloes remained more likely to deliver female calves than primiparous (OR = 2.18, 95% CI: 1.01-4.70; p = 0.047). The interaction between treatment group and parity was not significant (p = 0.235), so only the main effects were considered in the final multivariable model.

Effect of CSAOP and Other Factors on Gestation Length

The effects of CSAOP and other factors on gestation length are shown in Table **5**. The overall gestation length was 307.4 ± 4.8 days. The CSAOP supplementation, either alone or in combination with special feed, had no significant effect on gestation

Table 2: Univariable Logistic Regression Analysis for Predicting Factors Affecting Pregnancy Rate

Factors	Level	No. of pregnancy diagnosed buffaloes	No. of pregnant buffaloes	Pregnancy rate (%)	Odds ratio	95% CI		p-value
						Upper	Lower	
Treatment groups	CSAOP	53	40	71.7	Ref.			
	CSAOP & Special feed	47	38	74.5	1.15	0.47	2.80	0.756
	Special feed	53	35	77.4	1.35	0.56	3.25	0.504
	Control	48	41	83.3	1.97	0.75	5.19	0.168
Parity	Primiparous	62	43	69.4	Ref.			
	Pluriparous	139	111	79.9	1.75	0.89	3.46	0.107
BCS	>2.75	63	111	71.4	Ref.			
	≤2.5	138	43	79.0	1.46	0.73	2.92	0.281
Endo parasitic infestation	Positive	96	73	76.0	Ref.			
	Negative	105	81	77.1	1.06	0.55	2.04	0.854

Table 3: Univariable Logistic Regression Analysis for Predicting Factors Affecting Female Calf Birth Rate

Factors	Level	No. of pregnant buffaloes	Female calf (%)	Odds ratio	95% CI		p-value
Treatment groups	Control	40	42.5	Ref.			
	CSAOP	38	78.9	5.07	1.87	13.80	0.001
	CSAOP & Special feed	35	77.1	4.57	1.68	12.51	0.003
	Special feed	41	41.5	0.96	0.40	2.32	0.925
Parity	Primiparous	43	41.9	Ref.			
	Pluriparous	111	65.7	2.67	1.30	5.49	0.008
BCS	≤2.5	111	56.8	Ref.			
	>2.75	43	65.1	1.42	0.69	2.95	0.345
Endo parasitic infestation	Positive	73	58.9	Ref.			
	Negative	81	59.3	1.02	0.53	1.93	0.964

Table 4: Multivariable Logistic Regression Analysis for Predicting Factors Affecting Female Calf Birth Rate

Factors	Level	No. of pregnant buffaloes	Female calf (%)	Odds ratio	95% CI		p-value
					Lower	Upper	
Treatment groups	Control	40	42.5	Ref.			
	CSAOP	38	78.9	4.64	1.68	12.78	0.003
	CSAOP & Special feed	35	77.1	4.22	1.52	11.72	0.006
	Special feed	41	41.5	0.97	0.40	2.38	0.945
Parity	Primiparous	43	41.9	Ref.			
	Pluriparous	111	65.7	2.18	1.01	4.70	0.047

length in buffaloes. Among other factors, sex of the calf had a significant effect, with buffaloes giving birth to female calves exhibiting a shorter gestation period (306.2 ± 4.5 days) compared with those bearing male calves (309.1 ± 4.6 days; Estimate = −2.96 days, 95% CI: −4.43 to −1.49; p <0.001). However, parity, body condition score, and end oparasitic infestation status did not significantly affect gestation length.

DISCUSSION

Researchers have long tested various methods such as altering insemination time, supplementing minerals, adjusting vaginal and uterine pH, using sex-sorted semen, and applying chemicals to improve pregnancy rates and influence sex ratio in livestock. Except for sex-sorted semen, most approaches have shown inconsistent results, while existing technologies are costly and require specialised training. Therefore, affordable and reliable alternatives are needed. This study was thus aimed to evaluate the effect of CSAOP on pregnancy rate and sex ratio in buffalo.

In this study, no significant difference in pregnancy rate was observed among treatment groups, indicating that CSAOP supplementation did not affect conception rate. Although improved pregnancy rates following CSAOP administration in dairy cows were reported in India [23], our findings demonstrated no increase in pregnancy rate with its supplementation. This is the first study to evaluate CSAOP supplementation in buffaloes, and the comparatively lower pregnancy rate observed, relative to cattle, may be attributed to species differences in reproductive physiology, ovarian activity, and metabolic responsiveness. Similarly, none of the other factors, including parity, body condition score, or endoparasitic infestation, had a significant influence on estrus induction and pregnancy outcomes. Though BCS is considered a predictor of energy

Table 5: Univariable Linear Regression Analysis for Predicting Factors Affecting Gestation Length

Factors	Level	No. of pregnant buffaloes	Gestation length (Mean days±SE)	Estimate	95% CI		p-value
					Lower	Upper	
Treatment groups	Control	40	307.2±4.8	Ref.			
	CSAOP	38	306.4±4.6	-0.73	-2.86	1.40	0.500
	CSAOP & Special feed	35	307.9±4.8	0.79	-1.38	2.97	0.472
	Special feed	41	308.1±4.3	0.92	-1.17	3.01	0.384
Parity	Primiparous	43	307.6±4.4	Ref.			
	Pluriparous	111	307.3±4.9	-0.26	-1.95	1.44	0.77
BCS	≤2.5	111	307.2±4.9	Ref.			
	>2.75	43	307.8±4.4	0.61	-1.08	2.30	0.475
Endo parasitic infestation	Negative	81	307.5±4.9	Ref.			
	Positive	73	307.3±4.6	-0.13	-1.65	1.39	0.868
Sex of calf	Male	63	309.1±4.6	Ref.			
	Female	91	306.2±4.5	-2.96	-4.43	-1.49	<0.001

balance and has been shown to influence pregnancy rates [24], no such effect was observed in this study, likely because the buffaloes were grazed, maintaining a basal nutritional level, as further supported by the finding that feed supplementation did not affect pregnancy rate. Apart from this, consistent with our findings, none of the other factors had a significant effect on pregnancy outcomes in field-managed buffaloes [25, 26].

Previous studies have shown that vitamin-mineral and energy supplementation can enhance reproductive efficiency, particularly in herds facing nutritional stress or deficiency [27 - 30]. In this study, buffaloes were managed under a semi-intensive system, with free grazing during the day and stall feeding at night, ensuring a balanced intake of roughage and concentrate. Such management likely maintained adequate metabolic status, minimizing any additional benefit from supplementation. Moreover, buffaloes have lower metabolic rates and longer postpartum anestrus than cattle [31, 32], which may limit short-term reproductive responses to dietary interventions. Thus, the lack of improvement in pregnancy rate suggests that CSAOP, with or without nutritional supplementation, may not confer a further reproductive advantage when basal nutritional needs are already met. Future studies in nutritionally challenged herds could clarify whether its benefits emerge under conditions of deficiency or stress.

The female calf birth rate in buffaloes supplemented with CSAOP was significantly higher than that of CSAOP non-supplemented buffaloes in this study. Findings of this research are in close agreement with observations from a previous study [33]. Studies reported significantly higher female calf birth rates, i.e., 86.5%, 80%, and 82.03%, respectively, which are higher than the findings of this research [23, 34, 35]. The buffaloes supplemented with CSAOP are likely to alter the uterine environment, including the energy substrates present and their pH, potentially favouring the association of Y spermatozoa with ligands. The study in hamsters revealed that when the pH of the uterus was less acidic, the number of male offspring was lower, whereas in the later phases of the reproductive cycle, the pH of the vagina and uterus was very acidic, and therefore, a larger number of male offspring were born [27]. The change in pH of the uterus can be attributed to the diets provided to the cattle, while the pH of the vagina can be attributed to the hormonal condition of the cows [36]. Alterations in hormonal status can alter the secondary sex ratio at conception [37, 38]. However, as the present study did not directly assess physiological or biochemical mechanisms, actual pathways cannot be confirmed and need further study.

Similarly, parity was also associated with female calf birth, with pluriparous buffaloes producing a higher proportion of female offspring than primiparous buffaloes. The association of parity with female calf birth rate is inconsistent. In a study, an increase in the proportion of female buffalo calves was observed with increasing parity [39]. However, another study reported an increase in male calves with increasing parity of dams [40]. Therefore, further studies are needed to draw a definitive conclusion on this relationship. Except for parity, none of the other factors showed a significant association with female calf birth rate.

The average gestation length observed in this study was consistent with established reports in buffaloes [41] and was not influenced by CSAOP supplementation. The gestation length was significantly longer in buffaloes with male calves as compared to buffaloes with female calves in this study. Similar findings were reported in buffaloes and cattle, where male fetuses prolong gestation by 1-2 days [42-44]. Male calves generally have a higher birthweight than females, which requires a relatively longer intrauterine development period [45]. Since gestation length is positively correlated with calf birthweight, the increased fetal mass in males is a likely contributor to delayed parturition [42]. Moreover, placental development is sexually dimorphic: male conceptuses tend to allocate more resources to growth, with altered placental gene expression and nutrient transport capacity compared to females [46]. Fetal adrenal and gonadal hormones play a role in initiating parturition. Male fetuses exhibit higher androgen production, which can influence placental and maternal vascular signalling, potentially modulating the timing of parturition [47]. In addition, maternal adaptations to carry heavier male fetuses may delay the cascade of hormonal signals required for calving. Except for the sex of the calf, none of the other factors showed a significant association with gestation length.

CONCLUSION

Supplementation of CSAOP effectively increased the likelihood of female calf births without compromising pregnancy rate or altering gestation length in buffaloes. The additional provision of special feed did not further enhance the effect of CSAOP, indicating that CSAOP alone is sufficient to promote female-biased offspring. Moreover, parity influenced the calf sex ratio, with pluriparous buffaloes more likely to produce female calves. These findings suggest that CSAOP offers a promising, practical alternative for enhancing female calf production in dairy buffalo farming, potentially improving herd productivity and long-term economic returns. Future studies involving larger, controlled trials across diverse farm conditions and under nutritional and management challenges, including artificial insemination practices, are warranted to further validate these findings.

AVAILABILITY OF DATA AND MATERIALS

Data and materials for this research are available from the corresponding author upon request.

CONFLICT OF INTEREST

The authors declare no personal or financial conflicts of interest associated with this study.

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