

Comparative efficacy of oestrus synchronization protocols in buffalo (*Bubalus bubalis*)

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Abstract This study was conducted to test the efficacy of gonadotropic hormone (GnRH)-based synchronization protocols (Ovsynch, Heatsynch, and Ovsynch Plus) in buffaloes under field condition. Based on anamnesis and transrectal palpation twice at 10-day interval and serum progesterone (P₄) concentration, 150 anoestrous buffaloes and delayed pubertal heifers were selected to induce oestrus using GnRH-based protocols. These selected animals were randomly divided into three groups: group I: Ovsynch ($n = 50$), group II: Heatsynch ($n = 50$), and group III: Ovsynch Plus ($n = 50$) regimen. Before treatment initiation, blood samples were collected for P₄, beta-hydroxy butyric acid (β -OHB), and mineral estimation, in addition to the monitoring of oestrus signs. In this investigation, no significant difference ($P > 0.05$) in oestrus signs was deduced among three groups. Oestrus induction rate (OIR) was comparable ($P > 0.05$) among the groups (Ovsynch 82%, Heatsynch 86%, and Ovsynch Plus 88%). Conception rate (CR) following fixed time artificial insemination (FTAI) was slightly higher with Ovsynch Plus group (28%) as compared to Ovsynch (24%) and Heatsynch (18%) groups, though non-significant. Furthermore, serum glucose, β -OHB, macrominerals (calcium, potassium, and magnesium), and trace minerals (copper, zinc, and iron) remained comparable ($P > 0.05$) among the groups. In conclusion, all the protocols

(Ovsynch, Heatsynch, and Ovsynch Plus) are efficient in oestrus induction in anoestrous buffaloes under field condition with Ovsynch Plus protocol resulting in higher CR as compared to other protocols.

Keywords Buffalo · Oestrus · Synchronization · Anoestrus

Introduction

The major constraint to full exploitation of the productive potential of buffalo is its inherent low reproductive efficiency due to delayed puberty and sexual maturity, seasonality of breeding, prolonged postpartum anoestrus, silent oestrus, and postpartum uterine disorders (Das and Khan 2010). In India, incidence of anoestrus has been reported between 9.18 and 82.50% (Thakor and Patel 2013), whereas suboestrus varies from 3 to 73% in postpartum buffaloes (Kumar et al. 2013). Under field conditions, negative energy balance and aberrant mineral profile leads to delayed postpartum restoration of luteinizing hormone (LH) pulsatility, resulting into prolonged postpartum anoestrus (Kumar et al. 2010).

Use of gonadotrophic hormone (GnRH)-based hormone protocols for oestrus synchronization in cyclic animals has been tried in cattle and buffalo (Pursley et al. 1995; Paul and Prakash 2005). These protocols initiate ovarian cyclicity through endogenous production of progesterone (P₄) by inducing corpus luteum (CL) formation by the ovulation of dominant follicle. Ovsynch protocol has been used effectively in cyclic buffaloes with conception rate (CR) ranging from 33 to 64% after fixed time artificial insemination (FTAI) (Paul and Prakash 2005). However, CR following Ovsynch in acyclic buffaloes varied from 0 to 50% (Mohd et al. 2013). At the time of first GnRH injection, presence of large follicle diameter >8.5 – 9 mm has been reported to be a prerequisite for the

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success of Ovsynch program in anoestrous buffaloes (Karuppanasamy et al. 2017).

Hence, in an attempt to ensure similar ovarian follicular picture of all anoestrous buffaloes at the time of first GnRH injection, a new protocol “Ovsynch Plus” has been developed (Sharma et al. 2004). Another protocol viz. Heatsynch is similar to Ovsynch, except for the replacement of GnRH on day 9 by estrogen on day 8, resulting in 100% oestrus response (Ali et al. 2012). Therefore, the present study was designed to test the efficacy of these synchronization protocols (Ovsynch, Heatsynch, and Ovsynch Plus) in buffaloes under field conditions.

Materials and methods

Study location

The present study was conducted at Haryana state of India when the humidity was 75–85% and the ambient temperature ranges between 25 and 39 °C during summer and 1.1–15 °C during winter. The average annual rainfall is 459 mm and climate sub-type according Köppen climate classification is hot semi-arid climate (BSh).

Selection of animals and their management system

The study comprised of 150 anoestrous buffaloes (1–2 parity) and heifers aged between 4 and 5 years, with body condition score <3 maintained under field conditions. Anoestrous buffaloes averaged 173.80 ± 9.20 days (range 93–720 days) postpartum and delayed pubertal heifers averaged 42.32 ± 1.02 months (range 36.5–60 month) age at the beginning of the hormonal treatment. Anoestrus was diagnosed in the animals on the basis of anamnesis and conducting gynaeco-clinical examinations twice at 10-day interval. Further, the cyclicity confirmed either by transrectal ultrasonography or P₄ estimation at the start of treatment. The selected animals ($n = 150$) were randomly divided into three groups and treated with hormonal regimen as group I (Ovsynch; $n = 50$), II (Heatsynch; $n = 50$), and III (Ovsynch Plus; $n = 50$). Day of initiation of treatment was considered as day 0.

Blood sampling

Blood sample was collected by jugular venipuncture from each buffalo, in a serum activator polystyrene tube on day 0 of treatment in each group. All aseptic precautions were taken prior to collection. Blood glucose was estimated immediately after collection of blood samples. Blood samples were chilled on ice, transported to the laboratory, and centrifuged at 3000 rpm for 15 min to separate the serum. Serum was kept in sterilized vials and stored in a deep freezer at –20 °C until estimation of serum P₄, β-OHB, and mineral profile.

Hormone treatment protocols

Ovsynch regimen

In this protocol, 10 µg (2.5 ml) of GnRH analogue i.m. (Buserelin-Acetate, Receptal®, Intervet International GmbH, Germany) was administered on day 0 followed by an i.m. injection of 25-mg prostaglandin F_{2α} (PGF_{2α}, dinoprost tromethamine, Lutalyse™, Pfizer Animal Health, Belgium) on day 7. A second injection of 10 µg of GnRH was administered on day 9. Then two FTAs were carried out on day 9 and 10 of the treatment.

Heatsynch regimen

Under this treatment, buffaloes were administered an i.m. injection of 10 µg of GnRH on day 0 and PGF_{2α} on day 7. An injection of 1 mg of β-estradiol i.m. (Sigma Aldrich, USA) was administered on day 8. Then two FTAs were carried out on days 10 and 11 of the experiment.

Ovsynch Plus regimen

Buffaloes were administered pregnant mare serum gonadotropin (PMSG) 400 IU (Folligon®, Intervet) followed by GnRH 10 µg (i.m.) on day 3. An injection of PGF_{2α} was administered on day 10 followed by 10 µg GnRH 48 h later (on day 12). FTAI will be done on days 12 and 13 of treatment.

Oestrus induction, FTAI, and pregnancy diagnosis

Oestrus was assessed by behavioral signs, viz. excitement, bellowing, frequent urination, swollen vulva, chin resting on other animal, tail-raising, and mucus discharge. Ease of cervical passage during artificial insemination was also considered to judge the quality of oestrus. Oestrus induction rate (OIR) was calculated following induced oestrus and within 1 month of post-treatment. FTAI was performed in buffaloes using frozen/thawed semen from bulls of known fertility as per standard protocol. Pregnancy diagnosis was performed 60 days postartificial insemination (AI) in animals through per-rectal palpation. CR was deduced during FTAI and subsequent two AIs following spontaneous oestrus.

Hormone, metabolite, and mineral estimation

Serum P₄ was estimated in serum samples by liquid-phase radioimmunoassay (RIA) procedure using Iodine-125-based kits (code: RIAK-15V; Board of Radiation and Isotope Technology, BRIT, Navi Mumbai, India). Blood glucose estimation was done by using glucometer (ACCU-CHEK® Active). Beta-hydroxy butyric acid concentration was estimated by enzyme-linked immunosorbent assay kit (Sincere

Table 1 Serum progesterone (mean \pm SE) and diameter of largest follicle (mean \pm SE) on day 0 in anoestrous buffaloes treated with Ovsynch, Heatsynch, and Ovsynch Plus protocols

Groups	P ₄ (ng/ml)	Largest follicle diameter (mm)
Ovsynch	0.59 \pm 0.12	9.62 \pm 0.55 (40)
Heatsynch	0.45 \pm 0.09	9.71 \pm 0.69 (15)
Ovsynch Plus	0.46 \pm 0.10	8.50 \pm 0.48 (27)
Overall	0.50 \pm 0.06	9.27 \pm 0.33 (82)

Figures in parentheses indicate number of observations

Biotech Co., Ltd., Beijing, China) as per instructions. Sensitivity of kit was <1.0 ng/ml. Calcium, magnesium, potassium, zinc, copper, and iron contents in serum samples were estimated by Atomic Absorption Spectrophotometer (Electronics Corporation of India Ltd. (ECIL), Hyderabad, India, Model No. 4141).

Statistical analyses

Data was analyzed for using Chi-square test and one-way ANOVA with statistical software package (SPSS, Version 16). Results were considered significant at $P < 0.05$.

Results

Follicular profile

Transrectal ultrasound scanning was performed in 82 treated buffaloes (Ovsynch group (40); Heatsynch (15); Ovsynch Plus group (27)) on day 0 of treatment. Mean diameter (mm) of the largest follicle on day 0 in groups I, II, and III was 9.62 \pm 0.55, 9.71 \pm 0.69, and 8.50 \pm 0.48, respectively (Table 1). No visible CL was found in anoestrous buffaloes treated with different hormonal regimens.

Hormonal profile

Mean P₄ concentration (ng/ml) was 0.59 \pm 0.12, 0.45 \pm 0.09, and 0.46 \pm 0.10 in Ovsynch, Heatsynch, and Ovsynch Plus

Table 2 Oestrus induction response during induced oestrus by anoestrous buffaloes treated by Ovsynch, Heatsynch, and Ovsynch Plus protocols

Oestrus response	No. of responders			χ^2 and P value
	Ovsynch	Heatsynch	Ovsynch Plus	
Behavioral signs	29/50 (58)	34/50 (68)	26/50 (52)	2.71, $P > 0.05$
Mucous discharge	35/50 (70)	38/50 (76)	37/50 (74)	0.48, $P > 0.05$
Overall animals exhibited oestrus response	37/50 (74)	40/50 (80)	38/50 (76)	0.52, $P > 0.05$

Figures in parentheses indicate percentage

Table 3 Easy AI gun passage during FTAI in anoestrous buffaloes treated by Ovsynch, Heatsynch, or Ovsynch Plus regimens

Group	Easy AI gun passage (%)	χ^2 value	P value
Ovsynch	70 (35/50)	0.81	$P > 0.05$
Heatsynch	72 (36/50)		
Ovsynch Plus	64 (32/50)		

groups, respectively. There was no significant different ($P > 0.05$) in mean serum P₄ concentration among three groups (Table 1).

Oestrus signs

Oestrus was assessed by behavioral signs, viz. excitement, bellowing, frequent urination, swollen vulva, and vaginal discharge as observed by buffalo owner and results remained comparable between the three groups (Table 2).

Ease of AI gun passage during FTAI

Non-significant ($P > 0.05$) difference was found between the three groups with respect to ease of AI gun passage during FTAI (group I 70% (35/50); group II 72% (36/50); group III 64% (32/50)) (Table 3).

Oestrus induction rate

OIR in groups I, II, and III was 74 (37/50), 80 (40/50), and 76% (38/50), respectively. There was no significant difference ($P > 0.05$) in OIR during induced oestrus among three protocols. In anoestrous buffaloes, OIR remained comparable between the three groups (Ovsynch 71.79% (28/39); Heatsynch 80% (32/40); Ovsynch Plus 78.05% (32/41)). In heifers, 81.82 (9/11), 80 (8/10), and 66.67% (6/9) exhibited oestrus signs following Ovsynch, Heatsynch, and Ovsynch Plus treatments, respectively ($P > 0.05$). It was also observed that buffaloes and heifers returning to oestrus after 1 month posttreatment remained comparable between the three groups. Overall, OIR following treatment was 82 (41/50), 86 (43/50), and 88% (44/50) in Ovsynch,

Table 4 Oestrus induction and fertility response in anoestrous buffaloes treated with Ovsynch, Heatsynch, and Ovsynch Plus protocols

Parameter	Ovsynch	Heatsynch	Ovsynch Plus	χ^2 and P value
Oestrus induction rate (%)				
(a) During induced oestrus	74 (37/50)	80 (40/50)	76 (38/50)	0.52, $P > 0.05$
Buffaloes	71.79 (28/39)	80 (32/40)	78.05 (32/41)	0.81, $P > 0.05$
Heifers	81.82 (9/11)	80 (8/10)	66.67 (6/9)	0.73, $P > 0.05$
(b) Within 1 month posttreatment	30.77 (4/13)	30 (3/10)	50 (6/12)	1.29, $P > 0.05$
Buffaloes	36.36 (4/11)	25 (2/8)	55.56 (5/9)	1.72, $P > 0.05$
Heifers	0 (0/2)	50 (1/2)	33.33 (1/3)	1.28, $P > 0.05$
(c) Overall	82 (41/50)	86 (43/50)	88 (44/50)	0.75, $P > 0.05$
Buffaloes	82.05 (32/39)	85 (34/40)	90.24 (37/41)	1.138, $P > 0.05$
Heifers	81.82 (9/11)	90 (9/10)	77.78 (7/9)	0.54, $P > 0.05$
Conception rate (%) at FTAI				
Overall	24 (12/50)	18 (9/50)	28 (14/50)	1.42, $P > 0.05$
Buffaloes	20.51 (8/39)	20 (8/40)	29.27 (12/41)	1.22, $P > 0.05$
Heifers	36.36 (4/11)	10 (1/10)	22.22 (2/9)	2.044, $P > 0.05$

FTAI fixed time artificial insemination

Heatsynch, and Ovsynch Plus treatment, respectively ($P > 0.05$) among the groups (Table 4).

Conception rate

Overall, CR following FTAI was comparable between the three groups (Ovsynch 24% (12/50); Heatsynch 18% (9/50); Ovsynch Plus 28% (14/50)). In buffaloes, CR following FTAI was 20.51 (8/39), 20 (8/40), and 29.27% (12/41) in Ovsynch, Heatsynch, and Ovsynch Plus treatment, respectively ($P > 0.05$). In heifers, CR remained comparable ($P > 0.05$) between the three groups (Ovsynch 36.36% (4/11); Heatsynch 10% (1/10); Ovsynch Plus 22.22% (2/9)).

Serum glucose and beta-hydroxy butyrate (β -OHB) profile

Mean blood glucose and β -OHB level of the study animals on day 0 of treatment was 66.39 ± 0.92 mg/dl and 0.66 ± 0.04 mmol L⁻¹ with no significant difference ($P > 0.05$) among three groups (Table 5).

Table 5 Blood glucose and serum β -OHB (mean \pm SE) of anoestrous buffaloes treated with Ovsynch, Heatsynch, and Ovsynch Plus protocols

Group	Blood glucose (mg/dl)	β -OHB (mmol L ⁻¹)
Ovsynch	63.90 \pm 1.96	0.62 \pm 0.05
Heatsynch	67.06 \pm 1.58	0.66 \pm 0.06
Ovsynch Plus	68.22 \pm 1.01	0.69 \pm 0.08
Overall	66.39 \pm 0.92	0.66 \pm 0.04

Mineral profile

Mean level of macrominerals calcium, magnesium, and potassium was 11.04 ± 0.23 , 2.77 ± 0.15 , and 23.66 ± 0.49 mg/dl, respectively. Non-significance difference ($P > 0.05$) in peripheral levels of magnesium and potassium among three groups was observed, but a significant difference ($P < 0.05$) was in serum calcium level found between the three groups. Likewise, serum zinc, iron, and copper concentration were 1.34 ± 0.10 , 3.40 ± 0.10 , and 75.77 ± 1.53 μ g/ml, respectively, without significant difference ($P > 0.05$) among three groups (Table 6). It is evident that all study animals had normal metabolic and mineral profile at the start of experiment.

Discussion

Behavioural oestrus signs, oestrus discharge of secretions, and ease of passing AI gun observed in buffaloes treated with three protocols remained comparable. Yotov et al. (2012) reported similar findings in postpartum buffaloes treated with Ovsynch regimen. In this study, OIR of 74% using Ovsynch protocol was higher than Gupta et al. (2015) in postpartum buffaloes. Moreover, OIR during FTAI in Heatsynch regimen was found to be 80% in our study, which was discordant with Borhaniya et al. (2012) and Ali et al. (2012) in buffaloes. Lower response might be due to overlook of oestrus signs by farmers, individual variation, seasonal effect, and hormonal preparation used. Furthermore, OIR was slightly higher (78.05%) in anoestrous buffaloes as compared to those in heifers (66.67%), but it was comparable between buffaloes and heifers in other protocols. The lower response of these protocols in this study might be due to the fact that these GnRH-based protocols were primarily designed for oestrus synchronization in cyclic animals. Ovsynch

Table 6 Mineral profiles (mean \pm SE) of anoestrous buffaloes treated with Ovsynch, Heatsynch, and Ovsynch Plus protocol

Mineral	Ovsynch	Heatsynch	Ovsynch Plus	Overall
Calcium (mg/dl)	11.79 ^b \pm 0.32	11.67 ^b \pm 0.30	9.66 ^a \pm 0.48	11.04 \pm 0.23
Magnesium (mg/dl)	2.95 \pm 0.19	2.49 \pm 0.18	2.87 \pm 0.38	2.77 \pm 0.15
Potassium (mg/dl)	23.26 \pm 0.81	22.93 \pm 0.80	24.78 \pm 0.96	23.66 \pm 0.49
Zinc (ppm)	1.61 \pm 0.27	1.26 \pm 0.10	1.15 \pm 0.08	1.34 \pm 0.10
Iron (ppm)	3.59 \pm 0.22	3.33 \pm 0.16	3.29 \pm 0.14	3.40 \pm 0.10
Copper (μ g/dl)	78.86 \pm 2.74	76.03 \pm 2.62	72.42 \pm 2.55	75.77 \pm 1.53

^{a,b} Means with different superscripts in a row differ significantly ($P < 0.05$)

protocol has been developed for synchronizing oestrus in cyclic animals, as several studies scrutinize the effect of Ovsynch protocol in anoestrous buffaloes. The size and functionality of dominant follicle are important factors at the time of GnRH treatment, which determine ovulation in cow and buffalo. The dominant follicle should possess a diameter of >8.5 – 9 mm for GnRH action in Ovsynch protocol in anoestrous buffaloes (Karuppanasamy et al. 2017). Thus, prior to GnRH treatment, ovarian follicular size needs to be assessed by ultrasonography in order to achieve optimal ovulatory response following Ovsynch protocol in anoestrous buffaloes. Since, accurate follicular size assessment is difficult under field conditions with routine per-rectal palpation; enhancement of follicular growth prior to exogenous administration of GnRH is an important determinant to achieve an optimum fertility in Ovsynch protocol. Administration of PMSG 2–3 days prior to Ovsynch protocol enhances the dominant follicle size and ensuring the presence of one dominant follicle at the time of first GnRH injection (Sharma et al. 2004). These results indicate that Ovsynch Plus protocol is preferred choice as compared to Ovsynch and Heatsynch protocols for inducing oestrus in anoestrous buffaloes.

Overall, CR following FTAI in Ovsynch, Heatsynch, and Ovsynch Plus protocols was 24 (12/50), 18 (9/50), and 28% (14/50), respectively. CR following Ovsynch protocol was higher than acyclic cows (9%) and buffaloes (0–7%) following Ovsynch treatment (Azawi et al. 2012). However, higher rates (33 to 50%) following Ovsynch in anoestrous buffaloes have been reported (Mohd et al. 2013). Ovsynch protocol has been used effectively in cyclic buffaloes with CR ranging from 33 to 64% after TAI (Paul and Prakash 2005). In addition, CR of Heatsynch protocol was in contrast with study of Ali et al. (2012) and Mimmahmoudi et al. (2013) in anoestrous buffaloes (50–64%). But, our findings were comparable with Borhaniya et al. (2012) in cattle (16%). It was expected that buffaloes following estradiol-17 β administration in Heatsynch group would respond to its positive feedback effect. It is possible that first GnRH in Heatsynch protocol may not be able to induce ovulation and form CL leading to absence of P₄ priming and poor oestrus induction. CR using Ovsynch Plus protocol was 28%, which is agreed with the findings of Sharma et al. (2004) in postpartum anoestrous buffaloes (CR 28.6%) during non-breeding season. Overall, OIR in these three protocols ranged

between 74 and 80%, and CR was slightly higher with Ovsynch Plus protocol (28%) as compared to Ovsynch (24%) and Heatsynch (18%) protocols. This can be supported by the fact that for successful conception at FTAI, treated anoestrous buffalo must respond to both GnRHs which is evident in Ovsynch Plus protocol (Sharma et al. 2017). In addition, the role of season on the outcome of these three protocols should not be underplayed and needs to be investigated in future.

With respect to peripheral mineral and metabolic profile, serum calcium was similar to Abd Ellah et al. (2014) in buffaloes, but higher than Jayachandran et al. (2013). This disparity in serum calcium needs to be investigated further. Likewise, comparable serum magnesium among groups was reported by Abd Ellah et al. (2014) in buffalo. Further, peripheral potassium was in accordance with Chaurasia et al. (2010) in buffaloes. Mean copper concentration was within normal range among the three groups as reported by Akhtar et al. (2009). Also, peripheral zinc concentration showed no significant difference among three treatment groups which was concurrent with Jayachandran et al. (2013) in anoestrous buffaloes. Moreover, peripheral glucose and β -OHB showed no significant difference among three treatment groups being in agreement with Monteiro et al. (2012) in buffaloes. It is evident that blood metabolites including macro- and microminerals play an important role in fertility of buffalo (Khan et al. 2014; Kalasariya et al. 2016), but the exact mechanism of action and their interaction needs to be further investigated.

In summary, this study reports that GnRH-based protocols, viz. Ovsynch, Heatsynch, and Ovsynch Plus show comparable oestrus induction and CR in buffaloes under field conditions. Furthermore, the mineral and metabolic profile of study animals was within normal physiological range.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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