Kafrelsheikh Veterinary Medical Journal
(https://kvmj.journals.ekb.eg/)

Original Article

Fertility response of anestrous buffaloes to eCG2-Modified or Norgestomat plus eCG6- Modified-GnRH-based ovsync protocols during the breeding season

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Abstract

Objective: The aim of the current study was to evaluate the fertility response of anestrous buffaloes and associated follicular growth and hormonal profiles to eCG-Modified (eCG2-ovsync) or eCG plus Norgestomet ear implant-Modified (Norgestomet-eCG6-ovsync) GnRH-Based ovsync protocols.

Methods: Buffaloes (n=42) were randomly allocated into three treatment protocols: Basic-ovsync, Norgestomet-eCG6-ovsync and eCG2-ovsync (14 each). Each buffalo-cow in the Basic-ovsync protocol received two GnRH agonist injections on Days 0 (GnRHa1) and Day 9 (GnRHa2) and an injection of $PGF_{2\alpha}$ on Day 7 with TAI at 16 h after GnRHa2 injection. Buffaloes in Norgestomet eCG6-ovsync (n=14) received the same treatment as Basic –ovsync plus IM injection of 250 mg of long-lasting progesterone and S/C insertion of Norgestomet ear implant in the ear Conca on Day 0 and an injection of 500 IU eCG on Day 6. Buffaloes in eCG2-ovsync received the same treatment of Basic ovsync in addition to an injection of 1000 IU eCG on Day 2. Simultaneous transrectal ultrasonography (TUS) and blood sampling were performed on Days -10, 0, 7, 9 and 10 as well as on post-TAI Days 11 and 22.

Results: The largest follicle diameter (LFD) was significantly larger (p<0.05) in eventually diagnosed pregnant (EDP) than in eventually diagnosed non-pregnant (EDnP) buffaloes in all treatment protocols on Day 9. The serum progesterone (P4) concentration was significantly higher (p<0.05) in either EDP or EDnP buffaloes in Noegestomet eCG6-ovysync compared with other protocols on Day 7. There was a highly significant (r=0.74, P<0.01) positive correlations between corpus luteum volume (CLV) and LFD in EDP buffaloes.

Conclusions: It is concluded that modifying Basic-ovsync via P4 supplementation by injection of long-lasting P4 on Day 0 and insertion of Norgestomet ear implant from day 0 to day 7 plus an injection of eCG on Day 6 improve the fertility response in true anestrus in buffaloes.

Keywords: Buffalo, anestrus, Norgestomet, eCG, ovsync protocol.

1. Introduction

The productive performance of buffaloes is mainly influenced by the reproductive efficiency, which is reduced by late attainment of puberty, seasonality of calving, silent ovulation, long postpartum anestrus and subsequent calving interval (Barile, 2005; Borghese et al., 1993; de Carvalho et al., 2016). Buffaloes have inherent problem of summer anestrus and poor expression of estrus and therefore prolonged calving to conception interval (Kumar et al., 2019). Postpartum anestrus is characterized by insufficient pulsatile LH release to support dominant follicle development and maturation in buffaloes (Das and Khan, 2010). In true anovulatory anestrus, although ovarian follicular growth and turnover are observed, maturation and ovulation of the dominant follicle do not take place (Rhodes et al., 2003). Although ovsync TAI can be used in both cyclic and acyclic buffaloes, the fertility response was poor in acyclic buffaloes in terms of poor conception rate (De Rensis et al., 2005; Souza et al., 2015). The use of P4- supplemented GnRH-based ovsync protocol in anestrous cows resulted in conception rates similar to those obtained in cows inseminated following a first postpartum spontaneous estrus (Hanlon et al., 2000). In the treatment of true anestrus in buffaloes, the use of various forms of supplemental progesterone such as intravaginal devices (PRID and CIDR) as well as Norgestomet ear implant have been reported (Bartolomeu et al., 2007; Caesar et al., 2011; Naseer et al., 2012). Norgestomet ear implant was reported to restore ovarian activity resulting in ovulation in the majority of buffaloes with acceptable conception rates (Chhatry, 1998; Deka et al., 2010). The supplemental P4 in P4 modified-ovsync is thought to increase follicular fluid and serum concentration of E2, pulsatile release of LH and number of LH receptors, thereby enhancing the development and maturation of DF (de Carvalho et al., 2016).

Enhancing the growth of the dominant follicle with eCG might be a strategy to increase ovulation and pregnancy rates with ovsync TAI protocols in anestrus buffaloes (de Carvalho et al., 2016). eCG binds to both FSH and LH receptors and stimulates follicular growth in cattle (Soumano and Price, 1997). Treatment with eCG at the P4 device removal in P4-supplemented ovsync TAI increased ovulation rate and P4 concentration during diestrous following TAI resulting in higher conception rates in anestrous buffaloes (Baruselli et al., 2013; Carvalho et al., 2013). Previous studies recorded various ovulation and conception rates following the use of Norgestomet–eCG combination

for treating true anestrus in buffaloes (Caesar et al., 2011; Chhatry, 1998; Dodamani et al., 2011).

The current study tested the hypothesis that incorporating eCG alone on day 2 as in eCG2-ovsync protocol or on Day 6, one day before removal of Norgestomet ear implant, as in Norgestomet-eCG6 protocol would increase both the size and steroidogenic activity of the LF thereby ovulates in response to the GnRHa2 with a consequent positive impact on the conception rate. The aim of the present research work was to study the fertility response and associated follicular growth and hormonal profiles to modified Norgestomet eCG6-ovsync and eCG2-ovsync protocols in comparison with Basic-ovsync protocol (control) during the breeding season.

2. Materials and methods

The present study was carried out in the buffalo research station, MahalletMousa, Kafr El-Sheikh, Egypt, during the high breeding season (November-March). All experimental procedures, applied to the animals enrolled in this research work, were in accordance with the rules of the committee of animal care and use of the Faculty of Veterinary Medicine, Kafrelsheikh University.

2.1. Animals and management

Forty two anestrous buffaloes 60-180 days postpartum, 4-6 years old with a body condition score of 3.25±0.5 at a scale (1=lean to 5= obese) were enrolled in the current study. The animals were fed on a diet that met both maintenance and milk production requirements according to the recommendation of APRI (1997, unpublished data). They were milked twice daily. They were kept indoors in semi-sheltered yards, where 50% of the yard area was sheltered. They were free from diseases and vaccinated against infectious diseases according to the rules of the General Authority for Veterinary Services. Ovaries of the buffaloes not detected in estrous within 60-180 days postpartum, were examined by transrectal ultrasonography (TUS) two times at ten days interval on Day -10 and 0 (day of first GnRHa1 treatment). Only buffaloes, found to have no CL on the two ovaries in both two TUS, were enrolled in the study. Also, true anestrus was confirmed by the absence of changes in serum P4 concentration, which remained at basal level (<0.5 ng/ml) in the two blood samples taken simultaneously with two TUS examinations conducted on Days -10 and 0.

2.2. Study design

Buffaloes (n=42) were randomly allocated into three treatment protocols: Basic-ovsync, Norgestomet-eCG6-ovsync and eCG2-ovsync (14 each). Buffaloes in the Basic ovsync protocols received two IM injections of GnRH agonist (20µg Buserelin, Receptal®, MSD Animal health Company) on Days 0 (day of first GnRHa injection, GnRHa1) and 9 (day of the second GnRHa injection, GnRHa2) with an IM injection of 500µg Cloprostenol Sodium (Estrumate®, MSD Animal health Company) on Day 7 followed by TAI at 16 h after GnRHa2 injection. Buffaloes in Norgestomet-eCG6-ovsync received the same treatment as Basic-ovsyncplus S/C insertion of Norgestomet ear implant (Sychromate-B®,Ceva Company, it contains 3mg of potent progestagen and 3 mg estradiol valerate) in the ear Conca together with IM injection of 250mg long-acting P4 (Cidolut-Depot®, CID=Chemical Industries Development Company, Egypt)on Day 0. The Norgestomet ear implant was removed on Day 7 (day of PGF$_{2\alpha}$ injection). Also, 500 IU of eCG (Gonasir®, Laboratories, Hipra, S.A. Avda. la Selva, 135. 17170 AmerGirona, Spain) were administered by IM injection on Day 6. Buffaloes in eCG2-ovsync protocol received the same treatment regime as in the Basic- ovsync in addition to a single IM injection of 1000 IU eCG on Day 2.

2.3. Transrectal Ultrasonography

Transrectal ultrasonography (TUS) of ovaries was performed by the same operator using an ultrasound SonoScape A5 Vet (Sonoscape Co. LTD, Shenzen, China) supplied with a multifrequency linear transducer (3.0-8.0 MHZ). Two TUS of the ovaries were performed on Days -10 and 0 for diagnosis of anestrus. Absence of any luteal structure in the two TUS confirmed that buffaloes were suffering from true anestrus (Khan et al., 2018). Also, TUS was done on Days 0, 7 and 9 to estimate the diameter of the largest follicle (LFD). The presence of follicle >2.5cm in diameter on Day 9 (day of GnRHa$_2$ treatment) was defined as a cyst (Colazo et al., 2013) and were excluded from the study. Two buffaloes were found to have a cystic structure >2.5 cm in the eCG$_2$-ovsync group and excluded from the study. The ovaries were also scanned on post-TAI Days 11 and 22. The TUS on Day 11 aimed to determine the ovulation rate, which is defined as the number of buffalo with at least one CL 10 days after TAI (Murugavel et al., 2009). The TUS of ovaries on post-TAI Day 22 aimed to detect the volume (CLV) and diameter (CLD) of the corpus luteum. Pregnancy diagnosis was conducted by TUS of the uterus on Day 32 post-TAI. The observed reliable signs of pregnancy were visualizing the amniotic sac containing embryo proper and heartbeats. The conception rate was calculated by dividing the number of pregnant buffaloes by the total number of buffaloes subjected to FTAI.

2.4. Blood sampling

Blood samples were collected by jugular vein puncture using plain vacutainer tubes on the experimental Days -10, 0, 7, 9, 10 and post-TAI days 11 and 22 for P4 assay and on Days 9 and 10 for estradiol and insulin assays. Samples were kept in an inclined position for three hours in a refrigerator before being centrifuged for 20 minutes at 1500 xg. Serum samples were stored at -20c° until the hormonal assays were performed.

2.5. Serum progesterone and estradiol assays

Serum P4 and E2 concentrations were assayed by Radioimmunoassay (RIA) using Beckman coulter RIA progesterone and Beckman coulter RIA estradiol kits (Immuno TECH, S.r.o Radiova 1-10277, Prague, Czech Republic) respectively, according to the procedures described in the catalog enclosed with the kits. The inter- and intra- assay coefficients of variations were 8.66% and 8.15% for progesterone and 14.5% and 14.4% for estradiol, respectively. The average sensitivity was 9.58 ng/ml for progesterone and 9.58 pg/ml for estradiol.

2.6. Serum insulin assays

The serum insulin concentration was estimated using an Immunoradiometric kit (Insulin IMRA kit, Immunotech, S.r.o Radiova1-10227- Prague-Czech Republic), according to the manufacturer's instructions described in the catalog enclosed with the kit. The inter-and intra-assay coefficients of variations were 8.3% and 5.6% respectively. The average sensitivity was 4.55 IU/ml.

2.7. Statistical analysis

The data of LFD, serum concentrations of P4, E2, insulin and CLV in both EDP and EDnP buffaloes in all treatment protocols were presented as mean ± SEM. The obtained data were statistically analyzed by the SPSS/PC program (version 16.0; SPSS, Chicago, IL, 2007) using one way ANOVA. Differences were considered as statistically significant at $P< 0.05$. The significant differences among treatment protocols were estimated by multiple Duncan range test (Duncan, 1955). The data concerning the conception rates were analyzed by the Chi-square test. The correlations were estimated using Spearman's rank correlation

coefficient.

3. Results

3.1. The largest follicle diameter (LFD)

The LFD did not differ either among eventually diagnosed pregnant (EDP) or among eventually diagnosed non-pregnant (EDnP) buffaloes on Days 0 and 9. On Day 7, the LFD in EDP buffaloes showed a significant increase in eCG2-ovsync compared with Basic-ovsync (P<0.05) and Norgestomet-eCG6–ovsync (p<0.01). In contrast, the LFD in EDnP buffaloes on Day 7 showed a significant decrease (P<0.05) in Norgestomete-eCG6 –ovsync protocol compared with both Basic-ovsync and eCG2-ovsync.

Within Protocols, the LFD was significantly lager (P<0.05) in EDP compared with EDnP in all treatment protocols on Days 0 and 9. On Day7, it was observed that while the LFD did not differ between EDP and EDnP buffaloes in Norgestomet-eCG6-ovsync, it showed a significant increase in EDP compared with EDnP in Basic-ovsync (P<0.05) and eCG2-ovsync (P<0.01).Two buffaloes from the eCG2-ovsync protocol were excluded from the experiment due to the formation of a cystic ovary (Fig. 1).
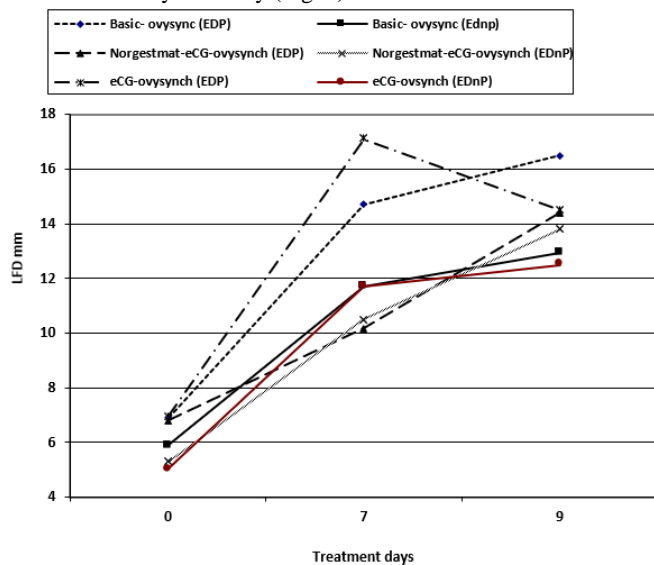

Fig.1. The largest follicle diameter (LFD) on Days 0, 7, 9 in Basic-ovisync, Norgestomat-eCG6-Ovsync and eCG2-Ovsync protocols in EDP and EDnP buffaloes. EDP=Eventually diagnosed pregnant buffaloes, EDnP= Eventually diagnosed non-pregnant buffaloes, Basic-ovsync= Ovulation synchronization protocol that was conducted by two injections of GnRHa on Days 0 and 9 with an injection of PGF2α on Day 7 followed by TAI at 16h after GnRHa2 injection, Norgestomet-eCG6-ovsnyc= A modified ovulation synchronization protocol similar to Basic –ovsync in addition to the insertion of Norgestomet ear implant and injection of 250mg of long-acting P4 on Day 0 and eCG (500 IU) injection on Day6 and eCG2-ovsync= A modified ovulation synchronization protocol similar to Basic Ovsync in addition to eCG (1000IU) treatment on Day 2.

3.2. SerumP4 concentration during treatment protocols

The serum P4 concentrations in the selected buffaloes on both day -10 and day 0 were at the basal level and did not exceed 0.5 ng/ml indicating ovarian acyclicity. On Day 7, the serum P4 concentration showed a significant (P<0.05) increase in EDP versus EDnP buffaloes in all treatment protocols (Fig.2). In EDP and EDnP buffaloes, the serum P4 concentrations in Norgestomet-eCG6-ovsync protocol were higher than their counterpart values in both Basic-ovsync and eCG2-ovsync protocols on Day 7. On Day 9, the serum

P4 concentrations in EDP were lower (p<0.05) in the Basic-ovsync group compared with their counterparts in either Norgestomet-eCG6-ovsync (p<0.01) or eCG2-ovsync (p<0.05). On Day 10, the serum P4 concentration in EDP buffaloes was higher in eCG2-ovsyncin comparison with Basic ovsync (p<0.05) and Norgestomet-eCG6-ovsync (p<0.01). Also, in EDnP buffaloes, the serum P4 concentrations was higher in Basic-ovsync in comparison with either Norgestomet- eCG6-ovsync (p<0.01) or eCG2-ovsync(P<0.05) (Fig. 2).

3.3. Post-TAI Serum P4 concentrations

On Day 11 post-TAI, although the serum P4 concentration in EDP was higher (P<0.05) in Norgestomt-eCG6-ovsync compared with either Basic-ovsync or eCG2-ovsync, it did not differ in EDnP buffaloes among all protocols. On Day 22 post-TAI, although there was a non-significant variation in serum P4 concentrations among protocols inEDnP buffaloes, there was a significant (P<0.05) decrease in Basic-ovsync compared with either Norgestomt-eCG6-ovsync or eCG2-ovsync in the case of EDP buffaloes. Within protocols, the serum p4 concentrations in EDP versus EDnP buffaloes showed a significant (p<0.01) increase in Basic –ovsync and highly significant (p<0.01) increase in either Norgestomet-eCG6-ovsync or eCG2-ovsync (Fig. 2).
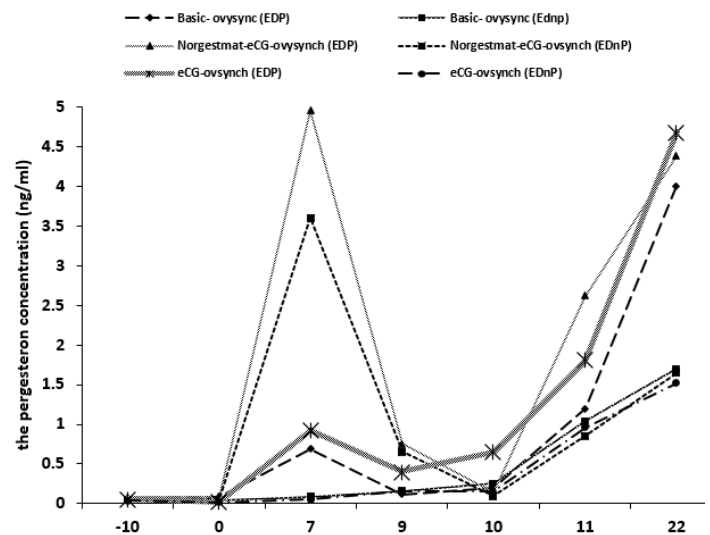

Fig.2. The serum progestrone concentrations on the respective days in Basic-ovsync, Norgestomat-eCG6-ovsync. and eCG2-P4-ovsync protocols in EDP and EDnP buffaloes. EDP=Eventually diagnosed pregnant buffaloes, EDnP= Eventually diagnosed non-pregnant buffaloes, Basic-ovsync= Ovulation synchronization protocol that was conducted by two injections of GnRHa on Days 0 and 9 with an injection of PGF2α on Day 7 followed by TAI at 16h after GnRHa2 injection, Norgestomet-eCG6-ovsnyc= A modified ovulation synchronization protocol similar to Basic –ovsync in addition to the insertion of Norgestomet ear implant and injection of 250mg of long-acting P4 on Day 0 and eCG (500 IU) injection on Day6 and eCG2-ovsync= A modified ovulation synchronization protocol similar to Basic Ovsync in addition to eCG (1000 IU) treatment on Day 2.

3.4. Serum estradiol concentration on Day 9 and 10

The serum E2 concentration, on Day 9, was higher in EDP versus EDnP buffaloes in either Basic-ovsync or Norgestomet-eCG6-ovsync (P<0.05) as well as in eCG2-ovsync (P<0.01). On Day 10, it was noted that while serum estradiol concentrations did not differ between EDP and EDnP buffaloes in either Basic-ovsync or eCG2-ovsync, it was higher (P<0.05) in EDP than EDnP buffaloes in Norgestomet eCG6-ovsync protocol (Fig. 3).
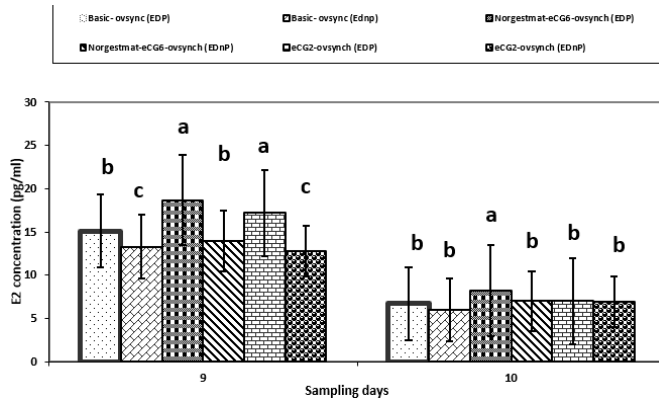
Fig.3. Serum E2 concentration on Days 9 and 10 in Basic-ovisync, Norgestmate-eCG6-ovisync and eCG2-Ovsync protocols in EDP and EDnP buffaloes. EDP=Eventually diagnosed pregnant buffaloes, EDnP= Eventually diagnosed non-pregnant buffaloes, Basic-ovsync= Ovulation synchronization protocol that was conducted by two injections of GnRHa on Days 0 and 9 with an injection of PGF2α on Day 7 followed by TAI at 16h after GnRHa2 injection, Norgestomet-eCG6-ovsnyc= A modified ovulation synchronization protocol similar to Basic –ovsync in addition to the insertion of Norgestomet ear implant and injection of 250mg of long-acting P4 on Day 0 and eCG (500 IU) injection on Day6 and eCG2-ovsync= A modified ovulation synchronization protocol similar to Basic Ovsync in addition to eCG (1000 IU) treatment on Day 2. Columns with different small letters are different at [a-b or b-c] P <0.05, [a-c] p<0.001.

3.4. Serum insulin concentrations

The serum insulin concentrations, on Day 9, were higher in EDP versus EDnP buffaloes in Basic-ovsync and eCG2-ovsync (P<0.01) as well as in Norgestomet-eCG6-ovsync (P<0.05). On Day 10, it was noted that while the serum insulin concentration did not differ between EDP and EDnP buffaloes in eCG2-ovsync, it was higher (P<0.01) in EDP compared with EDnP buffaloes in either Basic-ovsync or Norgestomet-eCG6-ovsync (Fig.4).
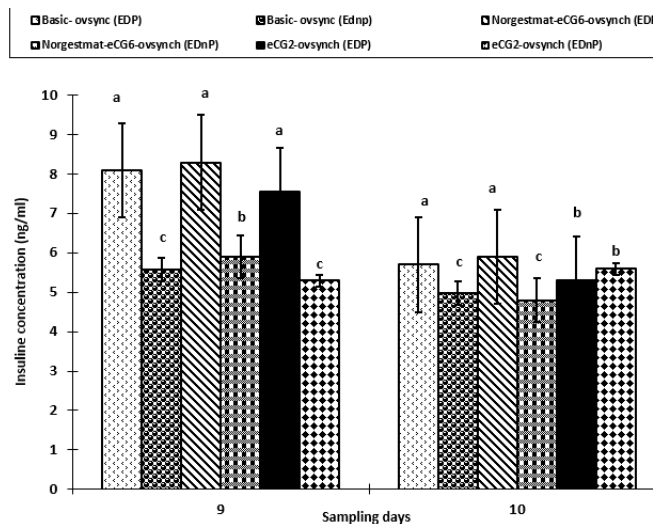

Fig.4: Serum Insulin concentrations on Days 9 and 10 in Basic-ovysync, Norgestmate-eCG6-ovysync and eCG2-ovysync protocols. EDP=Eventually diagnosed pregnant buffaloes, EDnP= Eventually diagnosed non-pregnant buffaloes, Basic-ovsync= Ovulation synchronization protocol that was conducted by two injections of GnRHa on Days 0 and 9 with an injection of PGF2α on Day 7 followed by TAI at 16h after GnRHa2 injection, Norgestomet-eCG6-ovsnyc= A modified ovulation synchronization protocol similar to Basic –ovsync in addition to the insertion of Norgestomet ear

implant and injection of 250mg of long-acting P4 on Day 0 and eCG (500 IU) injection on Day6 and eCG2-ovsync= A modified ovulation synchronization protocol similar to Basic Ovsync in addition to eCG (1000 IU) treatment on Day 2.

3.5. Diameters and volume of CL gravididatous on Day 22

The diameter of the corpus luteum (CL) was larger in EDP than EDnP buffaloes in Basic-ovsync and Norgestomet-eCG6-ovsync (P<0.05) as well as in EDP than EDnP in eCG2-ovsync (P<0.01) on the Day 22 Post-TAI. Among protocols, although the CL diameter showed non-significant variations in EDP buffaloes, it was smaller (P<0.05) in EDnP buffaloes in eCG2-ovsync compared its counterpart values in other protocols. The CL volume (CLV) was greater in EDP than EDnp buffaloes in Basic-ovsync (P<0.05) and in both Norgestomet-eCG6-ovsync and eCG2-ovsync (P<0.01). Among protocols, although the CLV did not differ among EDnP buffaloes, it was smaller (P<0.05) in Basic-ovsync in comparison with other protocols (Table1).

Table 1: Diameter and volume of corpus luteum gravididatous on Day 22 post-TAI.

Days of TUS	Basic-ovsync		Norgestomet-eCG6-ovsync		eCG2-ovsync	
	EDP	EDnP	EDP	EDnP	EDP	EDnP
Diameter of CL on Day 22	15.30±0.89ª	13.50±0.15ᵇ	16.00±0. 20ª	13.40±0.20ᵇ	18.60±0.51ª	10.20±3.40ᶜ
Volume of CL on Day 22	2.56±0.68ᵇ	1.60±0.15ᶜ	2.94±0.38ª	1.30±0.31ᶜ	3.40±0.27ª	1.40±0.44ᶜ

EDP= Eventually diagnosed pregnant buffaloes, EDnP= Eventually diagnosed non-pregnant buffaloes. Values within the same row differ at [a-b or b-c or c-d] p<0.05 [a-c] p<0.01 and [a-d]p<0.001.

3.6. Correlation

There was highly significant (r=0.73, P<0.01) positive correlations in EDP buffaloes and significant (r=0.60, P<0.05) positive correlations in EDnP buffaloes between the LFD and serum E2 concentration. Similarly, there was highly significant (r=0.86, P<0.01) positive correlations in the case of EDP buffaloes and significant (r=0.66, P<0.05) positive correlations in the case of EDnP buffaloes between the E2 and serum insulin concentrations. It was noted that while there was a highly significant (r=0.74, P<0.01) positive correlations between CLV and LFD in the case of EDP buffaloes, there were no correlations in EDnP buffaloes between CLV and LFD (Table2).

Table 2: Correlations between LFD and either CLV and serum hormone concentrations as well as among serum hormone concentrations themselves in EDP or EDnP.

Parameter	EDP				EDnP			
	Insulin	E2	CLV	LFD	Insulin	E2	CLV	LFD
LFD	0.29	0.73**	0.74**	1.00	0.51	0.60*	0.25	1.00
E2	0.86**	1.0	0.09	0.73**	0.66*	1.00	0.09	0.60*
CLV	-0.10	0.09	1.00	0.74**	0.42	0.09	1.00	0.25
Insulin	1.00	0.86**	-0.10	0.29	1.00	0.66*	0.42	0.51

EDP= Eventually diagnosed pregnant buffaloes, EDnP= Eventually diagnosed non-pregnant buffaloes. LFD=Largest follicle diameter, CLV=Corpus luteum volume, E2=estradiol. Values within the same row differ at *p<0.05, **p<0.01.

3.7. Conception rates

The conception rate in the Norgestomet-eCG6-ovsync group was higher (P<0.05) than either Basic-ovsync or eCG2-ovsync group (Fig.5). Also, the ovulation rate was higher (P<0.05) in Norgestomate-eCG6-ovsync compared with either Basic-ovsync or eCG2-ovsync (Fig. 5).
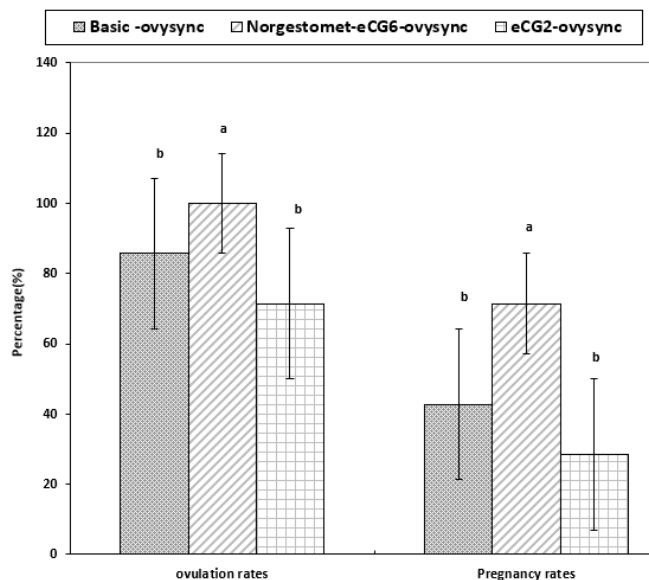
Fig. 5. The ovulation rates and pregnancy rates in Basic-ovsync, Norgestomat-eCG6-ovsync and eCG2-ovsync protocols. Figure 5: Ovulation and conception rates in Basic- ovsync, Norgestomet-eCG6-ovsnyc and eCG2-ovsyncBasic- ovsync= ovulation synchronization is conducted by two injections of GnRHa on Day 0 and 9 with an injection of PGF2α on Day 7 flowed by TAI at 16h after GnRHa2 injection. Norgestomet-eCG6-ovsnyc= Ovulation synchronization protocol similar to Basic –ovsync in addition to the insertion of norgestomet ear implant and injection of 250mg of long-acting P4 on Day 0 and eCG injection on Day6 and eCG2-ovsync=an ovulation synchronization similar to Basic Ovsync in addition to eCG treatment on Day 2. Columns with different small letters are different at [a-b] p<0.05.

4. Discussion

The present research work aimed to study the fertility response of anestrous buffaloes and associated follicular dynamics and hormonal profiles to eCG-Modified (eCG2-ovsync) or eCG plus Norgestomet ear implant-Modified (Norgestomat-eCG6-ovsync) GnRH-Based ovsync protocol. The LFD on Day 0 (day of GnRHa1 injection) was larger in EDP than EDnP buffaloes in all protocols, a finding that explains the occurrence of pregnancy in EDP buffaloes. The largest follicle (LF) in EDP buffaloes might ovulate or luteinize in response to GnRHa1 injection in either Basic-ovsync or eCG2-ovsync, resulting in low serum P4 as has been observed on Day 7. On the other hand, the smaller follicles in EDnP did not respond to the first GnRH injection, as was evidenced by the persistence of the basal serum P4 without change on Day 7 in these two protocols. The ovulation of the LF in response to GnRHa1 injection with subsequent increase in the serum P4 level is a prerequisite for the success of the ovsync protocol (Vasconcelos et al., 1999)while the anovulation of less responsive follicles in response to GnRHa1 injection is the reason for poor pregnancy rate following ovysync protocol in acyclic cows (López-Gatius et al., 2001; Wiltbank et al., 2002). The exogenous P4 supplementation in Norgestomet-eCG6-ovsync may be a resort to increase serum P4 concentration from Day 0 to Day 7, as was observed by the higher serum P4 concentration either in EDP or EDnP buffaloes on Day 7 compared with either counterpart values in the other two protocols. However, the non-significant difference in the serum P4 concentration between EDP and EDnP buffaloes in Norgestomet-eCG6-ovsync protocols on Day 7may be attributed to the masking effect of supplemented P4 in EDP and EDnP buffaloes (Ramoun et al., 2017). In agreement with our results, Nowicki et al., (2017) found that enhancing P4 concentration

prior to PGF2αin Basic GnRH-based ovysync protocol increased serum P4 concentration from day 0 to day 7 with subsequent substantial impact on the probability of pregnancy in Holstein dairy cows. Also,Bisinotto et al., (2010)reported that increasing serum P4 concentration during DF growth in an Ovsync protocol increased fertility to subsequent TAI.

In Norgestomet-eCG6-ovsync, the LFD in either EDP or EDnP buffaloes on Day 7 was smaller than their counterpart LFD in the other two ovsync protocols. This may be attributed to the persistence of the LF that was present at the time GnRHa1 injection and bypassed its effect and continued to grow at a slower rate due to the negative feedback effect of higher serum P4 level on the frequency and level of the tonic level of LH. Unlike many previous studies whereas eCG was injected at the time of the of Norgestomet ear implant (de Carvalho et al., 2016)or IPVD (P4 releasing intravaginal device)removal(Carvalho et al., 2020),eCG was injected one day before ear implant removal in Norgestomet-eCG6-ovsync in the current study to increase the interval of eCG effect to 3 instead of 2 days before ovulation thereby maximize its growth-promoting action on the LF.

Benefiting from the longer half-life period (6-10 days) of eCG, it was injected on the 2nd day in case of eCG2-ovsync to maximize its enhancing effect (for 7 days) on both size and steroidogenic activity of DF in the present study. The enhancing effect of eCG was observed in the present study by the increase in both size and estradiol production on Day 9 (Day of GnRHa2) in both Norgestomet-eCG6-ovsync and eCG2-ovsync in comparison with basic-ovsync. In agreement with our results in this regard, Khan et al., (2018) recorded an increase in both size and steroidogenic activity of LF following the incorporating eCG in Basic-ovsync during treatment of anestrous buffaloes. Also, the inclusion of eCG in ovsync protocols had positive effects on serum P4 concentration in the ensuing oestrus cycle (Khan et al., 2018; Souza et al., 2009). The increased serum P4 levels especially in EDP buffaloes on Days 11 and 22 post-TAI in both Norgestomet-eCG6-ovsync and eCG2-ovsync in comparison with Basic-ovsync confirmed the positive effect of eCG on P4 production in the estrous cycle following eCG-modified ovsync.

In the present study, the conception rate was improved in Norgestomet-eCG6-ovsync protocol (71.4%) and numerically decreased in the case of eCG2-ovsync (33.3%) in comparison with Basic-ovsync (42.8%). The CRs in the case of Norgestomet-eCG6-ovsync and Basic ovsync came in coincidence with Presicce et al., (2005) who recorded CRs of 70% and 40% for anestrus buffaloes treated with PRID plus eCG-ovsync and Basic-ovsync respectively. The higher CR in buffaloes submitted to Norgestomet-eCG6-ovsync protocol may be attributed to the highest serum P4 on Day 7 (day of PGF2αinjection), lowest serum P4 level on Day 10 (Day of TAI). In accordance with our explanation, Wiltbank et al., (2014)reported that high serum P4 during the growth of DF coupled with low serum P4 near the time of AI was associated with high P/AI. Bisinotto et al., 2010)found that P4-modified ovsyncis effective in resuming fertility in cows and a minimum level of 2-3 ng/ml of P4 is needed during DF growth in an ovsync protocol to achieve an AI in anovulatory cows. Also,de Carvalho et al., (2016)reported that high serum P4 (>1.0 ng/ml) levels at the time of PGF2α in an ovsync protocol were associated with Greater P/AI in buffaloes. Nonetheless, the higher CR in Norgestomet-eCG6-ovsync might be attributed to the synergistic effect between supplemental P4 and eCG on size and steroidogenic activity of LF as well as on CL formed following TAI (Baruselli et al., 2013; Carvalho et al., 2013). On the other hand, the decrease in the CR in eCG2-ovsync in comparison with Basic –ovsync (numerical) and Norgestomet-eCG6-ovsync (p<0.05) may be attributed to the high P4 level on Day 10 (Day

of TAI). The increased serumP4 level on Day 10 in eCG2-ovsync may be attributed to the presence of small luteinized follicle(s) that might not ovulate but get luteinized in response to GnRHa2. The longer effect of eCG on the ovary resulted in the successive development of follicles with various ages one of them was the follicle that gets luteinized in response to GnRH2.The higher serum P4 level around the time of TAI impairs the motility of the genital tract with a subsequent adverse effect on sperm transport to the site of fertilization decreasing the chance of fertilization (Bennett et al., 1988). Also, the high P4 level at that time decrease uterine wall thickness thereby affects uterine receptivity for Zygote and subsequently decrease CR (Souza et al., 2011).

However, the prolonged effect of eCG, in the presence of relatively low serum P4 concentration during the interval extending from day 2 to 9 in the case of eCG2-ovsync protocol resulted in overgrowth of LF in two buffaloes leading to the formation of cystic ovaries and these two buffaloes were excluded from the study. Our results in this regard came in coincidence with Braw-Tal et al., (2009)who concluded that cystic ovary develops due to the continued growth of the anovulatory follicle under the low systemic concentration of P4 in high-yielding dairy cows.

The positive correlation between LFD and CL volume in EDP buffaloes (r=0.74, P<0.01) confirms the relationship between the size of LF and CLV of CL formed following TAI. This came in accordance with Vasconcelos et al., (2001)who concluded that the CL size is related to the size of LF present at the time of GnRH2. Doubtless; the CLV is related to P4 production as has been observed by the high serum P4 levels on post-TAI Days 11 and 22 in EDP buffaloes. The positive correlation between serum insulin and E2 levels in EDP buffaloes(r=0.86, P<0.01) and in EDnP buffaloes (r=0.66, P<0.05) indicated the enhancing effect of insulin on the steroidogenic activity of the LF (Armstrong et al., 2001).

It is concluded that modifying Basic-ovsync via P4 supplementation by injection of long-lasting P4 on Day 0 and insertion of Norgestomet ear implant from day 0 to day 7 plus an injection of eCG on day 6 improve the fertility response in true anestrus in buffaloes.

Conflict of interest
The authors declare that they have no conflict of interest.

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