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EFFECT OF DIFFERENT HORMONAL PROTOCOLS ON CLINICAL SIGNS OF ESTRUS AND CONCEPTION RATES IN BUFFALOES

SUMMARY

The protocols PreSynch/OvSynch, OvSynch and OvSynch+PRID were applied in breeding season (IBS) and out of it (OBS) in 133 buffalo cows and OBS in 75 heifers. Gestation was diagnosed sonographically 45 days after timed artificial insemination (TAI). Dispersion analysis was conducted, including the factors: protocol; clinical signs of estrus (without CSE; patency of cervix; mucous discharge); age. Another 3-factor analysis solely on buffalo cows included the effect of season. The results show that the factor protocol has the most pronounced effect on TAI success ($P \leq 0.01$), significantly lowest being the pregnancy rate (p_i values) under PreSynch/OvSynch protocol – only 23.9%, compared to 40.7 and 47.4% under OvSynch and OvSynch+PRID, respectively. Although the effect of age is non-significant, in the heifers OvSynch+PRID and OvSynch show markedly higher results (50.0 and 52.9% respectively), while in the buffalo cows the differences are smaller, the OvSynch protocol having a relatively low pregnancy rate (38.3%), OvSynch+PRID – highest (45.0%), and PreSynch/OvSynch – lowest (30.4%). Season has non-significant effect, but the conception rate from OvSynch+PRID applied OBS is higher than IBS, so it can be used for overcoming the problematic seasonal anestrus. CSE is a significant source of variance of conception rates ($P \leq 0.05$), predictably the highest *pi* value belonging to the cases with mucus. The superiority of the OvSynch+PRID protocol finds expression in the highest incidence of full estrus (with mucus) in the lactating buffaloes (70%) and even more in the heifers (82%), as compared to PreSynch/OvSynch (56.5 and 52.2% respectively) and OvSynch (51.1 and 50.0%).

Keywords: buffaloes, conception rate, estrus signs, PRID, prostaglandin, gonadotropin

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INTRODUCTION

Management of herd replacement and profitability in buffalo farming are majorly based on age of first calving and calving interval, rather than on milk yield. This is proved with the economic weights of the traits in the Bulgarian Murrah (Peeva, 2000) and in the Italian Mediterranean breed (Barile, 2005). In particular, breeding efficiency of buffaloes is affected by late puberty, seasonality of calving, prolonged anestrus after calving, irregular cyclicity, low conception rates, etc. (Madan 1990). The great variability of estrus and ovulation is a challenge to AI, as well (Ohashi, 1994; Perera, 2011). Moreover, the reproductive process in the buffalo farms is specifically characterized by vaguely expressed signs of estrus and hence by poor timing and low success of artificial insemination (Alonso et al., 1992; Barkawi et al., 1993). In addition, the portion of animals in long anestrus is great, especially in the non-breeding season (El-Wishy, 2007). It is a characteristic of the species that high sexual activity comes in response to the reducing day light in late summer and early autumn in association with melatonin secretion. In the rest of the year buffaloes often exhibit anestrus which, on one hand, reduces reproductive efficiency and, on the other, results in a misbalanced milk production throughout the year (Zicarelli, 1997, 2007; Neglia et al., 2004).

Efficiency of protocols for estrus and ovulation synchronization has been established to increase the percentage of pregnant animals (Baruselli & Carvalho, 2005; Balamurugan et al., 2017). Further, combined administration of gonadotropins and prostaglandins has been introduced with the purpose to optimize the effect of these protocols by the inclusion of timed artificial insemination (De Rensis & López-Gatius, 2007; Hammam et al., 2009). Consequently, certain success has been achieved in eliminating the effect of breeding season (Carvalho et al., 2013; Baruselli et al., 2013). In buffalo breeding, the applied synchronization schemes (Paul & Prakash, 2005; Carvalho et al., 2007; Warriach et al., 2008) are of even greater importance, compared to cattle, in view of the above-mentioned species-specific peculiarities of reproduction. Namely, the new follicular wave they induce affords circumventing the species-problematic visual heat detection for AI timing (Barile, 2005). There are only few research works studying the presence of clinical signs of full estrus in synchronized buffaloes on global (Neglia et al., 2003) and national (Atanasov et al., 2012) scale, suggesting that mucous discharge can be used as an indicator for good conception rates. All this is especially important for the buffalo population of Bulgaria in view of the considerable weights of the reproduction traits, mentioned above (Peeva, 2000).

The objective of the presented field experiment was to study the efficacy of the application of three different protocols for estrus synchronization in buffalo heifers and buffalo cows of the Bulgarian Murrah breed expressed in manifestation of clinical signs of estrus and conception rates.

MATERIAL AND METHODS

The field experiment was carried out in the years 2021 and 2022 and included 75 buffalo heifers and 133 buffalo cows of the Bulgarian Murrah breed bred on the farm of Agricultural Institute – Shumen. The two categories of buffaloes are kept in tie-stall housing with exercise yards.

The diet of the buffalo cows includes 22 kg of maize silage, 3 kg of leguminous hay, 4 kg of cereal straw, and 5 kg of compound feed (*TopMix – 15% protein*) per capita per day. The diet of the heifers consists of 18 kg of silage, 3 kg of hay and 4 kg of feed. The buffaloes were selected to be up to sixth lactation and more than 60 days postpartum, and the heifers – to be roughly 22 months old with a live weight of about 380 kg. All included animals were in good body condition (BCS= 3–4) and free of obstetrical disorders and abnormalities in their prior parities (if any). On day 0, ovarian structures were examined sonographically and for treatment were chosen individuals with diameter of the follicles over 8 mm and no *corpus luteum*.

Three protocols of hormonal treatment for estrus induction/synchronization with timed artificial insemination (TAI) were applied in heifers and lactating buffaloes, as presented in Table 1. In the lactating buffalo cows, each of the protocols was applied both in breeding and non-breeding season, while in the heifers – out of season only. Gestation was also diagnosed sonographically on the 45th day after TAI. The echographic examinations were performed with a SonoScape A2 Vet (SonoScape Co. LTD, Shenzhen, China), a multi-frequency (7-12 MHz) linear probe and transrectal approach.

Table 1. Timing of hormonal administration within the three tested protocols

Day	hour	PreSynch/OvSynch (n_H= 46; n_c= 46)	OvSynch (n_H= 12; n_c= 47)	OvSynch+PRID (n_H= 17; n_c= 40)
1	8 a.m.	500 UI Synchrostim[1] i.m. + 5 ml Enzaprost T[2] i.m.	2 ml Ovarelin i.m.	PRID DELTA[4] vaginally inserted
4	8 a.m.	2 ml Ovarelin[3] i.m.		
8	8 a.m.		5 ml Enzaprost i.m.	PRID DELTA removal + 5 ml Enzaprost i.m.
10	4 p.m.		2 ml Ovarelin i.m.	2ml Ovarelin i.m.
11	8 a.m.	5ml Enzaprost T i.m.	TAI	TAI
	4 p.m.			TAI
13	4 p.m.	2 ml Ovarelin i.m.		
14	8 a.m.	TAI		

i.m. – intramuscular injection; n_H – number of buffalo heifers, n_c – number of buffalo cows; [1]Equine Serum Gonadotropin, *Ceva Sante Animale, France*; [2]Dinoprost Trometamol, *Ceva Sante Animale, France*; [3]Gonadorelin, *Ceva Sante Animale, France;* [4]Intrauterine device for cattle PRID delta 1.55 g Progesterone, *Ceva Sante Animale, France*

The criteria for the diagnosis of pregnancy were an enlarged uterine lumen, visualization of the embryonic vesicle, and foetal cardiac activity, as described by Fricke et al. (2016).

The artificial insemination and evaluation of the clinical signs of the genital apparatus associated with estrus were performed by an AI technician. The presence/absence of a clear cervical-vaginal mucous discharge was detected, as well as the passability of the cervical canal with a pipette (patency). Cryopreserved semen from tested buffalo bulls was used.

Examinations and treatments were carried out in compliance with the requirements and regulations of the Animal Welfare Act (AWA).

The set of data registered sonographically were processed via dispersion analysis of a non-orthogonal set of qualitative traits (Model-1), including the following factors and respective classes:

- Age – two classes: heifers; lactating buffaloes
- Protocol – three classes: PreSynch/OvSynch; OvSynch; OvSynch+PRID (as in Table 1)
- Clinical signs of estrus (CSE) as detected during TAI application – three classes: without CSE (lack of vaginal mucous discharge and low patency of the cervix); patency (good patency but lack of mucus); mucus (both presence of mucous discharge and good patency, herein referred to as expression of full estrus)

Another 3-factor analysis was conducted on buffalo cows only. Instead of age, Model-2 included the factor: season – two classes: in season (high breeding season); out of season (low breeding season). In-season TAI breeding was applied in the period from August 10th to November 21st. The rest of the year was considered out of season.

In the linear model, the gradations are presented as p_i values, resulting from the number of the individuals characterized by the qualitative trait pregnancy as diagnosed 45 days post TAI (Σm_x) out of the total number of individuals in the respective class (Σn_x). The effects of the singular factors included in the ANOVA, their co-effects and the all-factors effect (x) are expressed in coefficients of impact (η^2) and coefficients of significance (F), and the significance of the differences within gradations – in F_d-values.

RESULTS

Table 2 shows the raw data from the ultrasound examinations for the diagnosis of pregnancy 45 days after TAI, as well as the visual detection of the presence of vaginal mucus and the patency of the cervical canal during TAI. It is noteworthy that in TAI after application of the PreSynch/OvSynch protocol, 17.4% of the heifers have shown no visual CSE, but still one animal conceived (12.5%).

This indicates that this protocol had low success in the heifers, while under the OvSynch and OvSynch+PRID scheme all young animals manifested at least patency of cervical canal.

Accordingly, as indicated by the data in Figure 1, the pregnancy rate from the PreSynch/OvSynch protocol in the heifers was markedly lower as compared to the OvSynch and OvSynch+PRID synchronization schemes.

Table 2. Results from day 45 post TAI by protocols and by CSE and distribution by high or low season of breeding

Protocols within age groups	**CSE**	**n_x**	**m_x**	**Season, n_x/m_x**	
				High	**Low**
Buffalo heifers					
PreSynch/OvSynch	No CSE	8	1	-	46/8
	Patency	14	2		
	Mucus	24	5		
OvSynch	No CSE	0	0	-	12/6
	Patency	6	4		
	Mucus	6	2		
OvSynch+PRID	No CSE	0	0	-	17/9
	Patency	3	1		
	Mucus	14	8		
Lactating buffaloes					
PreSynch/OvSynch	No CSE	1	0	38/11	8/3
	Patency	19	4		
	Mucus	26	10		
OvSynch	No CSE	3	1	25/9	22/9
	Patency	20	5		
	Mucus	24	12		
OvSynch+PRID	No CSE	5	1	16/6	24/12
	Patency	7	2		
	Mucus	28	15		

n_x – number of individuals hormonally treated in the respective class;
m_x – number of the individuals characterized by the qualitative trait pregnancy 45 days post TAI out of n_x

In the buffalo cows under the PreSynch/OvSynch protocol, conception rate was also lowest, but with less pronounced differences with OvSynch and OvSynch+PRID (Figure 1). It is noteworthy that while in the heifers OvSynch+PRID and OvSynch lead to high results (conception rates of 50.0 and 52.9% respectively), in the buffalo cows OvSynch has lower success (38.3%).

With no CSE in the PreSynch/OvSynch protocol was only one buffalo cow (2.2%) and she did not conceive; in each of OvSynch and OvSynch+PRID scheme there was one animal, which constitutes respectively 6.4 and 12.5%, with pregnancy rates of 33.3 and 20.0 % (Table 2).

It is noteworthy that in OvSynch the results when patency is the only CSE detected are even lower, while when mucous discharge is present the conception rate is markedly higher.

The differences in the manifestation of full estrus are remarkable. Figure 1 indicates that the heifers with mucus constitute as much as 82.4% of all OvSynch+PRID treated animals, compared to 52.2 and 50.0% in the other protocols. Similar but slightly less-expressed dependency is observed in the buffalo cows – 70.0% in OvSynch+PRID, 56.5% in PreSynch/OvSynch and 51.1% in OvSynch.

Obviously, as Table 2 shows, the PreSynch/OvSynch protocol results in relatively good percentage of heifers with mucous discharge but also in poor follicular wave associated with poor conception rates (Figure 1). Figure 1 also shows that in both age groups the conception rates from full estrus are highest after application of TAI within the OvSynch+PRID protocol (over 50%), but in the lactating buffaloes OvSynch also has a high result (50.0%).

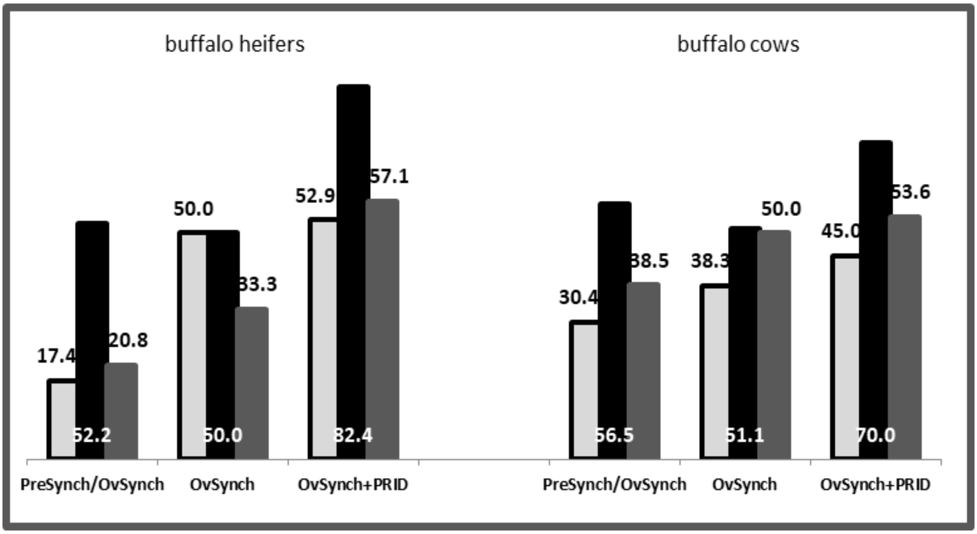

Figure1. Results based on raw data about overall conception rates (framed grey), incidence of full estrus (black) and conception rates from full estrus (dark grey) per protocol within age group, %

It is noteworthy that in the OvSynch scheme the heifers show good conception rates when their cervix has good patency and no mucus. Pregnancies in the buffalo heifers under the OvSynch+PRID are achieved majorly in the presence of mucous discharge (i.e., of both clinical signs), which applies also to the buffalo cows treated under the all three protocolsANOVA from the dispersion analysis in Model-1 indicates that the factor protocol has the most pronounced effect on conception rates – P≤ 0.01 (Table 3). The significantly lowest p_i-estimate belongs to the animals treated with PreSynch/OvSynch (Table 4), which is commensurate with the information in Figure 1 within age groups. CSE also has significant effect on the success of TAI (P≤ 0.05, Table 3), the highest p_i-value observed in the cases with mucous discharge during insemination.

Table 3. ANOVA from the dispersion analysis of conception rates, whole dataset – Model-1

Sources of variance	df	η^2	F	P
Age	1	0.0049	1.027	P> 0.05
Protocol	2	0.0463	4.894	**P≤ 0.01**
Clinical signs of estrus (CSE)	2	0.0373	3.948	**P≤ 0.05**
Age × Protocol	2	0.0505	5.340	**P≤ 0.01**
CSE × Protocol	4	0.0180	0.953	P> 0.05
CSE × Age	2	0.0595	6.287	**P≤ 0.001**
x	17	0.1016	1.265	P> 0.05
z	190	0.8984		
y	207	1.0000		

Table 4. Effect of the significant factors from ANOVA on conception rates, whole dataset – Model-1

Classes	Σn_x	Σm_x	p_i	F_d
Protocol				
1. PreSynch/OvSynch	92	22	0.239	1-[2, 3]*
2. OvSynch	59	24	0.407	
3. OvSynch+PRID	57	27	0.474	
Clinical signs of estrus (CSE)				
4. Without CSE	17	3	0.177	6-[4, 5]*
5. Patency	69	18	0.261	
6. Mucus	122	52	0.426	
Σ / Mean	208	73	0.351	

Σn_x – number of individuals in the class/gradation; Σm_x – number of individuals echographically diagnosed pregnant on day 45 post TAI out of Σn_x; Significance of differences among $\boldsymbol{p_i}$ values within gradation: 1-[2, 3]* and 6-[4, 5] at P≤ 0.05

Although Figure 1 suggested some differences between heifers and cows, the dependence of conception rates on protocol and CSE have similar trends in the two categories of buffaloes. In this context are the data in the ANOVA (Table 3), showing that age is not a significant source of variance of pregnancy rates.

On the other hand, the co-effects of age with the factors protocol ($P \leq 0.01$) and CSE ($P \leq 0.001$) are significant. The co-effect of protocol with CSE and the all-factors co-effect, expressed as *x*-value, are not significant.

The ANOVA under Model-2 (Table 5) shows that the factor protocol is not a significant source of variation of conception rates in the lactating buffaloes, which corresponds with the above observed smaller differences in conception rates between high and low breeding season in the buffalo cows than in the heifers (Figure 1).

Table 5. ANOVA from the dispersion analysis of conception rates in lactating buffaloes – Model-2

Sources of variance	df	η^2	F	P
Season	1	0.0137	1.794	P> 0.05
Protocol	2	0.0147	0.962	P> 0.05
Clinical signs of estrus (CSE)	2	0.0586	3.855	**P≤ 0.05**
Season × Protocol	2	0.0953	6.256	**P≤ 0.001**
CSE × Protocol	4	0.0504	1.653	P> 0.05
CSE × Season	2	0.0514	3.371	**P≤ 0.05**
x	17	0.1237	0.955	P> 0.05
z	115	0.8763		
y	132	1.0000		

This implies that the above established differences among protocols depend mostly on those in the heifers. More importantly, the analysis of variance indicates that season does not affect pregnancy rates significantly, as well. Table 2 also shows the timing of the protocols as per season. It is noteworthy that the conception rate after out-of-season TAI under the OvSynch+PRID protocol is higher (50.0%) than in the main breeding season (37.5%). The differences between the seasons in the protocols PreSynch/OvSynch and OvSynch are smaller, implying a tendency to diminish the impact of season on buffalo reproduction.

DISCUSSION

On the basis of the observation in the presented field trial that high conception rates are achieved in the cases with both clinical signs (full estrus) only, we share the opinion in other works with the OvSynch protocol in the Bulgarian Murrah that the presence of cervical mucus during AI can be used as an indicator for good conception rates (Atanasov et al., 2012), all the more that in our study the incidence of this clinical sign is much higher. Moreover, this rationale applies also to the OvSynch+PRID protocol where the proportion of buffalo cows and heifers with induced full estrus is definitely highest.

As in the breeds Murrah (Ghumen et a., 2014), Surti (Patel et al., 2022), Jafarabadi (Raval et al., 2021) and Italian Mediterranean (Presicce et al., 2004; De Rensis et al., 2005), field experiments on buffalo heifers and cows have resulted in very good estrus-inducing effect of the OvSynch protocol (under the same GnRH-

$PGF_{2\alpha}$-GnRH scheme), an additive favorable effect on conception rates afforded by progesterone treatment in anestrus in high and low breeding season.

Applying out-of-season treatment with PreSynch/OvSynch, in buffalo heifers was achieved high estrus induction and conception rate which were close to the results from the other two protocols (Sing et al., 2010). However, according to these and to other Indian authors, the sole application of this protocol does not provide good outcome, while in combination with progesterone inserts or prostaglandins it is much more effective (Saini et al., 1988; Andurkar et al., 1995; Kumar H. et al., 2010). Italian researchers came to the same conclusion, emphasizing the major economic impact of such protocols in buffalo heifers and pointing out that at earlier age the effect is relatively better due the usual lack of cyclicity at that stage of individual development (Barile et al., 2001). All these effects are implied also in the results from the Bulgarian Murrah heifers and buffalo cows from the present experiment.

The integral role of the timed components of the OvSynch+PRID protocol is described by the experiment of McDougall and co-researchers in dairy cows with the observation that increased concentrations of progesterone during the anestrous are important for incidence of estrus and for the luteal phase afterwards, hypothetically contributing for maturation of a dominant follicle and hence for prostaglandins secretion and ovulation (McDougall et al., 1992). The combination with prostaglandins at the beginning of the protocol was established to induce follicular atresia and a new follicular wave also in buffaloes (Baruselli et al., 2007).

When buffalo reproduction is concerned, season should be always taken in consideration. Having been included in the linear model with classes of the factor specific for the bubaline species within the conditions of Bulgaria, the effect of season is commensurate with the experience in the Italian Mediterranean breed, where especially in the recent decade the experiments with gonadotropins, progesterone and other exogenous hormones have demonstrated similar pregnancy rates after out-of-season breeding as in the high breeding season (Carvalho et al., 2013, 2016; Baruselli et al., 2013); and also with the experience in Murrah breed in India (Kumar P. et al., 2016). The lack of significant differences in the success of TAI by season is actually good news for the conditions in Bulgaria. It implies that the use of these protocols, and especially OvSynch+PRID, affords manipulation of the breeding season for better reproductive efficiency, hence profitability, of buffalo farming. It also contributes for better distribution of bulk milk throughout the year, in case there is misbalance between annual dynamics of production and prices of raw milk, as it is in Italy.

CONCLUSIONS

All three protocols tested in the present study show capacity to mitigate the impact of season on reproduction, having practical importance for overcoming the problematic seasonal anestrus, especially OvSynch+PRID. It can be summarized that in the Bulgarian Murrah buffaloes the follicular wave in response to the hormonal protocols is similar to that in other breeds in different conditions.

Namely, a favorable effect from the application of OvSynch+PRID in both age groups was established, and also of OvSynch in heifers, as well as of poor results from PreSynch/OvSynch. In our study, the superiority of the OvSynch+PRID protocol – as compared to the other protocols and also to other studies – finds expression in the higher incidence of full estrus especially in the heifers, which predetermines the high conception rate, despite the relatively high percentage of animals without CSE. In this way, its use in field conditions finds justification despite its labor-intensive application in practice. In this context, the presence of cervical mucus during AI can be confidently used as an indicator for high pregnancy rates under the protocols OvSynch and OvSynch+PRID.

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