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Efficacy of progesterone implants on induction of cyclicity in anestrous buffaloes

A JEROME[1], S K SRIVASTAVA[2], R K SHARMA[3] and S K PHULIA[4]

ICAR-Central Institute for Research on Buffaloes, Hisar, Haryana 125 001 India

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ABSTRACT

The present study was designed to study if estrus induction response in anestrous buffaloes is augmented by priming using fabricated progesterone implants. For this study, progesterone implants with dose (150 mg and 350 mg) were fabricated with non-biodegradable, biocompatible polymer following standard protocol. These implants were tested as subcutaneous ear implants in 8 acyclic buffaloes for 7 days. Based on the estrus induction response and serum progesterone profile across days, 350 mg P4 implants were used for trial 2. In trial 2, three groups (8 each), viz. 1 [control: no treatment], 2 [progesterone depot 500 mg i.m. on day 0, progesterone s.c. ear implant (350 mg) on day 4, PMSG 500 IU on day 6 and P4 implant removal on day 7 followed by estrus detection and AI], 3 [progesterone depot 500 mg on day 0 i.m.; PMSG 500 IU i.m. on day 6 followed by estrus detection and AI] were studied for estrus induction and fertility response. None (0.0%), 7 (87.5%) and 6 (75.0%) buffaloes were induced to estrus in groups 1, 2 and 3, respectively. Serum progesterone on day 7 was significantly higher in group 2 as compared to other groups. In group 2, four buffaloes ovulated out of which 3 conceived as compared to none in group 3. In summary, induction of estrus and ovulation in anestrous buffaloes by synergistic action of progesterone depot and implant ratify the potential scope of fabricated progesterone implants for inducing ovarian cyclicity in anestrous buffaloes.

Key words: Buffalo, Progesterone, Implant, Cyclicity, Anoestrus

Summer anestrus is an important cause of buffalo infertility in the tropics. In addition, post-parturient disorders, nutrition and production stress contribute to anestrus in buffaloes (Das and Khan 2010). These factors affect estrus length and intensity through increased cortisol secretion (Marai and Haeeb 2010). Furthermore, seasons alter the secretory pattern of reproductive hormones. It is evident that circulating progesterone (P4) has a positive effect on luteinizing hormone (LH) release and ovulatory response along with oocyte competence and embryo quality (Savio *et al.* 1993). However, in post-partum cattle and buffaloes, low circulating P4 concentrations have been associated with negative effect, both on follicular and oocyte maturation thereby affecting pregnancy outcome. This confirms that a minimum threshold of plasma P4 concentration is required for inducing ovarian rebound phenomenon for estrus induction and synchronization (Honparkhe *et al.* 2008). This is addressed by various P4 therapeutics primarily targeted for enhancing the circulating P4 level and induction of cyclicity (Wiltbank *et al.* 2011).

Various P4 based treatment regimens are used for estrus induction in cattle and buffaloes, and were effective in inducing ovarian cyclicity in anestrus cattle and buffaloes (Carvalho *et al.* 2014, Ramadan *et al.* 2014). Exposure of hypothalamus to P4 through these devices stimulates up-regulation of hypothalamic estradiol receptors thereby inducing LH peak release (Gümen and Wiltbank 2005). In particular, use of progesterone depot inducted estrus in cattle (Bharali *et al.* 2014), though being cost effective but less efficient as P4 concentration gradually decreases with time *in vivo*. This decrease in P4 levels affects the negative feedback mechanism to hypothalamaus and hinders the ovarian rebound phenomenon as evidenced by poor cyclicity induction (Kumar *et al.* 2014).

Therefore, for efficient estrus induction, higher plasmatic P4 is warranted to obtain the desired ovarian rebound. Besides, different P4 release devices have varied efficacy of P4 release depending on their surface area, shape, polymer used and P4 content (van Werven *et al.* 2013). Various polymers for steroidal hormonal delivery have been tried (Chen and Singh 2005). Polymer polydimethylsiloxane-co-ethylhydrosiloxane (PDMS) showed positive response due to its biocompatibility in several species (bovine, mice and human), efficiency in drug delivery and low cost (Chen *et al.* 2010). In this light, the present study was designed to test the hypothesis that the fabricated P4 implants augment plasmatic P4 levels for inducing cyclicity in anoestrus buffaloes.

MATERIAL AND METHODS

Study location and animals: The present study was conducted at ICAR-Central Institute for Research on

Present address: [1]Scientist (jerome210982 @gmail.com), [3,4]Principal Scientist (rksharmascientist@gmail.com, sphulia@gmail.com), APR Division. [2]Principal Scientist, Animal Reproduction Division, ICAR-IVRI (skivri6@gmail.com).

Buffaloes farm Hisar, (29.17 North latitude and 75.72 East longitude at 212 meters above mean sea level). The study was conducted during summer (May to August) as 2 trials (1 and 2) in 40 multiparous acyclic post-partum (> 90 days) buffaloes. The animals selected for the study aged between 4–6 years with body weight between 400–550 kg. Buffaloes under the present study were managed on semi-intensive system and were fed with *ad lib.* green fodder, wheat straw (2.0–2.5 kg), concentrate feed, mineral mixture daily. Approval of the Institute Animal Ethical Committee was obtained for carrying out all experimental procedures.

Monitoring of follicular dynamics: Ovarian activity in the buffaloes under study was determined by repeated transrectal ultrasonographic examinations on alternate days in all animals from 2 weeks before the start of the study by a single operator using a B mode ultrasound scanner (Toshiba, SSA 220, JustVision) equipped with an intraoperative 7.0 MHz microconvex transducer. Ovaries were scanned in several planes using the transducer to identify the ovarian structures. Position and size of follicles (≥3mm) were scanned and follicular locations were recorded. Acyclicity was confirmed by absence of corpus luteum during consecutive transrectal ultrasonography 10 days apart buffaloes under study. Ovulation was determined by the disappearance of a large follicle and subsequent appearance of a corresponding corpus luteum at the same location in epsilateral ovary.

Devising the polymer implants: Non-biodegradable polymer, viz. polydimethylsiloxane-co-ethylhydrosiloxane with curing agent (SLYGARD 184, Sigma-Aldrich, USA) (0.75 ml), was used for devising the implant. Furthermore, for suitability of using fabricated P4 implants as subcutaneous ear implant, the dimension of the fabricated implant was fixed as 2.5 cm (length) and 0.3 cm (diameter). Based on the study by Symons *et al.* (1974), implants with 2 doses, viz. 150 mg and 350 mg P4 were fabricated following the protocol described by Sabri *et al.* (2013). The polymer and hormone mixture was mixed thoroughly and the resultant mixture was completely outgassed and casted into desired implant molds. The poured mixture was cured at room temperature overnight and the solidified implants were cut out of the molds and stored at room temperature (20°C) till further animal trials.

In vivo *experimentation of progesterone implants*

Trial 1: Testing for dose determination of progesterone implants: For dose determination of progesterone implants, 16 acyclic buffaloes were selected, of which 8 acyclic buffaloes were implanted with 150 mg (group A) and remaining with 350 mg (group B) P4 implants. They were implanted subcutaneously as ear implants for 7 days and administered 500 IU PMSG (Pregnant Mare Serum Gonadotropin; Folligon®, Intervet, France) i.m. injection on day 6 and the implants were removed on day 7. Detection of estrus was done at the end of trials with per-rectal palpation along with ultrasonography and visual signs, viz. estrous behavior, mucus discharge, vulval swelling in morning and evening 24 h post end of treatment. Animals detected in heat were artificially inseminated using 0.25 ml frozen semen by AM-PM rule.

Trial 2: In vivo testing of progesterone implants: Acyclic buffaloes (n=24) were divided into 3 groups with 8 in each group [group 1: Control; group 2 and 3: treatment groups]. For the trial, group 1 [n=8]: control group (no treatment); group 2 [n=8]: 500 mg P4 (P-depot, Sarabhai Zydus Ltd, India) on day 0 i.m. injection followed by 350 mg P4 implant on day 4, 500 IU PMSG (Folligon®, Intervet, France) i.m. injection on day 6, removal of P4 implant on day 7 followed by AI at estrus detection; and group 3 [n=8]: 500 mg P4 (P-depot, Sarabhai Zydus Ltd, India) on day 0 as i.m. injection, on 6 day 500 IU PMSG as i.m. injection followed by estrus detection and AI. Estrus detection was done with per-rectal palpation along with ultrasonography and visual signs, viz. estrous behavior, mucus discharge, vulval swelling in morning and evening 24 h post end of treatment. Animals detected in heat were inseminated using 0.25 ml frozen semen sfollowing AM-PM rule.

Hormone estimation

Blood samples (8 ml) from the study animals were collected during the trial 1 and 2 (day 0, 2, 4 and 7) in BD vacutainer® serum and serum was harvested by centrifugation of the vacutainer tubes at 3,000 rpm for 15 min. Serum was stored at –20°C for P4 estimation. Progesterone estimation was done using Bovine specific ELISA kit (Cusa Biotech. Ltd., China) as per the kit protocol. The sensitivity of P4 was 0.12 ng/ml. The intra and inter–assay coefficient of variation of the kit used was <8 and <10% respectively.

Statistical analyses

Statistical analysis was carried as per standard (Snedecor and Cochran 1989) using SAS software. Repeated measure ANOVA was done to determine the difference in progesterone measured at different intervals in various study groups and the difference in mean was analyzed by Tukey's *post-hoc* test. The fertility response in different study groups was analyzed by Chi-square test. All the results were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

In the present study, an attempt was made to augment the P4 priming in anestrus buffaloes through fabricated implants for inducing cyclicity. Volume and surface area of the fabricated progesterone implants was 0.70 cm^3 and 5.27 cm^3, respectively (Fig. 1). Follicular profile and fertility response of trial 1 revealed that only 1 buffalo in group A showed estrus signs as compared to none in group B (Table 1), with nonsignificant difference between the 2 groups. But, P4 level differed significantly ($P<0.05$) on day 2 between the 2 groups and higher serum P4 level was maintained on subsequent days, though nonsignificant (Table 2). Comparative follicular profile with fertility response and serum P4 concentration in trial 2 is shown in

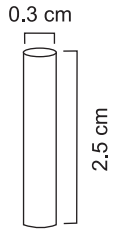

Fig. 1. Fabricated progesterone implant used in trials 1 and 2.

Tables 3 and 4, respectively. In trial 2, none of the control group buffaloes were induced to estrus as compared to 7 and 6 (P<0.05) buffaloes in groups 2 and 3, respectively (Table 3). With respect to follicular profile, follicular diameter at the end of treatment in groups 2 and 3 was significantly higher (P<0.05) than the groups 1. It was observed that more buffaloes ovulated in group 2 in comparison to other groups, though nonsignificant. Likewise, significant (P<0.05) difference in P4 level was observed between groups on day 2, 4 and 7. In addition, within each treated group (2 and 3), P4 level differed significantly (P<0.05) across days (Table 4). In this study, estrus induction response in treated groups (2 and 3) of trial 2 was significantly higher (P<0.05) than the control groups. This enhanced response to estrus induction might be due to the synergistic effect of progesterone priming in addition to the stimulatory action of PMSG. Studies have shown that combination of progesterone with PMSG have resulted in better estrus induction in buffaloes (Malik *et al.* 2010), which is in accordance with the results of this study. Furthermore, higher growth rate of the dominant follicle deduced in this investigation following PMSG is in accordance with Singh *et al.* (2004). Besides, it is noteworthy that pronounced behavioral estrus in group 2 justifies the positive role of PMSG with progesterone in inducing estrus signs and ovulation (Malik *et al.* 2011). From this study, it is evident that dose rate of 500 IU PMSG yielded better estrus induction and ovulation response as reported earlier (Fu *et al.* 2013). These findings corroborate the stimulatory role of PMSG on the quiescent HPG evident by the lower P4 levels during anestrus in buffaloes. Lower estrus response in control group might be due to absence of progesterone priming as evidenced by the serum progesterone concentration. With regard to P4 level, it should be noted that initial P4 supplementation on day 0 in treated group has increased the circulatory P4 level and possibly primed the hypothalamus. Nonetheless, this circulatory P4 level did not induce ovarian follicular rebound and ovulation in group 3. But, interestingly in group 2, P4 implant on day 4 along with P4 depot administration on day 0 resulted in better estrus, ovulatory response and conception rate. It is obvious that P4 implant on day 4 has enhanced the circulatory P4 level, which resulted in better

Table 1. Follicular profile and fertility response in trial 1

Characteristics	Group	
	A(N=8)	B(N=8)
Diameter largest follicle at start of treatment (mm)	11.82±0.30	12.07±0.50
Diameter largest follicle at end of treatment (mm)	12.33±0.42	12.05±0.10
Animal induced to estrus, n (%)	1 (12.5%)	0 (0%)
Animal showing vulvar swelling, n (%)	1 (12.5%)	0 (0%)
Animals showing mucus discharge and uterine tone, n (%)	1 (12.5%)	0 (0%)
No. of animals ovulated, n (%)	0 (0.0%)	0 (0%)

Table 2. Serum progesterone (ng/ml) in groups during trial 1

Group	Day 0(N=8)	Day 2(N=8)	Day 4(N=8)	Day 7(N=8)
A	0.38 ± 0.10^{a}	1.54 ± 0.21^{bA}	1.89 ± 0.29^{b}	1.21 ± 0.22^{b}
B	0.45 ± 0.09^{a}	2.82 ± 0.20^{cB}	1.90 ± 0.25^{b}	1.51 ± 0.13^{b}

Values expressed as mean±S.E; Values in a row with different lower superscripts differ significantly (P<0.05); Values in a column with different upper superscript differ significantly (P<0.05)

Table 3. Follicular profile and fertility response in trial 2

Characteristics	Group		
	1(N=8)	2(N=8)	3(N=8)
Diameter of the largest follicle at start of treatment (mm)	10.08 ± 0.58^{a}	12.36 ± 0.82^{b}	12.50 ± 0.80^{b}
Diameter of the largest follicle at the end of the treatment (mm)	10.25 ± 0.65^{a}	14.96 ± 0.51^{c}	11.60 ± 0.82^{b}
No. of animals induced into estrus (%)	0.0^{a} (0%)	7.0^{b} (87.5%)	6.0^{b} (75%)
No. of animals showing (%) vulvar swelling	0.0^{a} (0%)	7.0^{b} (87.5%)	6.0^{b} (75%)
No. of animals showing mucus discharge and uterine tone (%)	0.0^{a} (0%)	7.0^{b} (87.5%)	6.0^{b} (75%)
No. of animals ovulated (%)	0.0^{a} (0%)	4.0^{a} (57.1%)	1.0^{a} (16.6%)
Time of ovulation after end of the treatment (hrs)	-	35.01± 2.24	35.36± 1.51
Pregnancy rate (%)	0.0 (0%)	3.0 (37.5%)	0.0 (0%)

Values expressed as mean±S.E; Values in a row with different superscript differ significantly (P<0.05)

Table 4. Serum progesterone (ng/ml) in study groups during trial 2

Group	Day 0(N=8)	Day 2(N=8)	Day 4(N=8)	Day 7(N=8)
1	0.38 ± 0.12^{aA}	0.31 ± 0.15^{aA}	0.25 ± 0.20^{aA}	0.35 ± 0.13^{aA}
2	0.34 ± 0.08^{aA}	2.07 ± 0.24^{bB}	2.25 ± 0.25^{bB}	2.01 ± 0.18^{bB}
3	0.21 ± 0.05^{aA}	2.12 ± 0.24^{bB}	1.70 ± 0.25^{bB}	1.01 ± 0.22^{bC}

Values expressed as mean±S.E; a, b: Values in a row with different lower superscripts differ significantly (P<0.05); A, B, C: Values in a column with different uppers differ significantly (P<0.05)

estrus induction and ovulatory rate due to the progesterone priming and withdrawal effect.

From the study, it can also be inferred that serum P4 levels were significantly (P<0.05) higher in treated groups than control groups from day 2 to day 4 of trial. In addition on day 7, P4 levels were significantly (P<0.05) higher in group 2 as compared to other treated group. This can be attributed to the combined effect of progesterone release from P4 depot which was subsequently supported by P4 from implants. This higher P4 level during the implant withdrawal, along with PMSG, resulted in better estrus induction in group 3 in comparison to other groups. This is in consonance with De Rensis *et al*. (2005) citing the importance of progesterone priming followed by sudden withdrawal thereby facilitating ovarian rebound (Wiltbank *et al.* 2011). Likewise, this study also supports earlier studies of short term P4 supplementation (5 to 9 days) which successfully resulted in estrus induction (Day 2004). Our findings were in accordance with Carvalho *et al.* (2014) wherein both new and reused progesterone release device resulted in estrus induction in buffaloes confirming the sustained P4 release from polymer based implants. It was imperative to note that serum P4 level in group 2 was lower as compared to earlier report of progesterone release devices use (van Werven *et al.* 2013). This could be due to the difference in progesterone content, surface area, shape and route of application of the device and difference in assay procedure employed. Contrary to Kumar *et al.* (2010), lesser ovulatory response in group 3 confirms that gradual declining progesterone has less effect on ovarian rebound as compared to sudden withdrawal (Wiltbank *et al.* 2011). Furthermore, differences in follicular status, breed, management and nutrition status might contribute to this disparity.

From the present study, it is evident that the duration and level of progesterone priming plays a major role for inducing estrus, as this is needed by the hypothalamus for facilitating the positive feedback effect for estrus signs and ovulation. Though, estrus induction and ovulation was observed in implant treated group, comparable results with other groups suggest the use of more potent progesterone such as norgestomet in future trials. Besides, this is the first attempt to confirm the scope of using fabricated progesterone implants to augment the circulatory progesterone level for enhancing ovarian rebound in anestrous buffaloes.

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