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Reuse of norgestomet implants in an eCG-based superovulation protocol administered to Nelore (*Bos taurus indicus*) cows

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ABSTRACT

This study assessed the reuse of norgestomet implants in Nelore cows that were superstimulated with eCG. In a crossover design trial, eight cows were randomly divided into two experimental groups and twice superstimulated: Group 1 — half of the cows received a new norgestomet implant and 2 mg estradiol benzoate (EB) on Day 0; Group 2 — remaining cows received two once-used norgestomet implants and 2 mg EB also on Day 0. On Day 4 all cows received a single dose of 2000 IU eCG, and on Day 6 cows were treated with two doses of PGF$_{2\alpha}$ 12 h apart. Ovulation was induced with 12.5 mg pLH 12 h after implant withdrawal (on Day 7), and fixed-time artificial inseminations were carried out 12 and 24 h later. Seven days after pLH injection embryos were recovered and blood samples were taken to determine circulating progesterone. Ultrasound examinations were performed at pLH administration and at embryo recovery. The number of large follicles (≥ 8 mm) was greater (P<0.05) in Group 1 (17.1 ± 1.8) than in Group 2 cows (9.7 ± 1.6). The mean number of corpora lutea was greater (P<0.05) for Group 1 (13.8 ± 1.8) as compared to Group 2 cows (5.4 ± 1.0) and the percentage of large follicles that ovulated following pLH administration also differed (P<0.05) between treatments (80.3% and 53.8% for cows in Groups 1 and 2, respectively). However, no differences (P>0.05) were found between treatments (respectively, for Group 1 and Group 2 cows) in the follicle diameter (10.5 ± 1.6 and 10.2 ± 1.7 mm), corpus luteum (CL) diameter (15.7 ± 1.8 and 14.4 ± 1.7 mm), CL volume (2232 ± 1356 and 1893 ± 1828 mm^3), ova/embryos recovered (6.3 ± 1.1 and 4.0 ± 1.9), transferable embryos (4.0 ± 1.8 and 2.5 ± 1.0) and plasma progesterone concentration (36.1 ± 8.3 and 35.1 ± 6.9 ng/mL). In conclusion, the stimulatory effects on the ovaries provided by the use of two once-used implants were less intense than the ones verified after using a new implant.

1. Introduction

Embryo transfer (ET) is a reproductive biotechnology used to multiply the number of animals of high genetic value (Bó et al., 2004) to accelerate animal genetic improvement (Nogueira et al., 2002). Superovulatory (SOV) techniques are involved in ET programs to increase ovulation rate as well as the yield of transferable quality embryos (Armstrong, 1993; González et al., 2001). However, large variation in the SOV response still remains as a major hindrance to the routine production of embryos from cattle by conventional methods (González et al., 2001). Some variation can be attributed to hormone treatments (Mapletoft et al., 2002). The correlation between progesterone concentrations and the outcome of the SOV treatment continues to be a matter of controversy (reviewed by Britt and Holt, 1988). It is accepted that low

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circulating progesterone profiles are negatively related to the embryo yield and quality (Callesen et al., 1986, 1988; Greve et al., 1995), and that there is a threshold in progesterone concentration at several points of the SOV treatment, below (at follicular wave emergence and just prior embryo recovery) or above (at estrus) which the SOV response is impaired (Allen and Foote, 1988; Goto et al., 1987; Herrier et al., 1990).

In spite of strenuous research efforts to circumvent the variability, there have been few improvements in the super-ovulation of cattle over the last three decades. The average number of embryos recovered from superovulated cattle was 4.6 from 248 donors in 1979; 4.8 from 1485 donors in 1999 (Hasler, 2003) and 5.6 from 1192 donors in 2009 (Donaldson and Perry, 1983). This slight improvement in embryo production in the last 10 years occurred due to the use of intravaginal or subcutaneous progesterone/progestogen releasing devices, which made possible to initiate superovulation of donor cows at a random time of the estrous cycle (Mapletoft et al., 1999). More recently, the reuse of single devices that release progesterone or analogs to synchronize cows for fixed-time artificial insemination (FTAI) or fixed-time embryo transfer (FTET) have commonly been used (Almeida et al., 2006; Hernández et al., 2008; Maluf, 2002). However, recent unpublished results from our lab indicated the occurrence of breakthrough estrus in cows treated with a single once-used norgestomet implant. Indeed, 9.9% (8/81) of purebred Nelore cows exhibited estrus while the once-used implant was still in situ. This undesirable phenomenon was prevented by using two once-used implants in beef cows of the same herd.

The efficacy of reusing a progesterone/progestogen releasing device for superovulation of embryo donors has not been reported. Therefore, this study aimed to evaluate the ovarian response, embryo recovery and plasma progesterone concentration of Nelore cows subjected to superovulation protocols based on eCG administration and synchronized with either a new or two once-used norgestomet implants.

2. Material and methods

2.1. Location, animals and feed

The experiment was conducted at *Embrapa Pecuária Sudeste* (latitude 22°01′S; longitude 47°54′W), Southeastern Brazil. The local weather is tropical, type CwA in accordance with Köppen's classification, characterized by a rainy and hot summer, and dry winter. Non-lactating mature Nelore (*Bos taurus indicus*) cows (n = 8; 7 to 10 years old; 441 ± 19 kg body weight) were used in this study. All cows were considered sound after gynecological examination. Cows were managed under an intensive rotational grazing system based on highly productive artificially fertilized tropical pastures. All animals had free access to mineral supplement and water.

2.2. Experimental design and hormone treatment

In a crossover design trial, cows were randomly divided into two groups, and superstimulated twice with 35 days apart in such a way that any given cow was submitted to both treatments. Group 1: at a random stage of the estrous cycle, half of the cows received a new norgestomet implant in the auricular subcutaneous tissue, containing 3 mg norgestomet

(Crestar®, Intervet, São Paulo, Brazil), and 2 mg estradiol benzoate (Estrogin®, Farmavet Co., São Paulo, Brazil; i.m.) on Day 0 (7:00). Group 2: the other half of the cows received two once-used norgestomet implants, which had been previously used for nine days, and 2 mg EB i.m. (Day 0–7:00). From this day onwards, superovulation protocol did not differ between groups. On Day 4 (7:00) all cows received a single dose of 2000 IU eCG i.m. (Folligon®, Intervet) and on Day 6, two i.m. doses of 150 μg sodium cloprostenol (PGF$_{2\alpha}$ synthetic analog; Sincrocio®, Ourofino, Ribeirão Preto, Brazil) were administered 12 h apart (7:00 and 19:00). Implants were withdrawn 36 h after the first administration of PGF$_{2\alpha}$, and 12.5 mg of porcine LH (Lutropin-V®, Bioniche, São Paulo, Brazil) were administered i.m. 12 h after norgestomet implant removal. Cows were artificially inseminated twice, each time with a single dose of frozen semen of proven fertility from the same bull at 12 and 24 h after the administration of pLH (Fig. 1).

2.3. Ovarian ultrasonography

Numbers and diameter of antral follicles and corpora lutea were assessed by transrectal B-mode ultrasonography of the ovaries of all cows using a Mindray DP 3300, equipped with a 7.5 MHz transducer (Mindray Bio-medical Eletronics Co, Ltd., Shenzhen, China). Ultrasonography examinations were performed as described previously (Kinder et al., 1996). Ovulation rate was defined as the proportion of dominant follicles present on the ovary following superstimulation that ovulated in response to pLH administration. In Nelore cows, growing follicles ≥8 mm in diameter were considered large follicles, morphologically dominant (Mihm et al., 1994) and potentially pre-ovulatory. Ovaries were assessed on Day 8 (at the time of pLH administration) and on Day 15 (at embryo recovery), when ovulation rate was determined. Unovulatory follicles were defined as large follicles that failed to ovulate after pLH administration at the embryo recovery (D15). Diameters of detected corpora lutea were estimated after analyzing frozen images recorded from the ultrasound to a pen drive. Corpora lutea volumes were also estimated using the formula: $V = 4/3 \times \pi \times R^3$, as described by Sartori et al. (2002).

2.4. Embryo recovery

Seven days after the first FTAI, embryos were collected (D15) by a nonsurgical technique (Reichenbach et al., 2001). A total of sixteen uterine flushes were obtained with a phosphate buffered saline solution (PBS, Nutricell, Campinas, Brazil). Embryos were graded as transferable embryos (excellent, good or average morulae and blastocysts), degenerated, or unfertilized, according to Lindner and Wright (1983). The sum of transferable embryos, degenerated, and unfertilized were considered as ova/embryos recovered.

2.5. Plasma progesterone concentration

At the time of embryo recovery (D15) blood samples were taken by jugular venipuncture in heparinized, evacuated tubes. Samples were kept at 4 °C and centrifuged within 4 h of collection; plasma was removed and frozen at −20 °C until assay. Plasma progesterone concentration was determined using a commercial solid phase radioimmunoassay system
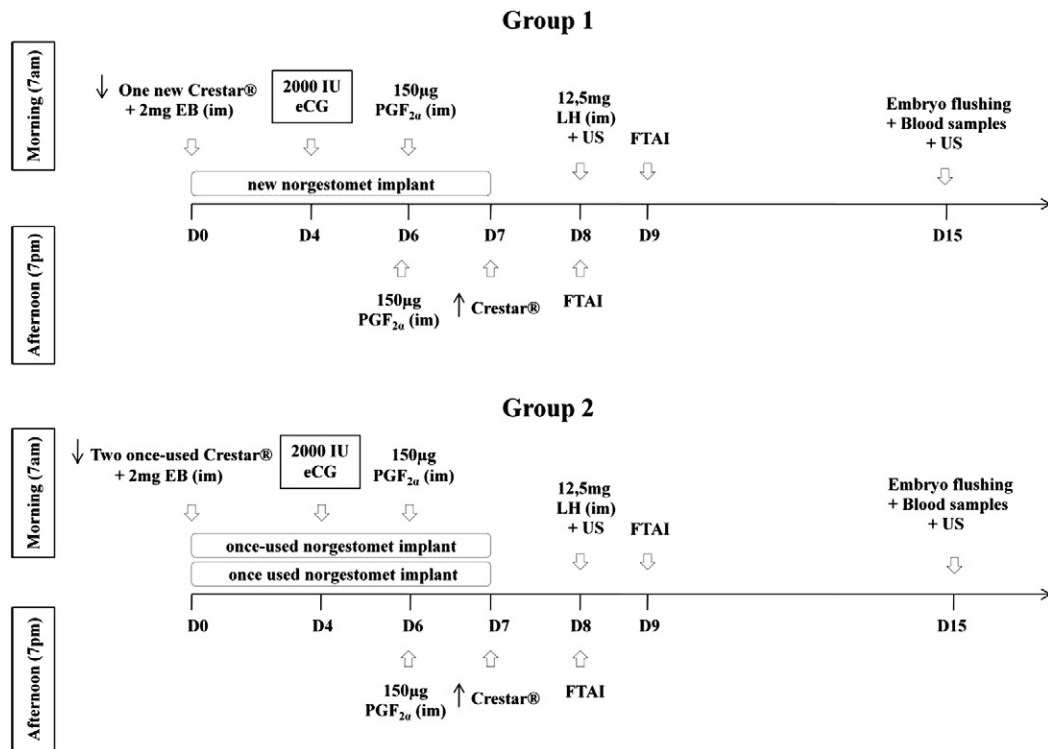
Fig. 1. Hormonal treatments used to induce eCG-based superovulation of Group 1 (one new norgestomet implant) and Group 2 (two once-used norgestomet implant) Nelore cows.

(Coat-A-Count, Diagnostic Products Corporation, Los Angeles, California, USA). The intra-assay CV was 5.60%, and the inter-assay CVs at concentrations of 2.5 and 6.0 ng/mL were 0.7 and 5.3%, respectively. Assay sensitivity was 0.01 ng/mL.

2.6. Statistical analysis

The unpaired *t* Test with Welch correction was used for the statistical analysis of continuous dependent variables (follicle diameter, CL diameter and volume and plasma progesterone concentration) as well as the discrete dependent variables (number of large follicles, number of CL, number of ova/embryos recovered and number of transferable embryos). Ovulation rate (proportion of dominant follicles present on the ovary following superstimulation that ovulated in response to pLH administration) was recorded as a ratio and analyzed through Fisher's exact test. The significance level of 5% ($P < 0.05$) was used and statistical analyses were performed with the GraphPad InStat 3 software (Graph Pad Software Inc., San Diego, CA).

3. Results and discussion

Main results are shown in Table 1. Follicle diameter on Day 8, CL diameter, CL volume, plasma progesterone concentration, number of ova/embryos recovered and transferable embryos did not differ ($P > 0.05$) between groups (Table 1). However, once-used implants reduced ovarian response of cows. Indeed, the number of large follicles and CL, as well as the ovulation rate

in cows from Group 1 was greater than those from Group 2 ($P < 0.05$; Table 1).

The SOV response is frequently measured by counting the numbers of CL (Cushman et al., 1999; Willett et al., 1953). In the present study, both treatments rendered an intermediate response (5–14 CL) according to the classification presented by Cushman et al. (1999). Previous reports described the role of hormonal treatment on the variation of the response after SOV challenge (Mapletoft et al., 2002; Martins et al., 2006, 2007; Murphy et al., 1984). Gonadotropin preparation, source, batch and biological activity were listed as sources of variation in the stimulation of ovaries (Murphy et al., 1984). In addition, a large variability in numbers of ovulations and transferable embryos has been proved to occur with eCG and other sources of gonadotropins, even at a constant dose (Elsden et al., 1978). In this study, mean number of ova/embryos recovered and transferable embryos was consistent (Table 1) with previous findings (Elsden et al., 1978; Monniaux et al., 1983).

Some authors described inconsistent stimulatory effects of eCG and have related such marginal response of eCG to its long half-life (Murphy and Martinuk, 1991), causing exaggerated follicular development, failure of ovulation (Gonzalez et al., 1994; Rubianes et al., 1996; Sudano et al., 2009; Ungerfeld et al., 1995), with continued follicular growth up to embryo collection and abnormal endocrine profiles coupled with poor embryo quality (Mikel-Jenson et al., 1982; Saumande et al., 1978, 1984). In the present study, ovulation failure occurred in one cow synchronized with two once-used implants, which

Table 1
Superovulation results and plasma progesterone concentration (mean ± SE, when applicable) of Nelore cows submitted to an eCG-based superstimulation protocol with one new or two once-used norgestomet implants.

	Group 1 (one new implant, n = 8)	Group 2 (two once-used implants, n = 8)
Large follicles at LH treatment; n	17.1 ± 1.8[a]	9.7 ± 1.6[b]
Follicle diameter at LH treatment; mm	10.5 ± 1.6	10.2 ± 1.7
Corpora lutea at embryo collection; n	13.8 ± 1.8[a]	5.4 ± 1.0[b]
Corpus luteum diameter at embryo collection; mm	15.7 ± 1.8	14.4 ± 1.7
Corpus luteum volume at embryo collection; mm^3	2232 ± 1356	1893 ± 1828
Ovulation rate; %	80.3[a]	53.8[b]
Ova/embryos recovered; n	6.3 ± 1.1	4.0 ± 1.9
Transferable embryos; n	4.0 ± 1.8	2.5 ± 1.0
Plasma progesterone concentration at embryo recovery; ng/mL	36.1 ± 8.3	35.1 ± 6.9

[a,b]Values with different superscripts in the same row are different ($P<0.05$).

was excluded from the data set for statistical analysis for such variables as ova/embryos recovered and transferable embryos. Nevertheless, performance of groups was not different in the production of embryos.

Protocols combining progestogens and different esters of estradiol resulted in most synchronous follicular wave emergence and initiate superstimulatory treatments at random stages of the estrous cycle in cows (Baruselli et al., 2006; Bó et al., 1995, 2006). Moreover, treatment with pLH 12 h after norgestomet implant removal synchronized ovulations, allowing for the application of FTAI in superstimulated *B. taurus indicus* cattle without the need of estrus detection and with no interference on the results (Baruselli et al., 2006). Such associations represented a breakthrough in conventional ET programs. However, hormonal treatment of SOV protocols is expensive and replacing FSH with eCG may reduce costs and labor. Another approach is the reutilization of previously used progesterone/progestogen releasing devices such as commercial norgestomet implants, which are very popular in Brazilian beef cattle operations. In the present trial, the combination of both strategies (i.e., stimulation with eCG and re-use of implants) allowed for a 19.5% cost reduction related to superovulation protocol. However, if the calculation is made based on the cost per transferable embryo recovered, the costs increased 28.9%.

Norgestomet (17α-acetoxy-11β-methyl-19-norpreg-4-ene-20, dione) is a modified 19-norprogesterone (Maluf, 2002). It is a progestogen with stronger biological activity than natural progesterone (Machado and Kesler, 1996). According to Machado and Kesler (1996) estrus suppression via the use of norgestomet silicone implants occurs due to the daily release of approximately 137–138 μg of norgestomet out of the implant. In another study, Kesler et al. (1995) showed that the norgestomet release peak occurs between Day 3 and Day 9, and that there is also a total release of 2.3 mg of norgestomet over the 9 days in which the implant remains in situ. Machado and Kesler (1996) reported that auricular silicone implants designed to contain 6 mg of norgestomet inserted in beef cows for 9 days displayed a decrease in the norgestomet load of 2.06 mg. However, there is no report in literature evaluating the hormone release rate from Crestar® implant, which contains 3 mg of norgestomet with different surface area from the ones in aforementioned studies. Indeed, some proportion of cows implanted with a single once-used device may display estrus in FTAI protocols (unpublished data from our lab). Therefore,

comparison between a new and a once-used device was abandoned and the decision to treat Group 2 cows with two once-used implants was reinforced. It was postulated that two once-used implant would have enough norgestomet to keep cows out of estrus and stimulate changes within the uterine environment which regulate receptivity and promote embryos' survival.

Nonetheless, Maluf (2002) assessed the use of one new implant, one once-used implant or two once-used implants of norgestomet and obtained no difference on the conception rate after FTAI for non-lactating (37.5; 34.0 and 39.5%) and lactating cows (44.0; 35.7 and 32.0%), respectively. Similarly, Almeida et al. (2006) reported no difference in the conception rate of cows treated with either one new or a once-used implant (48.3 and 48.7%, respectively). Furthermore, Dias et al. (2009) observed that the pregnancy rate was greater for the third-use treatment than those for the second-use and first-use CIDR treatments in Nelore heifers submitted to FTAI; and Claro Júnior et al. (2010) reported that a progesterone based treatment using a previously used CIDR in *B. taurus indicus* heifers resulted in greater follicular diameter, as well as a higher conception and pregnancy rates, than the new CIDR treatment. These studies support the reutilization of previously used progesterone/progestogen releasing devices for FTAI without difference in performance or in conception and pregnancy rates. However, the efficacy of reusing a progesterone/progestogen releasing device for superovulation of embryo donors observed in the present study was not as successful as the aforementioned studies with FTAI. Indeed, there was a significant difference in the superovulation response between a new or two once-used implants after considering Day 8 large follicles and Day 15 CL numbers, as well as ovulation rate. However, such difference did not affect ($P>0.05$) ova/embryos recovery and transferable embryos (Table 1); probably because of the limited number of cows per treatment. Ova/embryos recovery and transferable embryos were both reduced approximately 37%.

Sanchez et al. (1995) reported that an implant containing 3 mg norgestomet in mature heifers without a corpus luteum resulted in a typical LH pulse frequency for the follicular phase (approximately 1 pulse/h). However, two or four norgestomet implants suppressed LH pulse frequency to that of the luteal phase (Anderson et al., 1996), supporting the contention that circulating progesterone concentrations modulate LH concentrations and pulsatility and, in turn, growth of the ovulatory follicle (Adams et al., 1992). Circulating progesterone concentrations are

known to affect follicular development (Adams et al., 1992) and oocyte quality (Blondin and Sirard, 1995; Leibfried-Rutledge et al., 1987; Pfeifer et al., 2005). Several studies have shown that variations in circulating progesterone concentrations affect fertility after a synchronized breeding (Carvalho et al., 2008; Shaham-Albalancy et al., 2000).

Results herein described were in contradiction of the hypothesis that two once-used norgestomet implant would have the same SOV response of a single new implant in an eCG-based superovulation protocol administered to Nelore cows. The reduced SOV response in the two once-used implant treatment might be related with an abnormal, erratic, and lesser norgestomet release pattern which might have allowed for the emergence of an asynchronic follicular wave within the induced cycle, a phenomenon which could limit the superstimulatory effect of eCG and result in a reduced number of large follicles. Moreover, the lesser norgestomet release pattern probably allowed the presence of dominant follicles at the beginning of the superstimulatory treatment which also decreased the ovulation rate in Group 2 cows. This is in agreement with previous results indicating that the presence of dominant follicles at the start of superstimulation treatment is associated with a lower ovulation rate in cattle (Guilbault et al., 1991), primates (Goodman and Hogden, 1983) and ewes (Driancourt, 1987).

Furthermore, the unovulatory follicles observed at embryo recovery in the two once-used norgestomet implants group (present in 12.5% of cows) possibly were follicles that failed to be suppressed following EB administration, which also reinforced that circulating progesterone was low since the implant insertion. These observations support the hypothesis that the ovarian status at the beginning of treatment accounts for a large portion of the variability in the superovulatory response (Monniaux et al., 1983).

Despite the greater number of CL ($P<0.05$) observed in cows from Group 1 on D15, there was no difference ($P>0.05$) in plasma progesterone concentration between groups. Therefore, the reason why progesterone reached the same concentrations after both treatments is yet to be determined. Maybe, a possible explanation was that low progesterone concentrations, and consequently high LH pulse–frequency during the ovulatory wave were associated with overgrowth of the dominant follicle (Adams et al., 1992), which may improve CL function (Perry et al., 2007) of cows from Group 2. Moreover, the large number of CL must be considered in inhibiting progesterone secretion by luteal tissue from Group 1, i.e., greater progesterone concentration caused a down regulation of progesterone production (Moseley et al., 1979). Similarly, Garcia-Winder et al. (1988) observed that the use of a new norgestomet implant in superovulated beef cows reduced progesterone concentration compared with the untreated control group.

In conclusion, the administration of two norgestomet implants, previously used for nine days, was less effective to superovulate Nelore cows than the use of a new implant.

Acknowledgments

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