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Original Article - Veterinary Medicine

Effect of long-acting injectable progesterone supplementation, seven days after insemination, on embryo and fetal development in Nellore cows

[*Efeito da suplementação com progesterona injetável de longa ação, sete dias após inseminação, sobre o desenvolvimento embrionário e fetal de fêmeas Nelore*]

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ABSTRACT

This study evaluated the effects of long-acting injectable progesterone supplementation (P4LA) on embryonic and fetal development and birth weight in Nellore cows seven days after timed artificial insemination (TAI). One hundred and nineteen Nellore female cows became pregnant after the TAI protocol and were divided into two groups: P4LA with cows that received 150mg of P4LA, seven days after insemination, in a single dose, and the control group, with cows who did not receive any hormonal supplementation after TAI. Blood samples were collected on days 17 and 30 after TAI to determine P4 concentration. Embryonic and fetal measurements were performed at 30 and 45 days of gestation, respectively, with the aid of ultrasonography, measuring craniocaudal length and thoracic diameter. No difference was observed in P4 concentration between the groups supplemented with or without injectable P4 on days 17 (P=0.73) and 30 (P=0.62) after TAI. There was no significant difference in embryonic and fetal development or birth weight between the supplemented and non-supplemented groups (P=0.59, P=0.09, and P=0.64, respectively). Supplementation with injectable progesterone seven days after TAI did not interfere with the embryonic and fetal development of Nellore cows, nor did it affect birth weight.

Keywords: progestin, beef cattle, embryo measurement, fetal development, ultrasound

RESUMO

O presente estudo avaliou o efeito da suplementação de progesterona injetável de longa ação (P4LA), sete dias após a inseminação artificial em tempo fixo (IATF), em matrizes Nelores, sobre o desenvolvimento embrionário, fetal e o peso ao nascimento. Para tanto, 119 fêmeas Nelores que ficaram gestantes após protocolo de IATF foram divididas em dois tratamentos: grupo P4LA, com fêmeas que receberam 150mg de P4 injetável de longa ação, em dose única, sete dias após a IATF; grupo controle, com fêmeas que não receberam nenhuma suplementação após a IATF. Amostras de sangue foram coletadas nos dias 17 e 30 após a IATF, para determinação da concentração de P4. A mensuração embrionária e fetal foi realizada aos 30 e 45 dias de gestação, respectivamente, com o auxílio da ultrassonografia, pela mensuração do comprimento craniocaudal e o diâmetro torácico. Não foi observada diferença na dosagem de P4 entre o grupo suplementado ou não no dia 17 (P=0,73) e (P=0,62) após a IATF. Não houve diferença significativa entre o desenvolvimento embrionário e fetal dos grupos suplementados ou não (P=0,59; 0,09, respectivamente). Suplementação com progesterona injetável sete dias após a IATF não interferiu no desenvolvimento embrionário e fetal de matrizes Nelores, assim como no peso ao nascimento.

Palavras-chave: progestágeno, gado de corte, mensuração embrionária, desenvolvimento fetal, ultrassonografia

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INTRODUCTION

The search for better herd production rates has intensified because system efficiency is directly linked to profitability. This requires high reproductive efficiency in animals. Timed artificial insemination (TAI) has become increasingly popular, particularly among beef herds, and we seek to continuously improve this tool.

Hormonal treatments performed after TAI are increasingly used to obtain better pregnancy rates and reduce gestational losses; however, many studies are still needed to evaluate the efficiency of these protocols and their consequences. The use of progesterone after TAI has increased to meet these criteria (Couto *et al.*, 2019). However, the results remain quite varied, with null, positive, and negative results mainly correlated with the timing of P4 supplementation (Yan *et al.*, 2016).

The endometrium is responsible for the secretion and transport of substances called histotrophs, which help elongate the conceptus by affecting the proliferation and migration of the trophectoderm, and fixation and adhesion to the luminal epithelium of the uterine endometrium (Bazer *et* a*l.*, 2011). The indirect stimulatory effect of P4 on trophoblast elongation via the endometrium is indisputable because of gene expression in endometrial cells, which leads to changes in the composition of the uterine luminal fluid (ULF) to which the developing embryo is exposed (Faulkner *et al.*, 2013). Thus, the objective of the present study was to evaluate whether supplementation with P4LA, seven days after TAI (initial diestrus), can improve the uterine environment and stimulate not only the elongation of the conceptus but also embryonic and fetal development, thus improving the reproductive efficiency of the herd.

MATERIAL AND METHODS

This study was approved by the Animal Ethics and Use Committee (CEUA) of the Instituto de Medicina Veterinária, Universidade Federal do Rio de Janeiro (No. 6993220319). The experiment was conducted at a commercial beef cattle farm (Fazenda Reunidas Ingaiba) located in Mangaratiba-RJ, between November 2018 and January 2020.

During the study, four hundred and three females (nulliparous and pluriparous) were subjected to an ovulation synchronization protocol. All the animals were subjected to the same hormonal treatment for subsequent TAI. On the first day of the protocol (D0), an intravaginal progesterone device (1g of P4) was inserted and 2mg of estradiol benzoate was administered intramuscularly. On day 8 of the protocol, the P4 device was removed, and 500 µg of sodium cloprostenol, 400 IU of equine chorionic gonadotropin, and 1 mg of estradiol cypionate were intramuscularly administered. TAI was performed on D10 by the same technician.

A total of 119 pregnant dams (cows and heifers) were randomly selected for the experiment. After TAI, the animals were divided into two treatments according to body condition score, cyclicity, and presence of a calf so that each treatment had the same proportion of animals for each variable.

- Group P4 (GP4; n=60): females that received 150 mg of long-acting injectable progestin (Sincrogest® Injectable, Ourofino, Cravinhos/SP) intramuscularly, in a single dose, seven days after TAI.
- Control Group (CG; n=59): females that did not receive hormonal supplementation after TAI.

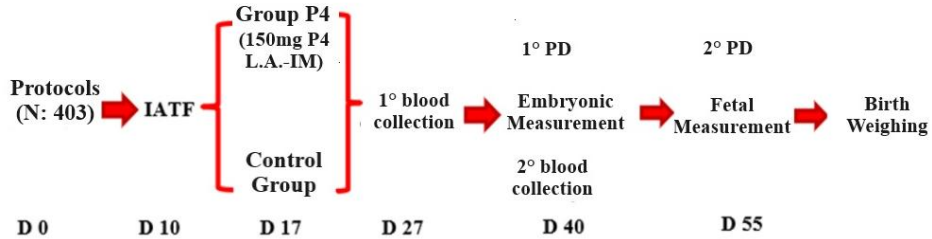

Figure 1. Schematic representation of the experimental procedures used to evaluate the effect of long-acting injectable progesterone (P4) supplementation, seven days after timed artificial insemination (TAI), on embryonic and fetal development of Nellore females, as well as birth weight. Intramuscular (IM); Pregnancy diagnosis (PD).

Blood samples were collected from 36 randomly selected animals (heifers and cows with calves) on days 27 and 40 after the start of the ovulation synchronization protocol (Fig. 1), 10 days after P4LA application. on the day of the first ultrasound evaluation of embryonic measurements. Blood samples were used to determine P4 concentration (Martins *et al*., 2019). For this purpose, 10 mL of blood was collected from the coccygeal artery in heparinized vacutainer tubes. The blood samples were centrifuged at 1500×*g* for 15 min, and the plasma was separated, stored in sterilized identification tubes, and kept at -20°C until the time of testing. P4 concentrations were determined by radioimmunoassay using commercial kits (ImmuChem, MP Biomedicals, Santa Ana, California, USA) approximately 60 days after collection. The sensitivity and intra-assay coefficient were 0.05ng/mL and 11%, respectively. All data were within the maximum and minimum points of the curve.

Embryonic measurements were carried out at 30 days of gestation with the aid of an ultrasound device (Mindray DP200, frequency 7.5 Mhz), and measurements were obtained from 119 pregnant females. As a parameter of embryonic development, the craniocaudal length (crown to rump length, CRL) was measured, which is the distance determined by a line between the anterior part of the skull (occipital bone) and the base of the tail (first coccygeal vertebra, as illustrated in Fig. 2) using a methodology adapted from Oosthuizen *et al*. (2018). Thoracic diameter (DTO) was assessed at 45 days of gestation by measuring the maximum distance between the lateral ends of the rib cage (i.e., between the vertebrae and sternum; Fig. 3), following the methodology described by Hunnam *et al.* (2009). The DTO measurement was carried out on only 17 females that had already been used to determine the CRL owing to the difficulty of the measurement and the excessively long time required, as the work was carried out on a commercial property.

The birth weight of the calves was also evaluated. Weighing at birth was performed by farm employees using weighing tape in the field on the day of the birth.

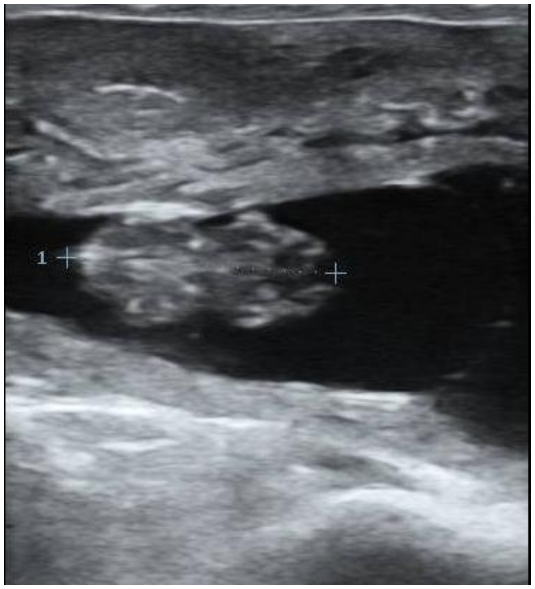

Figure 2. Ultrasound image of a bovine fetus showing craniocaudal length (CRL, crown-to-rump length) was defined as the distance from the anterior part of the skull to the base of the tail.

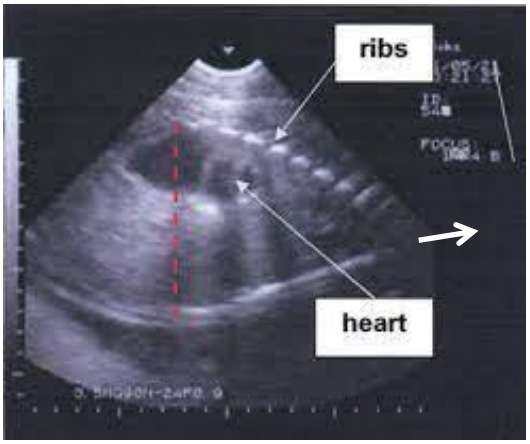

Figure 3. Ultrasound image of a bovine fetus showing measurements related to fetal thoracic diameter (DTO) (Source: Hunnam *et al*., 2009).

The quantitative variables [CRL (mm); P4 D17 (mg/L); P4 D30 (mg/L); DTO (mm); Birth weight (kg)] were compared using Student 's t test, at 5% of probability. Previously, the homoscedasticity of variance was assessed using Bartlett's test. All statistical analyses were performed using the R software (R Development…, 2021).

RESULT AND DISCUSSION

As shown in Table 1, there was no significant difference between the groups supplemented with or without P4LA, seven days after TAI, both for embryonic development and in the progesterone assay at 17 and 30 days after TAI.

The fact that no difference was observed in the concentration of progesterone on day 17 between the groups treated or not treated with P4LA may be related to the presence of only the residual concentration of exogenous P4 (<1.2ng/mL) 10 days after its application, as the P4 peak occurred 48 h after its application (6.54ng/mL), declined for up to 96 h, and then remained stable at residual levels for up to 240 h (Morotti *et al.*, 2018). Furthermore, P4LA supplementation 7 days after TAI probably did not affect the development of the corpus luteum (CL) and consequently did not interfere with P4 concentrations until day 30 of gestation. These results may be related to the timing of P4LA supplementation (seven days after TAI), which would not have been sufficient to induce early luteolysis or alter the uterine environment, leading to greater embryonic development.

Table 1. Craniocaudal length (CRL) at 30 days; progesterone concentration (P4) at 17- and 30-days post-timed artificial insemination, thoracic diameter (TOD) at 45 days, and weight at birth of calves from cows that received or did not receive progesterone supplementation (P4), seven days after timed artificial insemination

Variable	With P4	Without P4	*P* value
CRL (mm)	15.96±2.57	16.52±1.45	0.59
P4 D17 (ng/L)	5.81±1.90	5.43±2.46	0.73
P4 D30 (ng/L)	4.60±1.58	4.05±2.53	0.62
DTO (mm)	13.83±1.85	12.04±2.09	0.09
Birth weight (kg)	39.94±3.99	40.64±4.91	0.64

In cattle, P4 acts on the cells of the uterine luminal epithelium, negatively regulating their own receptors. P4 allows the positive regulation of estrogen receptors that induce the expression of oxytocin receptors (OXTR) and, therefore, stimulates the production of PGF2α and luteolysis. Studies have shown that the day of P4 supplementation can induce early luteolysis due to the acceleration of the negative regulation of progesterone receptors in the cells of the luminal epithelium of the uterus, leading to an acceleration of the expression of endometrial genes associated with the synthesis of PGF2α, specifically RE1 and OXTR (Batista *et al.*, 2019). In addition to being related to a probable decrease in LH levels normally released at this stage, which is essential for stimulating steroidogenesis and the formation of a developed luteal mass, P4 itself can inhibit its function and harm the early development of the CL (Batista *et al*., 2019).

O'Hara *et al.* (2013) supplemented heifers with an intravaginal P4 device 3–7 days after TAI and reported that CL weight and P4 concentration were lower in the supplemented animals 16 days after TAI; these results were associated with the early induction of luteolysis. They further reported that animals supplemented with progesterone had more elongated embryos and greater interferon-T (IFNT) levels than non-supplemented animals. However, this increase in IFNT production, provided by greater elongation of the conceptus, was not sufficient to overcome luteolytic signaling caused by P4 supplementation at the beginning of diestrus. Therefore, it was concluded that early luteolysis caused by P4 supplementation at the end of metestrus and the beginning of diestrus was harmful and compromised the fertility of the supplemented cows (Martins *et al.*, 2019).

The results of the present study are consistent with those reported by Yan *et al*. (2016), who observed that progesterone supplementation was harmful before day 3 by inducing early luteolysis and had no effect from day 7 onwards. These variations in results may be directly related to LH pulsatility in this initial phase, since there is an inverse relationship between P4 concentration and LH pulse, where the LC in primary development depends on LH receptors in theca cells and granulosa, thus making it vulnerable to hormonal variation (Niswender *et al.*, 1994). In other words, when supplementation with exogenous P4 occurs early (between 3–5 days), a

change in LH pulsatility may occur, thus affecting the development of the initial CL and, consequently, its production of P4.

Supplementation with P4 (after day 7) may be responsible for the lack of differences in embryonic development between groups treated with or without P4LA, as shown in Fig.4.

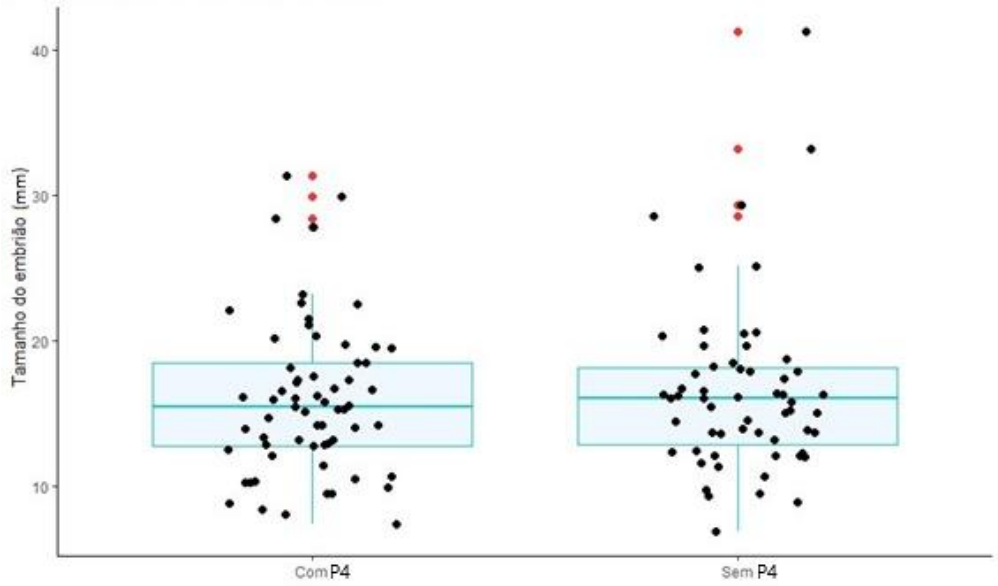

Figure 4. Box plot graph demonstrating the distribution of embryonic size (cranial-caudal length) measured at 30 days of gestation in the groups supplemented with or without progesterone seven days after TAI. No statistically significant differences were observed between the groups. (N=119)

In the present study, no differences in embryonic development were observed between animals supplemented with or without P4LA after TAI. This may be related to the late increase in P4, as preparation of the maternal system directly involves circulating concentrations of P4 (Forde *et al.*, 2011). The expression of genes, secretion of protein products, and active transport of other molecules that elongate the conceptus require negative regulation of the nuclear progesterone receptor in the luminal and glandular epithelia (Spencer and Hansen, 2015). In addition, Geary *et al*. (2016) showed that P4 induces the expression of several genes, specifically in the endometrial epithelium, which is stimulated by embryonic factors such as IFNT and prostaglandins. This results in a change in the environment and uterine luminal fluid, which promotes the survival, elongation, and implantation of the embryo to establish pregnancy. Therefore, low concentrations of circulating P4 during the early luteal phase may lead to altered gene expression in the uterine endometrial cells, suboptimal embryonic growth, and reduced pregnancy rates (Forde *et al*., 2011).

It was demonstrated that increased P4 in early diestrus altered maternal-embryo communication, and that an environment with high P4 concentrations was consistent with conceptus elongation. Furthermore, it was reported that the abundance of lipids in the ULF on day 14 post-insemination, in response to the increase in P4, where 47% of the lipids identified were linked to the biogenesis of the conceptus membrane, suggests that the secretion of ULF directly assists in the development of the conceptus membrane (Van Meer and De Kroon 2011). This suggests that the maternal lipid supply during the elongation window is primarily aimed at conceptual membrane biogenesis (Simintiras *et al*., 2019). Considering the above information, it is also possible to speculate that the increase in progesterone in the initial diestrus has an effect only on embryonic elongation and does not extend this effect until 30 days of embryo growth, which explains why no difference was observed between the embryonic development status of animals treated or not treated with P4LA.

Regarding DTO, P4 supplementation also had no effect on fetal development when comparing the groups supplemented or not supplemented with P4LA (P=0.09), as shown in Table 1, by evaluating the DTO at 45 days of gestation.

The results of the present study are in line with those reported by Stratman *et al.* (2020), who also found no significant correlation between embryonic and fetal development and circulating progesterone levels in cows and heifers when monitored using ultrasound measurements between days 33 and 45 of gestation. These authors stated that these results do not invalidate the results of previous studies with a similar focus, which indicated that progesterone affects embryonic growth, with most studies on embryonic development and progesterone being carried out during early pregnancy in the first two weeks after artificial insemination.

Fetal programming refers to the effect of the uterine environment on the health and well-being of offspring. The effects of fetal programming on neonates may be mediated by epigenetic modifications that regulate gene expression in the placenta and the fetus (Vickaryous & Whitelaw, 2005). Regarding maternal regulatory factors, estrogen and progesterone receptors have already been detected in placental cells, suggesting that these hormones play a role as local regulators of the growth, differentiation, and function of these structures (Mishra *et al.*, 2013). Furthermore, RNA of ADAM metallopeptidase with thrombospondin type 1 motif 1 was detected. *Thrombospondin I* (*ADAMTS1*) is highly responsive to P4 concentrations in the endometrium and placental tissues, suggesting that *ADAMTS1* may act as a key regulator of endometrial functions, regulating the necessary extracellular matrix remodeling, developmental processes, or both for implantation and development of the placenta in cattle, thereby improving embryonic and fetal development (Mishra *et al.*, 2013). However, no other studies have been carried out following the embryonic and fetal development of animals supplemented with progesterone during early diestrus. More studies are needed to evaluate the extent of the effects of progesterone supplementation on embryonic/fetal growth, and whether variations in the day of supplementation interfere with these results.

Burns *et al.* (2018) did not find a correlation between embryonic craniocaudal size and birth weight; however, they suggested a placental capacity to boost fetal growth because they found a relationship between birth weight and the size and thickness of placentomes at weeks seven and eight of pregnancy. Associated with the findings of these authors, as P4 did not influence embryonic and fetal development, it may not have influenced placental development, which could explain the lack of differences in the birth weights of the calves in the present study. However, no studies have evaluated the effect of progesterone supplementation on birth weight, highlighting the need for further studies in this area.

CONCLUSION

Supplementation with long-acting injectable progesterone seven days after TAI, under these conditions, did not affect the embryonic and fetal development of Nellore dams or the birth weight of calves.

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