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ANIMAL REPRODUCTION

An efficient shorter protocol for artificial induction of lactation in heifers

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ABSTRACT: Protocols to artificially induce cows and heifers into lactation are effective and commercially available in Brazil. However, these protocols demand long hormonal treatments, which is debatable since little research has been done in the subject. To further understand artificial induction to lactation (AIL) protocols, we conducted two experiments. In experiment 1, our objective was to better characterize steroidal profile during an AIL protocol and assess the estradiol (E2) and progesterone (P4) serum profiles of Jersey cows (n=6) subjected to a conventional protocol. In experiment 2, we aimed to compare milk production and serum E2 concentrations of Holstein heifers induced into lactation by the standard AIL protocol (15 injections of E2 and 8 P4 injections) and by a short protocol in which the number of E2 injections and, consequently, the overall dosage of E2, were reduced (8 injections of E2 and 8 P4 injections). We hypothesized that a short AIL protocol (8 days) would be as efficient as the long standard protocol of 15 days. Our hypothesis was confirmed, since we demonstrated that a shorter protocol was able to induce lactation in Holstein heifers without hindering milk production.
Key words: artificial induction to lactation, dairy cow, estradiol benzoate, injectable progesterone, intravaginal device.

Eficiência de um protocolo curto de indução artificial da lactação em novilhas

RESUMO: Protocolos que induzem à lactação artificial em vacas e novilhas são eficazes e estão comercialmente disponíveis no Brasil. No entanto, esses protocolos demandam longos tratamentos hormonais, o que é discutível visto que poucas pesquisas foram feitas sobre o assunto. Para entender melhor os protocolos de indução artificial à lactação (IAL), realizamos dois experimentos. Foi testada a hipótese de que um protocolo curto (oito dias) de IAL é tão eficiente quanto o protocolo padrão de 15 dias. No experimento 1 objetivou-se caracterizar o perfil esteroidal durante um protocolo de IAL e avaliou-se os perfis séricos de estradiol (E2) e progesterona (P4) de vacas Jersey (n=6) submetidas a um protocolo convencional. No experimento 2 foram comparadas a produção de leite e as concentrações séricas de E2 de novilhas holandesas induzidas à lactação pelo protocolo padrão de IAL (15 injeções de E2 e 8 injeções de P4) e por um protocolo curto, no qual o número de injeções de E2 e, consequentemente, a dosagem geral de E2 foram reduzidas (oito injeções de E2 e oito injeções de P4). A hipótese foi confirmada, demonstrado que um protocolo mais curto induziu a lactação em novilhas holandesas sem prejudicar a produção de leite.
Palavras-chave: indução artificial à lactação, vaca leiteira, benzoato de estradiol, progesterona injetável, dispositivo intravaginal.

INTRODUCTION

High milk production is strongly associated with declining fertility in dairy cows (BERRY et al., 2014). Hence, reproductive problems are among the main causes of culling in dairy herds (FETROW et al., 2006), which may reduce the profitability of the production systems. A strategy for avoiding the culling of infertile cows is artificial induction of lactation (AIL), through the administration of exogenous hormones that mimic the endocrine profile of females in the final period of gestation, allowing the production of milk in the absence of pregnancy (FREITAS et al., 2010, JORDAN et al., 1981, HARNESS et al., 1978, HENRICKS et al., 1972).

AIL protocols have been used and studied for many decades (MALPRESS & OWEN, 1947), and currently have efficacy varying between 70 and 100%. These protocols can induce between 65 and 78% of the milk production obtained from females in physiological lactation (SMITH & SCHANBACHER, 1974, HARNESS et al., 1978, MELLADO et al., 2006, FREITAS et al., 2010, LUZ et al., 2020). Although the AIL protocols have been developed almost 50 years ago, no major advances have been made regarding hormonal dosages, treatment/management periods, and administration routes. The scarcity of new data

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may be due to the existing restriction of the use of some hormones in animal production in many countries (LANE et al., 2008).

The commercially AIL available protocols in Brazil are based on the daily administration of high hormonal doses (estradiol, progesterone, prostaglandin, and corticosteroids) and lasting approximately 21 days of animal handling until the beginning of lactation (PESTANO et al., 2015). In these protocols, the administration of the hormones is usually intramuscular. Other routes; however, such as intravaginal route for progesterone (P4) administration, may also be able to provide satisfactory results (DAVIS et al., 1983).

The period of estradiol (E2) application and its commonly used high doses are also debatable, since a shorter protocol, with E2 treatment for only seven days has shown promising results (MACRINA et al., 2011, SMITH & SCHANBACHER, 1974). However, to our knowledge, there are no studies comparing milk production obtained using a long conventional (15 days) and short protocols (8 days). In a previous study by our group, using the conventional protocol, it was observed significantly higher E2 concentration in heifers submitted to AIL compared to heifers in the last two weeks of gestation (LUZ et al., 2019). In the same study, P4 concentrations in the induced group were also higher than the control group (pregnant heifers) during the pre-lactation period, when they received daily injections of long-acting P4, which indicated a possible cumulative effect of this hormone. Further data regarding the metabolism curves of exogenous P4 and E2 provided in the AIL protocols, either by injectable or intravaginal route, however, are scarce.

Therefore, it is important to improve the existing protocols and make them more effective in terms of production, responsiveness, and animal welfare, by avoiding accidental lesions by long periods of estrus expression, for example. Hence, it is essential to investigate the hormonal profile of animals submitted to AIL. Based on this knowledge, we can then seek to simplify the protocol by reducing the number of handlings to which the animals are exposed and also the labor and costs. The identification of the lowest effective hormonal dosage, which is capable of promoting the endocrine profile closest to that observed in the final period of a physiological pregnancy, by exploring the half-life of drugs is also key. Thus, this study evaluated the serum profile of P4 and E2 in long and short AIL protocols and also assessed whether a protocol with a lower number of E2 injections (short protocol) is able to induce lactation in Holstein heifers with milk yields comparable to the ones provided by the long conventional protocol of 15 days of E2 injections.

MATERIALS AND METHODS

Experiment 1 – Progesterone and Estradiol serum levels during AIL protocol

To evaluate the P4 and E2 curve during the AIL protocol, 11 non-pregnant and non-lactating Jersey cows, with an average weight of 482 kg (ranging from 444 to 513 kg) were used. Aiming to eliminate possible functional corpus luteum and avoid the influence of endogenous P4 at the beginning of the trial, all cows received two 7 days apart (D -7 and D0) i.m. injections of 0.25 mg of sodium cloprostenol (Sincrocio® Ourofino Saúde Animal, São Paulo, Brazil). The animals were allocated into two groups (Figure 1A): The IVD control group (IVD; n = 6) received only one intravaginal device (IVD) containing 1 g of P4 (DIB® Zoetis, Campinas, Brazil). The IVD was replaced by a new one 7 days later (14 days of exposure) to prevent the animals from manifesting estrus. The induced cows (AIL; n = 5) were submitted to an AIL protocol that started with 0.25 mg/kg i.m. P4 (Fagron do Brasil Farmacêutica, São Paulo, Brazil) and estradiol benzoate (EB; 0.1 mg/kg) (Fagron do Brasil Farmacêutica, São Paulo, Brazil) from D0 to D6, followed by administration of EB alone (0.1 mg/kg) from D7 to D14. In addition, cows from the AIL group received three i.m. injections of dexamethasone on D19 to D21 (0.05mg/kg) (Cortiflan® Ourofino Saúde Animal, São Paulo, Brazil) and an i.m. injection of 0.25 mg of sodium cloprostenol on D17. Blood samples were collected by coccygeal venipuncture using a vacuum system (Vacuplast®, Brazil) on days 0, 2, 4, 8 and 12 of the AIL protocol. The tubes were centrifuged at 5000x g for five minutes to accelerate the separation of the serum, which was then stored in -20 ºC until the time of the evaluations. The serum levels of P4 (Elecsys Progesterone III Cobas Assay; Roche Diagnostics, Mannheim, Germany; REF 07092539; sensitivity: 0.21 ng/mL; intra-assay CV: 8.6%) and E2 (Elecsys Estradiol III Cobas Assay; Roche Diagnostics, Mannheim, Germany; REF 06656021; sensitivity: 11.8 pg/mL; intra-assay CV: 9.1%) were determined by electrochemiluminescence. All the samples were run in a single P4 or E2 assay. The animals were kept with ad libitum access to water, natural pasture, and mineral salt during the experiment.

Experiment 2 – Milk production of heifers submitted to long conventional or short AIL protocols

In experiment 2, the milk yield of heifers artificially induced with lower E2 dose and fewer injections was compared to a conventional protocol. For this purpose, 10 repeat breeder Holstein heifers, that did not conceive after three or more breeding,

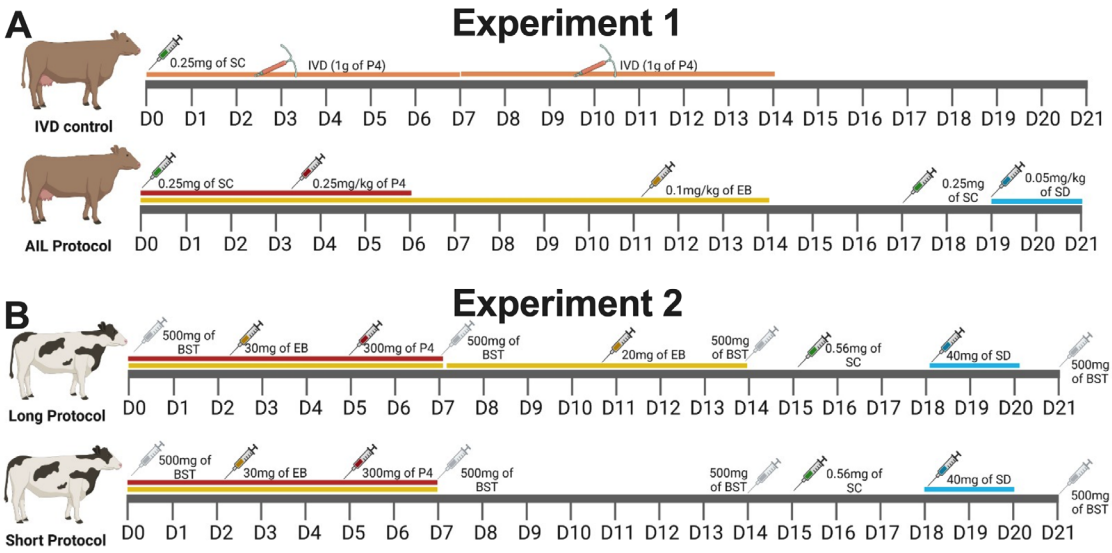

Figure 1 - (A) Hormonal protocols used in Experiment 1. All the cows received two 7 days apart (D -7 and D0) i.m. injections of 0.25 mg of sodium cloprostenol. Serum progesterone concentration was evaluated in Jersey cows subjected to an artificial induction to lactation (AIL) protocol (AIL; n = 5) or not (IVD; n = 6). Estradiol concentration was evaluated only in estradiol-treated cows (AIL; n=5). (B) Hormonal protocols used in Experiment 2 to compare milk production in Holstein heifers after the standard (LongP; n = 5) or the short protocol (ShortP; n = 5) AIL protocol. IVD: intravaginal device; SC: sodium cloprostenol; P4: progesterone; EB: estradiol benzoate; SD: sodium dexamethasone; BST: bovine somatotropin.

with an average age of 30 months and an average body weight of 430 kg were used. The heifers were allocated in two groups (Figure 1B): Long conventional Protocol (LongP; n = 5) and Short Protocol (ShortP; n = 5). The LongP consisted of i.m. injections of 30 mg of EB (Gonadiol® Zoetis, Campinas, Brazil) and 300 mg of P4 (Sincrogest® Ourofino Saúde Animal, São Paulo, Brazil) daily, from D0 to D7. From D8 to D14, the heifers only received daily doses of 20 mg of EB. On D15, the animals received an i.m. injection of 0.56 mg of sodium cloprostenol (Sincrocio® Ourofino Saúde Animal, São Paulo, Brazil). From D18 to D20, 40 mg of sodium dexamethasone (Cortiflan® Ourofino Saúde Animal, São Paulo, Brazil) was i.m. injected daily. On days 0, 7, 14, 21, and then every 14 days after the start of milking, the animals received a dose of 500 mg of bovine somatotropin (BST) (Lactotropin® Elanco Saúde Animal, São Paulo, Brazil). On D21, the milking period of the heifers began, which lasted for 260 days. Milkings were performed twice a day with a 12-hour interval. The ShortP differed from the LongP protocol only by omitting the administration of 20 mg of EB from D8 to D14. The animals were not handled in this period. The milk production of heifers was measured using the automatic milking parlor system (Afimilk Agricultural Cooperative, Afikim, Israel), on days 22, 29, 36, 43, 50 and then fortnightly for the next 260 days. On days 0, 5, 10 and 15, blood samples were collected from the coccygeal vessels using vacutainer tubes with clot activator and 25 x 0.70 mm needles (Vacuplast®, Brazil). The blood was then centrifuged to obtain the serum, which was used to measure serum estrogen levels using electrochemiluminescence (Elecsys Estradiol III Cobas Assay; Roche Diagnostics, Mannheim, Germany; REF 06656021; sensitivity: 11.8 pg/mL; intra-assay CV: 6.3%).

During the lactation period, twice a day, the heifers were fed with a mixed ration containing 5kg of concentrate (20% protein) and 7kg of corn silage, and mineral salt. Animals from both groups were kept together, in the same paddocks, and had free access to pasture (oats/ryegrass in the cold season, and Tifton, in the hot season). The experiment was conducted in a single replicate, that means that the heifers were kept under the same grazing condition throughout the experiment.

Statistical analysis

The statistical analysis was performed using the Graphpad Prism 8 software. To compare

the curves of serum concentrations and milk production between the groups in both experiments, a two-way ANOVA was used, to assess the effects of the group, day (or moment) and group/day interaction. $P < 0.05$ was considered statistical difference.

RESULTS

Experiment 1 – P4 and E2 serum levels during AIL protocol

Overall, P4 levels were higher on the AIL group (Figure 2A). However, on day 12, AIL group had lower ($P < 0.001$) P4 levels (0.5 ng/ml) than IVD group (2.8 ng/ml). E2 levels, conversely, were maintained over 2000 pg/ml during the 12 days of protocol (Figure 2B). Estradiol concentration in IVD group (control) was not evaluated because the cows were not treated with estradiol and were maintained with IVD containing progesterone, which decreases follicular growth and estradiol synthesis and inhibits estrus. All the cows from AIL group responded to the hormonal treatment with the development of the mammary gland and milk secretion; although, milk production was not evaluated.

Experiment 2 –Milk production of heifers submitted to a shorter AIL protocol

As expected, on D5 and D10, the serum E2 levels were over 2000 pg/ml and did not differ between the groups (Figure 3A). On day 15, however, the E2 levels were lower ($P < 0.001$) in the ShortP (386.1 pg/ml) than in the LongP (2007 pg/ml), since the heifers were no longer receiving EB injections in ShortP (Figure 3A). The lower levels, however, did not affect the milk production. The milk yield of the heifers from both LongP and ShortP did not differ ($P = 0.99$) throughout the milking period nor at any day of evaluation (Figure 3B). On day 62 of lactation the average (±standard error) milk production was 15.2±1.2 and 15.6±1.3 L/day for LongP and ShortP, respectively.

DISCUSSION

The shorter AIL protocol described in the study was able to induce all the heifers into lactation at the same production levels than the standard AIL protocol. All heifers reached at least 14 L/day of milk at some point during lactation. As both groups had similar lactation curves, the period of estradiol administration on an AIL protocol and hence its overall doses can be reduced with no effect on the milk yield of heifers. In addition, we also demonstrated the estradiol and progesterone profiles during a standard AIL protocol.

AIL protocols are commercially available in Brazil. These protocols are used by producers mostly when milk prices are high. Although the AIL protocols available are effective and able to induce a milk yield around 75-85% of a physiological

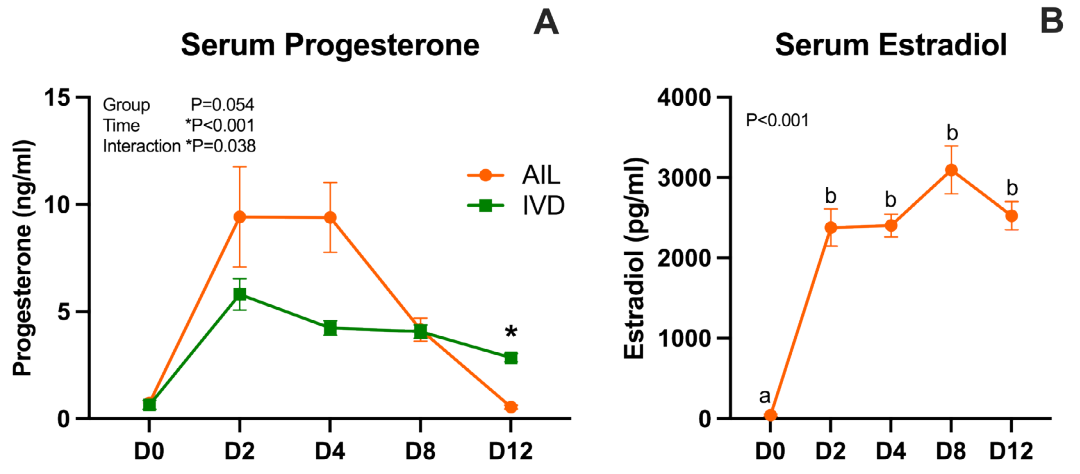

Figure 2 - (A) Serum progesterone levels (ng/ml) of Jersey cows subjected to an artificial induction to lactation (AIL) protocol (AIL; n = 5) or not (IVD; n = 6). * represents significant difference ($P < 0.05$). (B) Serum estradiol concentration (pg/ml) of Jersey cows subjected to an artificial induction to lactation protocol. Different letters represent significant difference ($P < 0.05$). Serum estradiol concentration was not evaluated in IVD group (control cows) because they were not treated with estradiol and were maintained only with an IVD containing progesterone.

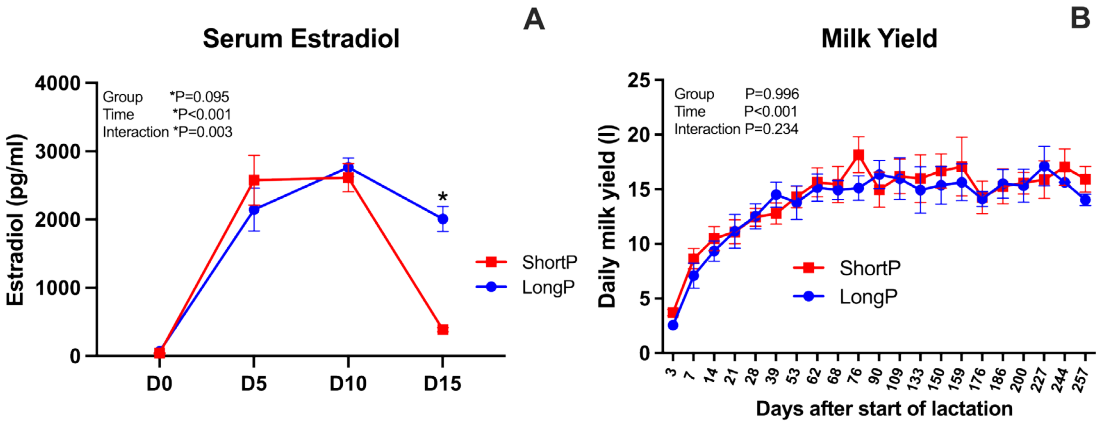

Figure 3 - (A) Serum estradiol levels (pg/ml) of Holstein heifers artificially induced into lactation with the standard (LongP; n = 5) or the short protocol (ShortP; n = 5). * represents significant difference (P < 0.05). (B) Daily milk yield (l) of Holstein heifers artificially induced into lactation with the standard (LongP; n = 5) or the short protocol (ShortP; n = 5).

lactation (FREITAS et al., 2010, LUZ et al., 2020), these protocols demand high dosage of hormones, especially E2. These dosages are debatable since little research has been done in the subject. Although we did not have a natural lactation group, the milk production we observed seems to be within the literature range of around 75% of a natural lactation from a Holstein heifer (FREITAS et al., 2010, LUZ et al., 2020). In a previous study from our group (LUZ et al., 2020), using the same long AIL protocol, heifers submitted to AIL produced 74.54% of the total volume produced by contemporaneous primiparous cows.

In the first experiment, we evaluated the P4 and E2 profile of animals submitted to a standard AIL (long-term) protocol. E2 concentrations remained above 2000 pg/mL from the second to the 12th day of the protocol, similar to that demonstrated by HENRICKS et al. (1972) in pre-calving pregnant cows with concentrations ranging from 4000 pg/mL to 2000 pg/mL (between days -14 to -1 in relation to calving). A study using an AIL protocol based on 17β Estradiol and P4 reported a peak of estrogen concentration of 5000 pg/mL (DELOUIS et al., 1978). A peak of E2 (1246.6 pg/mL) was also observed in induced heifers in a study conducted by our group; however, lower E2 concentrations were observed in pregnant heifers (93.0 pg/mL and 75.8 pg/mL at 21 and 14 days prepartum respectively) (LUZ et al., 2019). As for P4 concentrations, in the latter mentioned study the induced heifers showed a peak in P4 concentration on day 7 (14.14 ± 1.5 ng/mL) which can be attributed to a cumulative effect of long-acting injectable P4. Our results, however, reported slightly lower levels at days 2 and 4 (~9 ng/ml) and which further decreased (~4 ng/ml) at day 8. These levels are closer to the ones reported for pregnant cows and heifers in the last 14 days of gestation, which are lower than 4 ng/mL (HENRICKS et al., 1972, LUZ et al., 2019).

Since E2 levels reached during the long conventional AIL protocol can be higher than the ones reached during the end of pregnancy (LUZ et al., 2019), and a 7-day protocol using β Estradiol was reported able to induce lactation (MACRINA et al., 2011), we sought out to compare a shorter protocol (with less E2 applications) and the AIL protocol usually used in Brazil. Decreasing the number of E2 treatments, however, could have a negative effect on the milk yield of the induced animals, since E2 is the main inducer of the milk production due to its stimulatory effects on prolactin production (MEANEY & O'KEANE, 2002). Hence, it was important to compare the milk yield of heifers artificially induced to lactate by both short and the long standard protocol, at the same time, in the same system, using EB.

The data showing no differences in the milk production of the heifers induced by the ShortP, demonstrates that improvements can still be done in the AIL protocols, especially by reducing the administration of E2. E2 use is restricted in some countries, due to environmental and health concerns raised on the last decades (KAVLOCK et al.,

1996), even though effects from possible exposure to sex steroids from food been scarcely assessed (MALEKINEJAD & REZABAKHSH, 2015). Also, consumers are becoming more interested in the origin of their food and are especially concerned about hormones. In Brazil, estradiol benzoate and cypionate are used, mainly in estrous synchronization protocols (BARUSELLI et al., 2017). The use of these molecules may be positive, as long as they are used responsibly and at minimal levels. In the ShortP there is also a greater interval between the last E2 administration and the beginning of milking. In fact, E2 serum concentrations were already statistically lower 7 days before milking in this group. This protocol also represents less financial spending and less handlings and injections on the heifers, which consequently represent less stress for these animals. It is important to consider that these data are from nulliparous Holstein heifers and may not be the same for multiparous cows or animals from other breeds, due to differences in the steroid's metabolism.

AIL protocols can also be an alternative to prevent the culling of genetic superior young cows and heifers due to infertility. Besides providing a lactation from cows and heifers unable to conceive, some studies report a fertility recovery of some cows after the AIL protocols (FREITAS et al., 2010, LUZ et al., 2019, LEYVA & MELLADO, 2009, MAGLIARO et al., 2004), even though the mechanisms involved in this phenomenon have not yet been elucidated. The heifers from Experiment 2 presented a 40% conception rate when synchronized at 60 days post-AIL, showing the potential of AIL as a "therapeutic" procedure to restore fertility. The same pregnancy rate (40%) was observed in a previous study from our group (LUZ et al., 2019) after timed artificial insemination in repeat-breeder heifers (n = 20) induced to lactate with the same long AIL protocol used in the present study.

The improvement of AIL protocols by reducing the number of E2 injections allows the elimination of seven animal handlings, reducing the residues of this hormone in the environment. Another possibility for improving protocols, which should be investigated in future studies, is the reduction in the number of treatments with injectable P4, thus taking advantage of the long action of the drug. The optimization, aiming at practicality, efficiency, and profitability, will reflect in a more rational use of AIL.

CONCLUSION

Our results demonstrated the progesterone curve during an artificial induction of lactation (AIL) protocol in contrast to the curves produced by intravaginal devices, as well as the estradiol curve during AIL protocols. Also, the use of a short protocol, under the experimental conditions of this study, was able to induce lactation in nulliparous Holstein heifers, with production equal to a long conventional AIL protocol.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

JVVI and HSP wrote the paper and performed animal handlings, SFVJ, FCO, FPM, CRCB and LCG worked in animal handling and sample/data analysis, RF, ADV, MTR, RGM and BGG worked on study design and conception and revised the manuscript.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

All procedures adopted were approved by the Animal Experimentation Ethics Committee of the Universidade Federal de Pelotas (23110.010524/2014-71 and 23110.007347/2016-15).

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