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Dissertação

**Aspectos metabólicos, produtivos e reprodutivos de novilhas em lactação
induzida ou fisiológica**

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Pelotas, 2018

**Aspectos metabólicos, produtivos e reprodutivos de novilhas em lactação
induzida ou fisiológica**

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Resumo

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O início da lactação é um período de intenso desafio, marcado por diversas adaptações fisiológicas e homeorréticas em que novilhas e vacas, enfrentam uma série de alterações hormonais e metabólicas. Assim sendo, o objetivo deste trabalho foi avaliar o status energético, inflamatório, hepático e hormonal de novilhas induzidas a lactação comparadas com primíparas, além da mensuração de aspectos produtivos e reprodutivos. Para este trabalho foram selecionadas 60 novilhas da raça Holandês, idade média de $32 \pm 0,6$ meses, subdivididas em dois grupos: Grupo Controle (n=30) novilhas prenhas, acompanhadas desde 21 dias pré-parto até os 224 dias em lactação (DEL) e Grupo Indução (n=30) novilhas vazias, submetidas a um protocolo de indução de lactação, acompanhadas desde o início do protocolo até os 224 DEL. Para avaliação do metabolismo destes animais foram realizadas coletas de sangue em dois momentos: momento pré-início de lactação (semanas -3, -2 e -1) e momento pós-início de lactação (semanas 1, 2 e 3), para avaliação do status energético (glicose e ácidos graxos não-esterificados) e inflamatório (paraoxanase-1 e albumina), perfil hepático (ALT e GGT) e perfil hormonal (cortisol, progesterona e estradiol). Além disso, o controle leiteiro foi realizado do início da lactação até os 224 DEL. A taxa de prenhez foi avaliada das novilhas aptas a reprodução (Controle, n=13 e Indução, n=20) e inseminadas até os 49 DEL e, a partir daí, foram considerados todos os demais animais para cálculo do intervalo parto-concepção / início da lactação-concepção até os 240 DEL. As análises estatísticas foram realizadas no programa NCSS 2005. Os resultados dos metabólitos, hormônios e produção, foram analisados através da análise de medidas repetidas, análise de frequência para taxa de prenhez e análise de sobrevivência para os dados de intervalo parto-concepção / início da lactação-concepção. A indução de lactação foi eficiente para induzir a lactação em 100% das novilhas submetidas ao protocolo, as quais produziram 74,54% da produção de leite das novilhas primíparas em lactação fisiológica. No momento pré-lactação as novilhas do Grupo Indução apresentaram maiores concentrações de NEFA do que as novilhas do Grupo Controle, diferentemente do que foi observado no momento pós-lactação, onde o Grupo Controle apresentou maiores concentrações ($P<0.001$). Em ambos os períodos as concentrações de albumina e ALT foram inferiores no Grupo Indução, e as concentrações de PON e GGT superiores, em relação ao Grupo Controle ($P<0.05$). Quanto aos resultados das análises hormonais, as concentrações de cortisol e progesterona não diferiram entre os grupos em nenhum dos períodos estudados ($P>0.05$), contudo, as concentrações de estradiol foram maiores no Grupo Indução durante o período pré-lactação ($P<0.001$). Desta forma o momento pré-lactação foi um período de maior desafio do que o início da lactação para novilhas induzidas em relação ao status energético e inflamatório. O perfil hepático e performance reprodutiva não apresentaram alterações. Assim, a indução de lactação demonstrou ser um procedimento viável, não afetando negativamente o metabolismo dos animais proporcionando resultados produtivos compatíveis com a lactação fisiológica.

Palavras-chave: metabolismo; protocolo hormonal; status energético

Abstract

LUZ, Gabriela Bueno. **Metabolic, productive and reproductive aspects in heifers of induced or physiological lactation.** 2018. 37p. Dissertation Master degree in Sciences. Graduate Program in Animal Science. Federal University of Pelotas, Pelotas, 2018.

Early lactation is a period of intense challenge, characterized by several physiological and homeorhetic adaptations in which heifers and cows undergo a series of hormonal and metabolic changes. Therefore, the objective of this study was evaluate the energetic, inflammatory, hepatic and hormonal status of dairy heifers induced lactation in comparison to primiparous, beyond the measurement of productive and reproductive aspects. Were selected sixty Holstein heifers, average age 32 ± 0.6 months, subdivided into two groups: Control Group ($n=30$) pregnant heifers, accompanied for 21 days before expected calving day until 224 days in milk (DIM) and Induction Group ($n=30$) non-pregnant heifers, submitted to lactation induction protocol, accompanied from the beginning of the protocol until 224 DIM. For the evaluation of metabolism of these animals, blood collections were collected at two moments: pre-lactation period (weeks -3, -2 and -1) and post-lactation period (weeks 1, 2 and 3) aiming to evaluate the energetic (glucose and non-esterified fatty acids) and inflammatory status (paraoxonase-1 and albumine), hepatic profile (ALT and GGT), as well as, hormonal profile (cortisol, progesterone and estradiol). Besides that, the milk production was controlled until 224 DIM. The pregnancy rate was evaluate of heifers suitable to reproduction (Control, $n=13$ and Induction, $n=20$) and inseminated until 49 DIM, and from there, taking in consideration the overall cows in both groups and evaluated the conception moment until 240 DIM. Statistical analysis were performed using NCSS 2005 software. The metabolites, hormones and milk yield data, were analyzed using repeated measures; frequency analysis for pregnancy rate; and survival analysis for the data of interval from calving-to-conception (Control) / beginning of lactation-to-conception (Induction). The induction of lactation was efficient to induce the lactation in 100% of the heifers submitted to the protocol, and product 74.54% of the production of the primiparous heifers in physiological lactation. At pre-lactation period, induced heifers presented higher concentrations of non-esterified fatty acids (NEFA), differently what has been observed at post lactation period, where the Control Group presented higher NEFA concentrations ($P<0.001$). In both moments albumine and ALT were lower for the Induction Group, and the paraoxonase-1 and GGT concentrations were higher, than what we found in Control Group ($P<0.05$). Regarding the results of the hormonal analyzes, cortisol and progesterone concentrations did not differ between groups in any of the periods, however, estradiol levels were higher for the induced heifers at pre-lactation moment ($P<0.001$). Thus, pre-lactation time was a more challenging period than the beginning of the lactation for heifers induced in relation to energetic and inflammatory status. The hepatic profile and reproductive performance. Therefore, lactation induction is a physiological viable alternative to have dairy cows producing milk, having no negative impact on either energetic and inflammatory status, as well as comfort and welfare of the animals.

Key-words: energetic status; hormonal protocol; metabolism

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1 Introdução geral

Nas últimas décadas, na bovinocultura leiteira, a seleção genética de animais para alta produção de leite tem sido associada negativamente com um acentuado declínio da fertilidade (Butler, 2003), comprometendo, desta forma, a renovação do plantel pelo aumento do descarte involuntário de animais por ineficiência reprodutiva. Um levantamento realizado no Brasil em diferentes rebanhos e com mais de 2000 animais avaliados demonstrou que o descarte por falhas reprodutivas representou 27,7% do total de animais em 6 grandes fazendas produtoras de leite (Silva et al., 2008). Portanto, as falhas na eficiência reprodutiva estão entre os fatores que mais afetam a lucratividade da pecuária leiteira, envolvendo custos diretos associados à criação ou reposição de animais no plantel e os custos indiretos na manutenção de animais improdutivos dentro do sistema (Pritchard et al., 2013).

Dentre as categorias presentes no sistema produtivo de leite, as novilhas são aquelas que proporcionam principalmente o incremento genético do rebanho e a sua entrada na vida produtiva proporcionará ao produtor o descarte de animais velhos e menos produtivos (Santos e Lopes, 2014). Assim, espera-se que ao atingir a puberdade esses animais estejam aptos a ter uma concepção, iniciando o retorno do capital investido nas suas fases de cria e recria. Porém, uma porcentagem de animais mesmo tendo as características necessárias para iniciar sua vida reprodutiva, como idade e peso adequado, falham em conceber após sucessivas tentativas de inseminações, levando o produtor a realizar o seu descarte.

Como forma de evitar o descarte deste animal e o prejuízo associado, tem-se disponível uma estratégia que é o protocolo de indução artifical de lactação, realizado através da aplicação de hormônios específicos, que mimetizam a endocrinologia do terço final da gestação e promovem o início de uma lactação na ausência de gestação (Erb et al., 1976).

Os eventos relacionados à reprodução e lactação em vacas leiteiras fazem parte do mesmo processo fisiológico e são desencadeados pela atuação sinérgica de determinados hormônios. A interação endócrina que ocorre durante os últimos dias de gestação acaba atuando também na glândula mamária nos eventos de mamogênese, lactogênese e galactopoiese. Hormônios metabólicos, fatores de crescimento, prolactina e, especialmente, os hormônios reprodutivos, são necessários

para o desenvolvimento da glândula mamária (Lamote et al., 2004). A mamogênese refere-se à proliferação epitelial da glândula mamária, ocorre ao longo da gestação e é dependente e estimulada por estrógenos e progesterona (Svennersten-Sjaunja e Olsson, 2005). Já a lactogênese, é o processo que ocorre do pré-parto até o parto, e está envolvida com a síntese dos componentes do leite, diferenciação e multiplicação das células alveolares mamárias (Trott et al., 2012). A galactopoiese, é o evento responsável por manter a lactação, sendo dependente de estímulos neurais e endócrinos, e diversos hormônios galactopoieticos como: prolactina, hormônio do crescimento (GH), glicocorticoides, insulina e ocitocina (Tucker, 2000). Desta forma, o protocolo de indução de lactação, objetiva simular o perfil endócrino ao qual vacas gestantes estão expostas, para propiciar o início da lactação.

Atualmente, os protocolos mais utilizados tem duração de 21 dias de manejo, com aplicações de estradiol, progesterona, prostaglandina e cortisol (Freitas et al. 2010, Pestano et al., 2015). Durante os primeiros oito dias do protocolo, a associação de estrógeno com progesterona, desempenha uma atuação sinérgica no desenvolvimento da glândula mamária, ductos lactíferos, diferenciação alveolar e multiplicação das células epiteliais mamárias (Collier et al., 1984). Isso simula o que ocorre durante a gestação, nos dias anteriores ao parto, em que há uma queda nos níveis de progesterona e elevação nos níveis de estradiol, induzindo a um grande crescimento alveolar e liberação de prolactina (Tucker, 2000), onde durante o protocolo de indução artificial de lactação, se simula através de aplicações isoladas de estradiol. A liberação de prolactina, inicialmente induzida pelos níveis de estradiol, ainda pode ser incrementada e induzida pela administração de prostaglandina associada à corticoide (Sawyer et al., 1986), onde fisiologicamente em vacas no pré-parto estes hormônios estarão presentes envolvidos na luteólise e diferenciação do epitélio da glândula mamária, respectivamente (Ecco e Berber, 2014).

Apesar dos grandes benefícios que a indução de lactação proporciona ao produtor, ainda há uma limitação referente a menor capacidade produtiva dos animais quando comparado a animais em uma lactação fisiológica. Como forma de amenizar este problema e buscando um incremento na capacidade de produção de vacas induzidas, Magliaro et al. (2004) e Macrina et al. (2011) avaliaram a viabilidade da incorporação da somatotropina bovina (bST) aos protocolos. O bST é um hormônio que mimetiza a ação do GH, sintetizado naturalmente pelo animal, e atua no direcionamento dos nutrientes para a síntese láctea, através da mobilização de

reservas corporais e maior produção de IGF-1 (Bauman, 1999). Nos protocolos utilizados atualmente, recomenda-se a aplicação de uma dose de bST a cada 7 dias, onde vacas que receberam aplicações de bST tiveram um acréscimo de 17,8% e 36%.

Os protocolos de indução de lactação vem sendo testados desde a década de 40 (Walker e Stanley, 1941), contudo, devido a maioria dos países desenvolvidos não permitirem a utilização de hormônios na produção de bovinos, os estudos e pesquisas realizados a respeito dos protocolos, evolução e resultados que se tem até o momento são limitados. Ainda é um procedimento que envolve muitos manejos, elevadas doses hormonais e apresenta grande variabilidade de resposta individual dos animais, e os protocolos comumente utilizados apesar de semelhantes, variam de acordo com autores e empresas em relação a duração e aplicações (Pestano et al., 2015). Desta forma, fica evidente a necessidade da realização de mais estudos desmistificando e investigando as possíveis interferências e benefícios causados para a saúde humana e animal, bem como a otimização dos resultados proporcionados.

Com o intuito de avaliar além do metabolismo dos animais induzidos como também a viabilidade econômica da indução de lactação, realizamos um levantamento de custos com as novilhas avaliadas nesta dissertação (dados ainda não publicados), levando em consideração os custos com manejo nutricional, sanitário e reprodutivo, novilhas induzidas apresentaram um lucro em torno de 40% em relação a novilhas paridas na primeira lactação, produzindo ao longo da lactação (305 dias) 72% da produção das novilhas em lactação fisiológica. Apesar de aparentemente não ser um lucro tão expressivo, a indução de lactação pode ser considerada uma alternativa interessante principalmente em situações de animais com problemas reprodutivos e ineficientes para o sistema, como nos casos das fêmeas “repetidoras de serviço” (*repeat breeders*), que falham em conceber após três ou quatro serviços. Assim ao serem submetidas ao protocolo, iniciam a vida produtiva, sendo possível que produzam em torno de 70 a 78% do volume de leite produzido de fêmeas em lactação fisiológica (Mellado et al., 2006), tornando-se produtivas.

Escassos são os trabalhos que se tem até o momento, e estes, relatam e discutem apenas resultados de produção e composição do leite, e algumas sugestões sobre possíveis efeitos na fertilidade. A respeito da composição de leite de vacas induzidas, com base em diversos trabalhos, a indução parece ter pouco ou nenhum efeito sobre a proteína, gordura e lactose (Narendran et al., 1974; Magliaro et al., 2004). Quanto aos possíveis efeitos sobre a fertilidade, estudos relatam taxas de

prenhez variando de 41,4% (Freitas et al., 2010) a 71% (Mellado et al., 2006), em vacas repetidoras de serviço da raça Holandês submetidas a inseminação artificial durante a lactação, após o protocolo de indução de lactação.

O que ainda nenhum trabalho buscou investigar, são os possíveis efeitos do protocolo no metabolismo de novilhas e vacas induzidas, sobre os parâmetros de fertilidade, imunidade, produção de leite, balanço energético e metabolismo, tendo apenas informações especulativas. Uma vez que os protocolos de indução artificial de lactação são cada vez mais utilizados na pecuária leiteira brasileira, é necessário investigar melhor a interferência do protocolo hormonal nos desafios encontrados na lactação e as limitações relacionadas ao manejo e bem-estar dos animais induzidos bem como, se apresenta melhor resultado para determinada categoria. Embora as fêmeas induzidas à lactação não enfrentem a transição de vaca gestante não-lactante para lactante, evento típico do periparto, passam de não-lactantes para lactantes.

Para vacas leiteiras o periparto é uma fase crítica de intenso desafio de alta demanda energética para desenvolvimento final do feto e início da lactação, onde ocorrem eventos fisiológicos e homeorréticos de resistência à insulina, reduzida ingestão de matéria seca induzindo a lipomobilização e consequente balanço energético negativo (Grummer, 1995). Com isto, há intensa mobilização de gordura, aumento das taxas de gliconeogênese e das concentrações sanguíneas de ácidos graxos não-esterificados (AGNES) (Ingvartsen e Moyes, 2015), tornando-se mais suscetíveis a ocorrência de desordens no pós-parto (Ospina, 2010).

Além disso, neste período as fêmeas enfrentam quadros de imunossupressão uma a duas semanas antes e duas a três semanas depois do parto, momento em que também deve ocorrer a recuperação uterina concomitante a ocorrência de mudanças drásticas na circulação das concentrações de progesterona, estrógenos e um pico de cortisol, contribuindo substancialmente para o comprometimento do sistema imune neste período e maior risco de ocorrência de enfermidades (LeBlanc, 2010).

Levando em conta estas considerações a respeito de fêmeas periparturientes, e utilizando como modelo experimental novilhas, por serem animais que ainda não iniciaram a vida produtiva e, também por serem um modelo homogêneo, expostas e submetidas às mesmas condições, o objetivo deste trabalho foi avaliar a interferência do protocolo de indução de lactação no status energético (AGNES e glicose), inflamatório (paraoxanase-1 e albumina), hepático (ALT e GGT) e hormonal (cortisol,

progesterona e estradiol) de novilhas induzidas comparadas com novilhas prenhas, além da mensuração de aspectos produtivos e reprodutivos.

2 Artigo

Artigo formatado conforme normas da revista **Journal of Dairy Science**.

Induction of lactation in dairy heifers: metabolism, productive and reproductive aspects

1 **Induction of lactation in dairy heifers: metabolism, productive and reproductive aspects**

2

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15

ABSTRACT

17 The objective of this study was evaluate the glucose, non-esterified fatty acid (NEFA),
18 paraoxonase (PON-1), albumin, ALT, GGT, cortisol, progesterone and estradiol concentrations
19 of dairy heifers induced to lactation in comparison to primiparous, beyond the measurement of
20 milk yield and pregnancy rate. Were selected 60 Holstein heifers, 32 ± 0.6 months of age,
21 subdivided into two groups: Control Group (Control, n=30) pregnant heifers, accompanied
22 since 21 days before expected calving day until 224 days in milk (DIM) and Induction Group
23 (Induction, n=30) non-pregnant heifers, submitted to a lactation induction protocol,
24 accompanied from the beginning of the protocol until 224 DIM. For the evaluation of
25 metabolism of these animals, blood samples were collected at two moments: pre-lactation
26 period (weeks -3, -2 and -1) and post-lactation period (weeks 1, 2,3 and 4) aiming to evaluate
27 the energetic (glucose and non-esterified fatty acids) and inflammatory status (paraoxonase-1
28 and albumin), hepatic profile (ALT and GGT), as well as, hormonal profile (cortisol,
29 progesterone and estradiol). Besides that, the milk production was controlled until 224 DIM.
30 The pregnancy rate was evaluate of heifers suitable to reproduction (Control, n=13 and
31 Induction, n=20) and inseminated until 49 DIM. The induction of lactation was efficient to
32 induce lactation in 100% of the heifers submitted to the protocol, producing 74.54% of the
33 production of the primiparous heifers in physiological lactation. At pre-lactation period,
34 induced animals presented higher concentrations of NEFA than the Control heifers, differently
35 from the observed at post lactation period, when the Control presented higher NEFA
36 concentrations. In both moments albumin and ALT were lower in Induction, and paraoxonase-
37 1 and GGT concentrations were higher, than what we found in Control. Regarding the results

38 of hormonal analyzes, cortisol and progesterone concentrations did not differ between groups
39 in any of periods, however, estradiol levels were higher for the induced cows at pre-lactation
40 moment. Based upon the parameters investigated for evaluation of the lactation induction,
41 during the pre-lactation moment, the induced heifers showing an interesting increase of NEFA
42 and higher PON-1 levels. The hepatic profile and reproductive performance did not present any
43 changes. Therefore, lactation induction is a physiological viable alternative to have dairy cows
44 producing milk, having no negative impact in the metabolism of the animals.

45

46 **Key words:** energetic status, hormonal protocol, inflammatory, hepatic profile

47

48

49 INTRODUCTION

50

51 In the last decades, the genetic selection of dairy cows for high milk yield has been
52 negatively associated with a marked decline in fertility (Butler, 2003), implicating, thus, the
53 herd renovation because the increase of culling rates of animals with reproductive inefficiency.
54 The reproductive failures are considered one of the main factors of culling (Pryce et al., 2004)
55 and are among the costliest factors of dairy farms, involving directs costs associated the rearing
56 or bring of an animal in the farm, beyond the indirect costs with maintenance of unproductive
57 animals in the system (Pritchard et al., 2013).

58 A strategy to minimize these problems of discarding females at productive age and to
59 enable these cows with high genetic merit for milk yield to remain in the property, is the use of
60 protocols of lactation artificial induction of lactation (AIL), allowing the beggning of a lactation
61 in the absence of gestation (Freitas et al., 2010). The conventional hormonal protocol lasts for
62 approximately 21 days (Freitas et al., 2010), with daily applications of hormones (estradiol,
63 progesterone, prostaglandin and corticoids), which make it possible to simulate the hormonal
64 levels produced in the last days of cow pregnancy.

65 Most of the previous papers evaluated the productive and reproductive aspects of those
66 animals, and report satisfactory results to the AIL protocol, reaching from 85 to 100% of cows
67 initiating natural lactation, as well as, producing around 70 to 78% in relation to cows in
68 physiologic lactation. Also, it has been reported an improvement in fertility, with pregnancy
69 rates varying from 41.4% to 71% during the induced lactation (Mellado et al., 2006; Freitas et
70 al., 2010). However, limited information referring to the metabolism and endocrinology of these
71 animals induced to lactation are available.

72 The physiological adaptations that occur in periparturient dairy cows during the
73 transition for an animal pregnant and non-lactant to non-pregnant lactant, is already well
74 elucidated. Peripartum is a critical period, occurring physiological events and homeorhetic
75 adaptations, characterized by decreased energy intake and lipomobilization in the early
76 lactation. Besides, females confront immunosuppression, aggravated by dramatic changes in
77 circulating progesterone, estrogen and cortisol concentrations (LeBlanc, 2012). Accordingly,
78 the objective of this study was evaluate the glucose, non-esterified fatty acid (NEFA),
79 paraoxanase (PON-1), albumin, ALT, GGT, cortisol, progesterone and estradiol concentrations
80 of heifers submitted to the lactation induced protocol in comparison with pregnancyheifers
81 during the transition period, beyond the measurement of milk yield and pregnancy rate.

82

83

84

MATERIALS AND METHODS

85

86

Animal Housing and Care

87

All procedures involving animals in the study were approved by the University of Pelotas Animal Care and Use Committee and by the University of Pelotas Research Committee.

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90

Experimental Design and Treatments

91

Sixty heifers Holstein, average age 32 ± 0.6 old months, body score between 3-3.5 (1 = emaciated, 5 = obese) were selected and subdivided into two groups: Control Group (Control; n=30), pregnant heifers, followed since 21 days prepartum until 224 days in milk (DIM); and Induction Group (Induction n=30), non-pregnant heifers, submitted to artificial induction of lactation protocol, according illustrated in the Figure 1. From the 1st to 8th day 30 mg of estradiol benzoate (Sincrodiol® Ourofino Saúde Animal, São Paulo, Brasil) were injected daily, together with 300 mg of progesterone (Sincrogest® Ourofino Saúde Animal, São Paulo, Brasil). From the 9th until 14th, animals received a daily dose of 20 mg of estradiol benzoate (Sincrodiol® Ourofino Saúde Animal, São Paulo, Brasil). On the 16th day, 0.56 mg of sodic cloprostenol (Sincrocio® Ourofino Saúde Animal, São Paulo, Brasil) was given and from 19th to 21st day, daily injections of 40 mg of dexamethasone sodium phosphate (Cortiflan® Ourofino Saúde Animal, São Paulo, Brasil) were given to heifers. Also, on days 1, 8, 15 and 22, the animals received a dose of 500 mg of sometribove zinc (Lactotropin® Elanco Saúde Animal, São Paulo, Brasil).

105

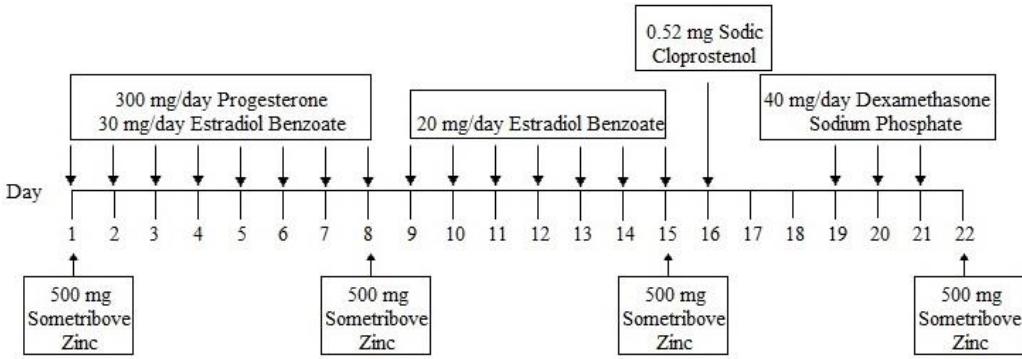


Figure 1. Protocol of induction of lactation applied to Induction Group during 21 days.

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On the 22nd day, milking began and heifers were milked twice a day until 224 DIM.

110

Previously to protocol initiation, induced heifers were pre-synchronized with two 11-day prostaglandin shots to ensure that all were synchronized, at the same stage of the estrous cycle, and under the similar hormonal conditions. Table 1 shows the diet composition of daily rations for both groups in the late 21 days before lactation and during lactation.

111

112

Table 1. Nutrient composition of daily rations for Control Group and Induction Group in 21 days before lactation and during lactation.

Component	21 days pre-lactation	Lactation
Dry Matter (DM), Kg	49.4	49.6
Net energy of lactation (NEL), MJ	1.53	1.68
Crude protein (CP), % DM	14.96	15.75
Fat, % DM	2.46	3.41
Neutral detergent fiber (NDF), % DM	43.26	35.74
DCAD (meq/100g)	4.11	-

113

Blood sampling and analysis of biochemical, hormonal and inflammatory markers

114

In both groups, weekly blood collections were performed, from 21 days before the beginning of lactation (weeks: -3, -2 and -1) until 28 days in milking (weeks: 1, 2, 3 and 4). Blood was collected from the coccygeal arteriovenous complex by vacutainer system, into two tubes without anticoagulant for posterior evaluation of the energetic, inflammatory and hormonal parameters and one tube with sodium fluoride for evaluation of the glucose levels.

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124 Immediately after blood collection, the samples were centrifugated, processed and frozen (-
125 20°C), in eppendorf tubes, properly identified, until further analysis.

126 Serum of induced (n=30) and control (n=30) heifers were analyzed at weeks -3, -2, -1,
127 1, 2 and 3 for paraoxonase-1 activity (PON-1), non-esterified fatty acids (NEFA), albumin,
128 ALT, GGT and glucose. The PON-1 activity was analyzed by spectrophotometry according to
129 methodology described by Browne et al. (2007). Samples for the other metabolites were
130 analyzed in the automatic biochemical analyzer Labmax Plenno (Labtest Diagnóstica ®, Minas
131 Gerais, Brazil), by spectrophotometry, programmed for biochemical and immunochemical tests
132 with specific reagents for each analysis.

133 Subgroups from induced (n=5) and control (n=5) heifers were ramdomly for hormonal
134 dosages evaluating cortisol at the weeks -3, -2, -1, 1, 2 and 3. Progesterone and estradiol were
135 evaluated at the weeks -3, -2, -1, 1 and 4. These analyses were made with specific kits in the
136 Access2 Immunoassay System (Beckman Coulter®, California, EUA) equipment, by
137 chemiluminescence.

138

139 ***Milk production and reproduction evaluations***

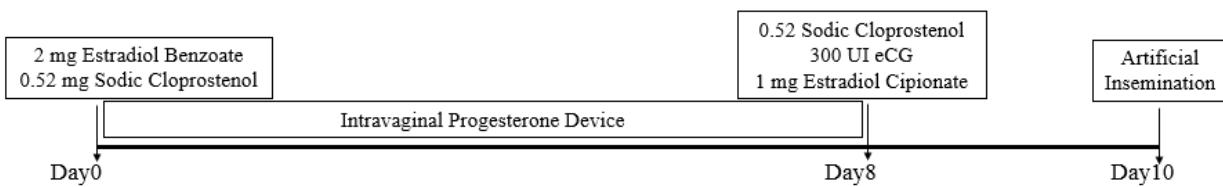
140 Milk production evaluation was carried out twice day, checking the individual milk
141 yield by the digital reader (De Laval). Lactation was monitored weekly until the 28st week and
142 after this monthly until the 8th month for posterior comparation of the milk yield among groups.
143 Once the animals were in the same milking herd, both groups were subject to the same routine
144 herd management protocols regarding milking, breeding, and health care.

145 Heifers from both groups were subject reproductive to weekly evaluations, from the
146 beginning of the lactation until the 35 days of lactation. Uterine examinations using ultrasound
147 equipment, as well as, was used to evaluate uterine content and a vaginoscopy to check opening
148 and staining of cervix mucosa.

149 Both groups were eligible for breeding after a 42-d voluntary waiting period. Heifers
150 were considered allowed to reproduction whether status of adequate nutritional (BCS ≥ 3),
151 uterine involution (Control Group), translucent mucus, and overall healthy condition were in
152 accordance. Therefore, cows in adequate reproductive condition were subject to hormonal
153 protocol of timed artificial insemination (Figure 2).

154 Cows were assigned to receive 2 mg of estradiol benzoate (Sincrodiol® Ourofino Saúde
155 Animal, São Paulo, Brasil), 0.52 mg of sodic cloprostenol (Sincrocio® Ourofino Saúde Animal,
156 São Paulo, Brasil) and an insertion of an intravaginal device containing 1 g of progesterone
157 (Sincrogest® Ourofino Saúde Animal, São Paulo, Brasil) on day 0 of the protocol. At day 8,

158 0.52 mg of sodic cloprostenol (Sincrocio® Ourofino Saúde Animal, São Paulo, Brasil) was
 159 injected, intravaginal device was removed and 300 UI of equine corionic gonadotropin
 160 (SincroEKG® Ourofino Saúde Animal, São Paulo, Brasil) and 1 mg of estradiol cipionate
 161 (SincroECP® Ourofino Saúde Animal, São Paulo, Brasil) were also given. At day 10th, timed
 162 artificial insemination was performed. Pregnancy diagnoses were performed by ultrasound
 163 (Mindray M5VET - Shenzhen Mindray Bio-Medicinal Electronics Co., Ltd) using linear
 164 transductor of 7.5 MHz, at 31 to 35 days post AI. Cows that were not able to reproduction until
 165 49 DIM were subject to the same reproductive herd management of the farm.
 166



167
 168 Figure 2. Protocol of timed artificial insemination used in the females considered suitable to
 169 reproduction.

170

171 Statistical analysis

172 Biochemical and hormonal data were analyzed as repeated measures in a completely
 173 randomized design, considering the main effects of group (Control and Induced) over time (e.g.,
 174 week -3 to 0 and 1 to 3 related the lactation beginning) and random effect of cow. As an
 175 exception estradiol and progesterone, after start lactation, were analyzed just at the first and
 176 fourth week. Therefore, the following statistical model was analyzed via Repeated
 177 Measurements ANOVA of NCSS 2004 and PASS 2005 (NCSS and PASS. Number Cruncher
 178 Statistical Systems. Kaysville, Utah):

$$179 Y_{ijkl} = \mu + M_j + T_k + MT_{jk} + c_l(b_i) + e_{ijkl},$$

180 where: Y_{ijkl} is the dependent continuous variable, μ is the overall mean, M_j is fixed effect of
 181 group ($j = \text{control vs. induction}$), T_k is the fixed effect of time (6 weeks), MT_{jk} is the interaction
 182 between group and time, $c_l(b_i)$ is the random effect of cow, and e_{ijkl} is the random residual error.

183 Milk production was analyzed by ANOVA considering the main effects of group
 184 (Control and Induced), week and their first order interaction until 224 DIM.

185 Reproductive performance data consisted of pregnancy rate and the interval calving-to-
 186 conception (Control group) or beginning of lactation-to-conception (Induction group). Analysis
 187 of frequency was utilized to the pregnancy rate considering data from animals breed until 42

188 DIM. Significance was determined at $P \leq 0.05$, and tendencies were determined at $P > 0.06$ to $P \leq 0.10$.

190

191

192 RESULTS AND DISCUSSION

193

194 *Milk production*

195 The protocol of lactation induction obtained 100% success rate, where all the induced
196 heifers responded to the protocol and beginning lactation. From the 1st to the 32nd lactation week,
197 induced heifers produced 74.54% of the control group (physiological lactation), showing an
198 average milk production of 19.17 kg and 25.48 kg, respectively. Even is found in this study,
199 Mellado et al. (2006), evaluated 98 artificially induced Holstein cows, and observed 78% of
200 milk yield in comparison with calved cows.

201 Initially the induced heifers started production average 8.72 Kg per animal and in the
202 last week average production of 28.37 Kg. Even as found in this study, Mellado et al. (2006)
203 and Freitas et al. (2010) also related the lactation peak more later for induced cows.

204 As it was expected, the average milk production of Control Group was higher ($P < 0.05$),
205 which is similar to other studies (Jewell et al. 2002; Freitas et al. 2010), whose found induced
206 cows producing between 65 to 77.2% in relation to cows in physiological lactation.

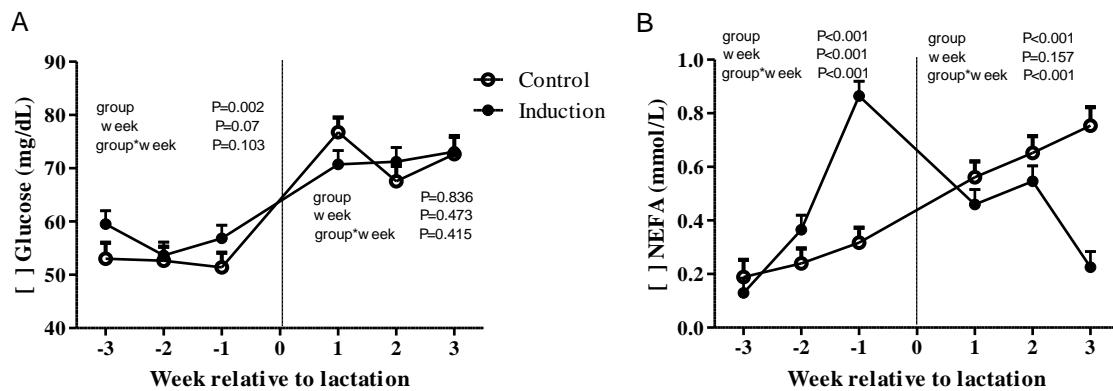
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208 *Glucose concentrations and non-esterified fatty acids (NEFA)*

209 Glucose levels were different ($P < 0.01$) among groups, only at pre-lactation moment
210 (Figure 3A), when induced heifers had higher glucose levels (56.66 ± 0.92 mg/dL) than Control
211 animals (52.34 ± 1.02 mg/dL), with no effect ($P > 0.05$) of weeks and interaction week and
212 groups. During the transition period, glucose acts as an important energetic nutrient, been that
213 approximately 85% of the total blood glucose is directed to the mammary gland (Bickerstaffe
214 et al., 1974). Previous researches have demonstrated that during the prepartum the glucose
215 plasmatic concentrations remain stable, increasing in the parturition, and decreasing
216 immediately after it. The increase observed after lactation start could be caused by the
217 increment of the glucagon and glucocorticoids in both groups that promote the glycogen
218 mobilization of liver to support lactation (Kunz et al., 1985; Vazquez- Anon, 1994; Grummer,
219 1995). Our results indicate that even before and after lactation, glucose levels in both groups
220 kept within the physiological standard levels.

The NEFA concentrations were different ($P<0.001$) among groups either pre and post-lactation (Figure 3B). Induced heifers had a peak of lipomobilization one week before lactation start, which could be related to acute energy demands of mammary gland and other tissues involved in lactation, as well as, the intense daily routine of induction protocol. In Control heifers seems that NEFA has a linear increment according energy demands increase during pre-lactation period and it continues during an adaptation of tissues to produce more milk.

227



228
229 Figure 3A-B. Glucose and NEFA concentrations in the moments pre-lactation (weeks -3, -2, -
230 1) and post-lactation (weeks 1, 2 and 3).

231

232 During peripartum, the neuroendocrine system interacts in different ways to try to
233 maintain the homeostasis and the hormones as insulin, somatotropin, cortisol and estrogen have
234 oscillations playing an important role in the lipolysis regulation (Bauman and Vernon, 1993;
235 Bell and Bauman, 1997). Thus, the endocrine regulation causes an intense mobilization of
236 corporal tissue, primarily adipose tissue, to provide the nutrients required for maintenance and
237 milk production, causing initially, an increase of circulating NEFA concentrations. The severity
238 of lipomobilization reached is associated to immunosuppressive conditions and diseases risk
239 during the transition period (LeBlanc et al., 2012). Furthermore, according to our study, it can
240 be postulated that for induced heifers, the pre-lactation period is more challenging and cause more
241 lipomobilization than the onset of lactation, playing a different role and interacting later with
242 immunity and other systems.

243

244 **Albumin and paraoxonase-1 (PON-1)**

245 The acute phase proteins (APP) have been used as important inflammatory biomarkers
246 predictors in dairy cows during the transition period (Huzzey et al., 2011). Our study used
247 albumin and PON-1 to evaluate the inflammatory status in induced and calving heifers. Both
248 predictors are, negative acute phase proteins, therefore their levels are low when an

inflammatory episode is beginning. Induced heifers had higher ($P<0.05$) PON-1 concentrations than control heifers in both moments (Figure 4A). Thus, suggesting that induced females have a lower inflammatory condition than control heifers during the pre-lactation period. Physiological process as pregnancy in dairy cow, has a huge influence in the lipid metabolism and liver function, which during the transition period drives to highly vulnerable to oxidative stress. Thereby, alterations in PON-1 serum activity (as an enzyme involved with oxidative protection) could be a diagnosis marker utilized for detect diseases in cows during peripartum (Kulka et al., 2014). Higher PON-1 levels in induced heifers, at pre-lactation period, indicated that the protocol did not caused inflammatory conditions in the animals. However, an apparent decline from week -1 to week +1 indicates that lactation is more a challenging than induction itself. Low levels of PON-1 activity for calving heifers during prepartum period indicate that the gestation is more challenging for animals regarding the inflammatory status, than the lactation induction, and may be an indicator that pregnant animals are more likely to be affected by diseases than induced cows.

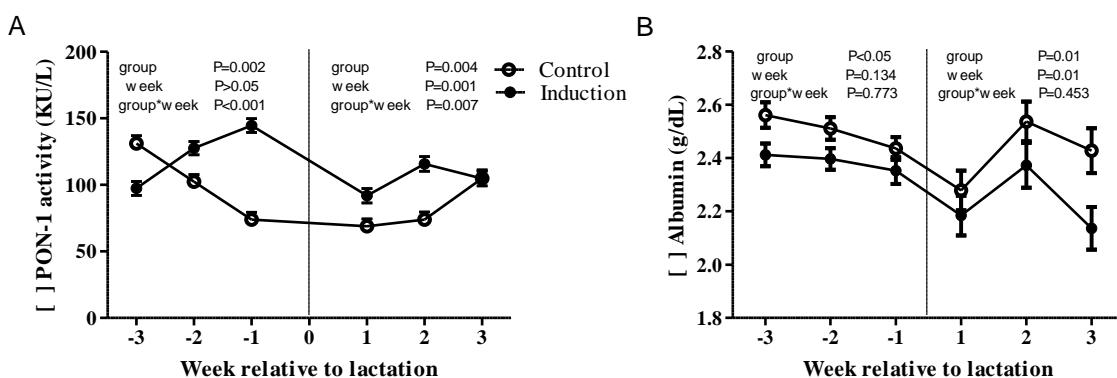


Figure 4A-B. Concentration of PON-1 activity and albumin for induced and control heifers in the moments pre-lactation (weeks -3, -2 and -1) and post-lactation (weeks 1, 2 and 3).

Differently from the observed for PON-1, albumin levels were higher ($P<0.05$) for control group than for induced, at both moments (Figure 4B). The albumin is the major component of total protein of the blood plasma, corresponding to approximately 50 to 65% of the all circulating proteins, and these concentrations can be affected by the nutrition, hepatic function, amino acids bioavailability, dehydration and weight loss (González, 2000). Considered as a negative acute phase protein, it demonstrates in lowers levels lipomobilization and immunosuppression situations. It is important to emphasize that although the concentrations have been lower for induced heifers in comparison to controls, both groups showed lower levels in comparison with physiological concentrations of 2.7 to 3.8 g/dL for the species (Kaneko et al., 1997). Thus, not only pregnancy (Control Group), as well as, lactation

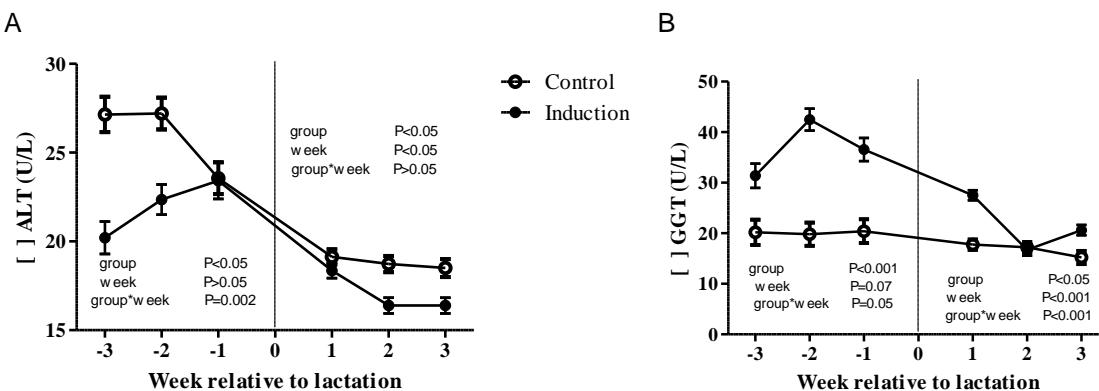
277 (Induced Group) represent periods of greater susceptibility albumin oscillations. Moreover, in
 278 the pre-lactation moment, the lower albumin concentrations in induced heifers can be associated
 279 with high NEFA concentrations, indicating a lipomobilization moment for those animals, which
 280 can be explained by the daily routine and preparation for the beginning of lactation.

281
 282 **Hepatic enzymes: ALT and GGT**

283 It is reasonable to believe that the AIL protocols would result in an intense hepatic
 284 metabolism, due to the daily use of high doses of hormones, although this hypothesis has not
 285 been investigated in dairy cows yet. Therefore, with the objective to evaluate the hepatic profile
 286 and occurrence of possible muscular damages in the animals submitted to AIL in comparison
 287 to the pregnant heifers periparturient, were evaluated in this study the hepatic enzymes ALT
 288 and GGT. These enzymes are markers presented in high levels in acute process, proportional to
 289 the found lesions (Gonzalez and Silva, 2006). Radavelli et al. (2016), described elevated ALT
 290 and GGT levels in sheeps submitted to AIL protocol.

291 Weekly serum concentrations of ALT in Control heifers, remained higher ($P<0.05$)
 292 than in Induction group during both periods (pre and post-lactation) (Figure 5A), while GGT
 293 had an opposite behavior higher (Figure 5B). However, levels for both enzymes were in
 294 accordance with the physiological values for the specie (ALT 0-38 U/L and GGT 0-39 U/L).

295



296

297 Figure 5A-B. Concentration of ALT and GGT for control and induced heifers in the moments
 298 pre-lactation (weeks -3, -2 and -1) and post-lactation (weeks 1, 2 and 3).

299

300 As previously reported for Vieira et al. (2010) and Araújo et al. (2014), bST injections
 301 could be the responsible for higher GGT levels in induced heifers, probably due to the
 302 application of bST, an important hormone utilized for to increase the production of the animals
 303 submitted to lactation induction, with a market influence in nutrients partition (Bauman, 1993).

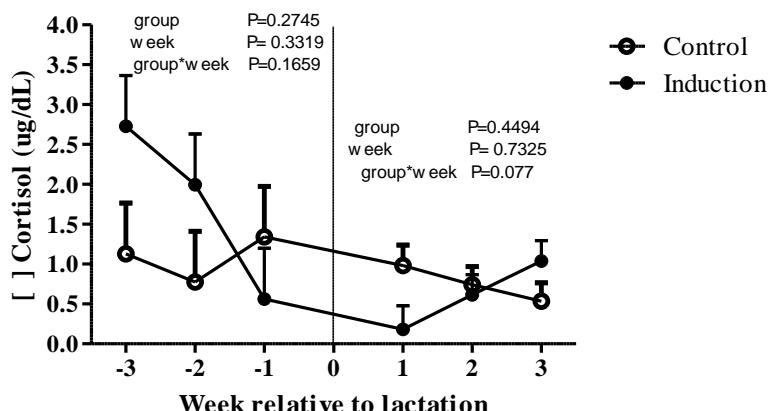
304 Although it was not possible establish a physiological explanation for this event, it may be due
 305 to the hepatic metabolism required after bST utilization.

306

307 ***Hormonal profile: cortisol, progesterone and estradiol***

308 Cortisol in dairy cows release can be one of the primary adaptive mechanisms to adverse
 309 conditions, acting for short term of mobilization of body reserves and regulation of
 310 inflammatory responses (Gross et al., 2015). Our results showed similar ($P>0.05$) in both groups
 311 at pre- and post-lactation period (Figure 6). Initially, it was expected that cortisol concentrations
 312 would be higher in induced heifers, once AIL is a protocol with intensive daily management,
 313 and involves three corticoid injections in the last days, which could increase cortisol
 314 concentrations. Therefore, considering cortisol levels, our results showed that AIL protocol
 315 seems not has negative impact. Another point to considered, is that the dexamethasone doses
 316 used during the protocol were sufficient to simulate the levels found during the last 21 days of
 317 gestation of a heifer, been the ideal dose for not exceed the levels physiologically produced.

318



319

320 Figure 6. Concentration of cortisol in the moments pre-lactation (weeks -3, -2 and -1) and post-
 321 lactation (weeks 1, 2 and 3) of the control and induced animals.

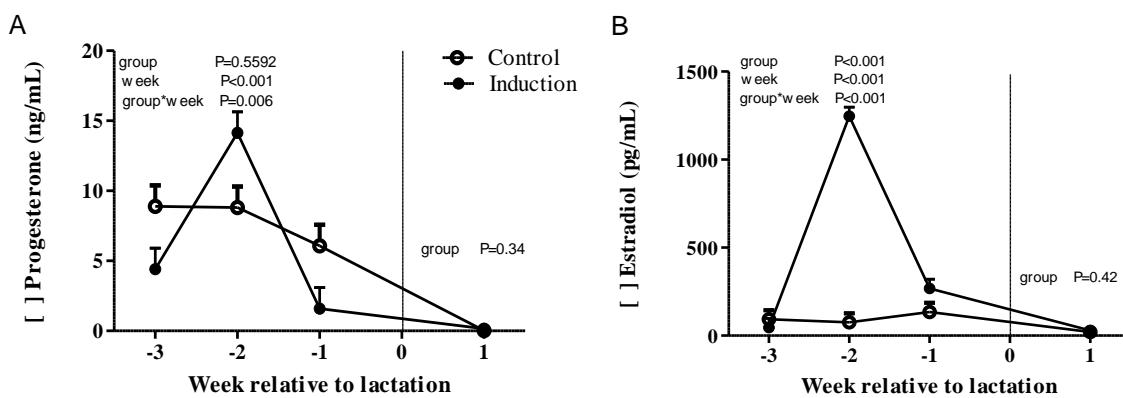
322

323 Progesterone levels were similar ($P>0.05$) at either pre and post-start of lactation
 324 moments among groups. During the pre-lactation, induced heifers had higher progesterone
 325 ($P<0.001$), once during the first seven days of the protocol they received progesterone daily.
 326 There was a peak (from 4.4 ± 1.5 ng/mL at week -3 to 14.14 ± 1.5 ng/mL at week -2), indicating
 327 a cumulative effect of this hormone (Figure 7A), which was already expected, because it was
 328 used a long-acting drug. In Control heifers, progesterone concentrations were elevated because
 329 pregnancy, and them it was a marked decline near the parturition (Chew et al., 1979). Control
 330 heifers, showed a continuous decline until parturition, highlighting an accentuated drop at the

331 last prepartum week (6.08 ± 1.50 ng/mL to 0.01 ± 0.41 ng/mL in the first week postpartum).
 332 Concomitant with this decrease there is an increase in prolactin, while the estrogen
 333 concentrations remain increasing (Smith et al., 1973), which are physiological mechanisms to
 334 enhance lactation.

335 Estradiol concentrations were different ($P < 0.01$) among groups only in the pre-lactation
 336 period, because induced heifers had estradiol levels increased during the first two weeks,
 337 corresponding to the daily applications of estradiol within the protocol. At the first week (-3)
 338 before AIL, the average basal level of estradiol for induced heifers was 46.2 pg/mL, reaching
 339 to 1246.6 pg/mL, after ten days of daily injections (Figure 7B). Heifers of control group, in the
 340 same period, had concentrations of 93.0 pg/mL (week -3) to 75.8 pg/mL (week -2). These results
 341 demonstrate that the recommended doses used in this recommended protocol utilized could be
 342 re-adjusted, considering the possibility of using lower estradiol dose and a longer range of
 343 application. Furthermore, the isolated applications of estradiol, can cause intense estrus
 344 manifestation, when progesterone is not part of the protocol (in our study we use a long acting
 345 progesterone). Estrous signs could be a negative behavior, once it can cause accidents or
 346 traumas in induced heifers. In our study, only 3/30 induced heifers showed estrus during
 347 the protocol. During the lactation period, there was no difference between the groups, nor
 348 between weeks ($P > 0.05$). Physiologically, in cows during the final period of gestation, there is
 349 an increase in plasma concentrations of estradiol, which decrease immediately after the
 350 parturition (Chew et al., 1979), however, control group had no difference ($P > 0.05$) between the
 351 weeks. It is important emphasize that, in both groups, the progesterone and estradiol
 352 concentrations were basal, upcoming to zero, in the first week after the beginning of lactation,
 353 demonstrating the safety of milk produced from that moment on the absence of residues.

354
 355



356
 357

358 Figure 7A-B. Concentrations of progesterone and estradiol for control and induced heifers in
359 the moments pre-lactation (weeks -3, -2 and -1) and post-lactation (week 1).

360
361 The hormonal dynamics, with progesterone reduction and estradiol increase that
362 occur in this final period of gestation, is essential to trigger the events of mamogenesis and
363 lactogenesis. It is well established that the estrogens stimulate the mammary ducts growth and
364 the progesterone and estrogens combination demonstrate synergistic action, stimulating the
365 lobe-alveolar development of the mammary gland. Besides these hormones, it is also important
366 the secretion of prolactin and GH by the anterior pituitary (Folley, 1952; Lyons, 1958).

367

368 *Reproductive Performance*

369 As previously described by Mellado et al. (2006), at second week of lactation, 16/30
370 (53.33%) of Induction Group heifers, had follicular cysts. Cysts are normally caused by
371 hypothalamic failures in response to estradiol and progesterone (Silvia et al., 2002). High
372 hormone doses used in induced heifers, could cause these failure in the sensibility and feedback
373 of the hypothalamic-pituitary-gonadal axis.

374 Progesterone and estradiol evaluation performed in the first week after the beginning of
375 the lactation, aimed to evaluate the metabolization and physiological concentration of the
376 hormones used during the protocol. On the other way, the measurement performed in the fourth
377 week, was to evaluate the return to cyclicity of the heifers in both groups. There was a tendency
378 ($P=0.06$) for higher levels of progesterone in the induced heifers (Induction Group = 1.07 ± 0.23
379 ng/mL and Control Group = 0.38 ± 0.28 ng/mL). Therefore, it shows a potential higher luteinic
380 activity for induced heifers, where 42% of the animals showed progesterone concentrations
381 above 1ng/mL, while just 12.5% of the Control heifers had it. Studies evaluating the return to
382 cyclicity considered animals with progesterone concentrations above 1 ng/mL cyclic, while in
383 our study the animals in the Control group, still did not demonstrate luteal activity. Estradiol
384 concentrations did not differ ($P=0.13$) between the groups, where Induction (220 ± 1.62 pg/mL)
385 and Control (180 ± 2.05 pg/mL) heifers were similar.

386 Around 42 days of lactation (voluntary waiting period of the farm), 33 heifers (Control
387 Group, n=13 and Induction Group, n=20) were considered suitable to reproduction, and were
388 inseminated according to the protocol described previously. The overall pregnancy rate was
389 47.75%, where induced heifers obtained a pregnancy rate of 40% for induced heifers and
390 55.55% for Control heifers. Our result was similar to that found by Freitas et al. (2010) in

391 repeated breeder cows, where 29 cows were inseminated after the induction of lactation
392 protocol and these 41.1% became pregnant.

393 It should be pointed out that, 40% of induced heifers becoming pregnant with only one
394 insemination, corroborating what has already been presented in other studies, which suggest a
395 positive effect of induced lactation on fertility. It is possible that the high serum concentrations
396 of estradiol reached during the protocol of induction, at the pre-lactation moment, have
397 interfered in the uterine environment and follicular population of these animals (Sawyer et al.,
398 1986; Pestano et al., 2015). Furthermore, it is possible that the hormonal combination used has
399 a central effect on the synthesis and release of gonadotrophins and / or an effect on the ovarian
400 follicular population.

401

402

403

CONCLUSIONS

404

405 Based upon the parameters investigated for evaluation of the lactation induction, during
406 the pre-lactation moment, in relation to the energetic status, the induced heifers showing an
407 interesting increase of NEFA, and about the inflammatory predictor, higher PON-1 levels,
408 indicating that the protocol did not cause inflammatory conditions. The hepatic profile and
409 reproductive performance did not present any changes. Therefore, lactation induction is a
410 physiological viable alternative to have dairy cows producing milk, having no negative impact.

411

412

413

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414

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418

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3 Considerações finais

A indução de lactação é uma estratégia economicamente viável e que não demonstrou interferir negativamente no metabolismo das novilhas induzidas quando comparadas a novilhas no periparto. O status energético e inflamatório dos animais demonstrou-se mais desafiado uma semana antes do início da lactação, provavelmente devido aos manejos diários do protocolo, em que durante 21 dias os animais eram conduzidos para o manejo das aplicações. Além disso, em relação aos hormônios utilizados dentro do protocolo, nota-se que as concentrações de estradiol podem ser reavaliadas, visto que atingiram concentrações extremamente elevadas, e que possivelmente em menores doses exerçeriam o efeito desejado. Contudo, mesmo que em altas doses, não foram capazes de exercer alterações prejudiciais no perfil hepático dos animais. E quanto as possíveis interferências na performance reprodutiva, o protocolo proporcionou o retorno à reprodução das novilhas, que até então eram consideradas com problemas de fertilidade e eram improdutivas dentro do sistema.

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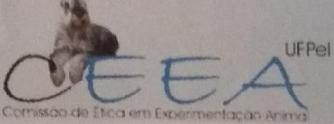
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Anexos

Anexo A – Carta de aprovação do Comitê de Ética em Pesquisa




 Pelotas, 04 de abril de 2017

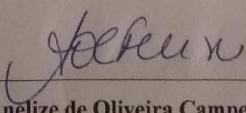
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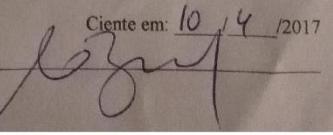
Certificamos que a proposta intitulada “**Protocolos de indução da lactação em novilhas: aspectos metabólicos, reprodutivos e de bem-estar**” registrada com o nº 23110.008716/2016-89, sob a responsabilidade de **Cassio Cassal Brauner** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua execução pela Comissão de Ética em Experimentação Animal, em reunião de 27/03/2017.

Finalidade	(X) Pesquisa	() Ensino
Vigência da autorização	05/04/2017 a 01/12/2019	
Espécie/linhagem/raça	Bovina/Holandês	
Nº de animais	60	
Idade	2-3 anos	
Sexo	Fêmeas	
Origem	Granja 4 irmãos S/A – Município de Rio Grande/RS	

Solicitamos, após tomar ciência do parecer, reenviar o processo à CEEA.

Salientamos também a necessidade deste projeto ser cadastrado junto ao COBALTO para posterior registro no COCEPE (código para cadastro nº **CEEA 8716-2016**).


M.V. Dra. Anélize de Oliveira Campello Felix
Presidente da CEEA

Assinatura do Professor Responsável: 
 Ciente em: 10/4/2017