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Injectable supplementation of butaphosphan through differents pharmaceutical forms on postpartum metabolism and milk production in dairy cows

Suplementação injetável de butafosfan através de diferentes formas farmacêuticas e os seus efeitos no metabolismo de vacas leiteiras e a produção de leite

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Abstract: The aim of this study was to evaluate the effects of different pharmaceutical forms of Butaphosphan on milk production and the metabolism in dairy cows during the postpartum period. After *in vitro* and pharmacokinetic assays, thirty-six multiparous cows belonging to the Holstein breed, were randomly divided into three groups: Group BUT (n=12), that received an aqueous solution of Butaphosphan (150 mg/mL); Group BUTSR (n=12), that received a sustained-release formulation of Butaphosphan (150 mg/mL) and Group Control (CL ; n=12), that received saline solution (NaCl 0.9 %). All the groups received three subcutaneous doses of 30 mL in the neck region, on the day of parturition (day 0) and 3 and 7 days after parturition. Blood samples were collected on days 0, 3, 7 and 10, postpartum. Daily milk production was evaluated from day 11 to 60, postpartum. The animals of the BUTSR group presented greater (P=0.01) milk production than the other groups. It was observed that the BUTSR and BUT groups showed higher blood levels of calcium (P=0.01) than the animals in the CL group. The BUTSR group obtained higher milk production compared to other groups, demonstrating that this pharmaceutical form has great potential for a future product and could be an alternative for the market. More studies are needed to better understand the action of Butaphosphan on the metabolism of dairy cows in the recent postpartum period.

Keywords: Butaphosphan; Sustained Release; Holstein Cow.

Resumo: O objetivo deste estudo foi avaliar os efeitos das diferentes formas farmacêuticas contendo Butafosfan sob a produção de leite e o metabolismo de vacas leiteiras durante o pós-parto. A partir de ensaios *in vitro e in vivo,* duas formas farmacêuticas foram testadas em animais de produção. Trinta e seis vacas da raça Holandês, foram divididas aleatoriamente em três grupos: Grupo BUT (n=12), que recebeu solução aquosa de Butafosfan (150 mg mL^{-1}); BUTSR (n=12), que recebeu uma formulação de liberação prolongada de Butafosfan (150 mg mL^{-1}) e o Grupo CL (Controle; n=12), que recebeu solução fisiológica de cloreto de sódio. Todos os grupos, tiveram administração de três doses subcutâneas de 30 mL. A aplicação foi realizada no dia do parto (dia 0) e nos dias 3 e 7 após o parto.

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Amostras de sangue foram coletadas nos dias 0, 3, 7 e 10 para avaliação de parâmetros metabólitos e a produção de leite foi avaliada do dia 11 aos 60 dias pós-parto. O grupo BUTSR apresentou maior produção de leite (P=0.01), em comparação aos demais grupos. Observou-se que os grupos BUTSR e BUT apresentaram maiores níveis sanguíneos de cálcio (P=0.01) que os animais do grupo CL. O grupo BUTSR obteve maior produção de leite em comparação aos demais grupos, demonstrando que essa forma farmacêutica possui grande potencial, podendo ser uma alternativa para o mercado. Mais estudos são necessários para melhor compreender a ação do Butafosfan no metabolismo de vacas leiteiras no pós-parto recente.

Palavras-chave: Butafosfan; liberação prolongada; Gado Holandês

1. Introduction

Dairy cows in the early postpartum period require elevated energy requirements for the production of colostrum and milk as well as for their own maintenance[1]. However, this requirement is generally not adequately catered to with the intake of dry matter intake (DMI), resulting in a negative energy balance (NEB)[1].

In order to attenuate NEB and possibly prevent these metabolic disorders, several strategies may be employed, including intake of phosphorus-based supplements, administered either orally or injected, which may improve energy metabolism[2]. Butaphosphan is a type of organic phosphorus known to be rich in bioavailability, of which 100 mg provides 17.3 mg of P in the form of [1- (butylamino) – 1 – methylethyl] - phosphonic acid [3,4]. Phosphorus is extremely important, as a constituent of the plasma membrane's nucleic acids, energetic molecules such as adenosine triphosphate (ATP), and adenosine diphosphate (ADP), in the recovery of energy balance and mineral homeostasis of animals [5,6].

Under normal circumstances, successive injections are administered because of the relatively short plasma half-life of Butaphosphan [2,7,8], making the protocol unfeasible when working with large numbers of animals. Owing to this, new strategies have been developed regarding the use of this compound, such as those consisting of sustained-release properties that are defined by the ability to release a particular compound at a predetermined quantum and time lapse. These systems may be formed by hydrogels, three-dimensional polymeric structures, capable of promoting crosslinking [9,10].

The present work is the first to administer Butaphosphan via a sustained-release formulation (Butaphosphan SR), which is biodegradable and that provides dairy cows with similar or better energy support than the commonly used multiple protocols of Butaphosphan associated with cyanocobalamin [2]. The aim of this study was to evaluate the effects of differents pharmaceutical forms of Butaphosphan on milk production and the metabolism in dairy cows during the postpartum period.

2. Material and Methods

In vitro tests of the sustained-release formulation

The sustained-release formulation was previously tested *in vitro*. In keeping with intellectual property regulations, the composition of the formulation will not be detailed in this document. Formulations were prepared according to Good Pharmaceutics Practices, using inputs (adjuvants, active substances and other materials) obtained from qualified and certified suppliers. Prior to the study *in vitro* tests were carried out in triplicate to validate the formulations, including properties such as gelation and drug-release profile.

The sustained-release formulation is characterized by being a thermosensitive hydrogel. For these tests, a concentration of 150 mg mL^{-1} of Butapho**s**phan was used. The gelation temperature was determined by the method called "tube inversion" [11,12]. For this, 5 mL aliquots of the formulation were poured into testes tubes, which were placed in a water bath that had a temperature thermostat. Each solution was progressively subjected to a temperature rise at a rate of 2 °C every 2 min, at which time the tubes were inverted to observe the state of the formulation, whether it remained liquid or became solid. When the formulation stopped flowing, after inverting the tube and on it becoming solid, the temperature displayed on the thermostat was recorded, as the gelation temperature.

A Butaphosphan release test was also carried out over an extensive period of 120 hours, by adding 5 mL aliquots of the formulation into test tubes, which were placed in a water bath at 39 ºC under agitation (Visomes Plus), so that the formulations could form the hydrogel. Thereafter, 15 mL of phosphate-saline buffer (PBS) with pH 7.4 was added into the test tubes with the formulation, to mimic the body fluids [12]. Next, every 24 hours, all the medium was removed and a new medium was added, the amount of Butaphosphan released in the medium was evaluated through absorbance analysis by UV spectrophotometry (FEMTO 700 Plus), at 254 nm. Concomitantly, 15 mL of pre-warmed PBS (39°C) was returned to the test tube. From the analysis of the different concentrations of the solution, the calibration curve was constructed. The concentration (x) and peak area (y) showed the following linear equation:

$$Y = 1715.1 X + 1.2387.$$

In vivo tests of the sustained-release formulation

The pharmacokinetic study was also performed at the Brazilian Animal Research Center, located in the municipality of Amparo, State of São Paulo (22°40´59.3´S; 46°52´06.5´W). Five Holstein cows were used with 116 to 404 DEL, 459 to 657 kg and age 2 to 6 years. Before starting the study, the animals were acclimatized over a period of 5 days, in which clinical examinations were performed daily. If any abnormality was observed in the animals during this period, they would be excluded from the study. All animals had a body condition score (ECC) equal to 4, according to the scale of 1 to 5 of Wildman et al., [13]. During this period, no medication or vaccines were allowed.

The animals received a single application of the sustained-release formulation at a concentration of 10 mg/kg of Butaphosphan, in the neck region, through the subcutaneous route. Blood samples (tubes Vaccutainer® containing EDTA; 5 mL) were collected through the mammary vein in the following periods: 0 hour (pretreatment), 4, 8, 16, 30 minutes and 2, 5, 8, 12, 24, 48, 72, 96, 120, 144, 168, 192 e 216 hours after administration. Afterwards, centrifugation was performed to obtain the plasma which was stored under refrigeration at −20 °C for further analysis.

Pharmacokinetic analyses were carried out at the laboratory LabFor – Laboratorial Analysis Ltd (Campinas, SP). From the results referring to the chromatographic analysis the following pharmacokinetic parameters of the formulation were defined: peak plasma concentration (Cmax), time to peak plasma concentration (Tmax) and area under the curve (AUC) from plasma concentration versus time between 0 and 96 hours (AUC0-96 h).

Study with production cows

Animals and management. The Committee of Ethics in Animal Experimentation of the Federal University of Pelotas approved all the procedures performed in this study (4983-2017). The study was carried out in one of the largest commercial dairy farms in the south of Rio Grande do Sul, (32.8°16'S, 52.8°32'W), Brazil, which has 500 lactating animals, with an average milk production of 30 kg/day.

Thirty-six Holstein multiparous cows were allocated into a compost barn system and were fed with a ration three times daily and *ad libitum* access to water and were milked twice daily. The ration was formulated to meet the nutritional requirements according to Nutrient Requirements of Dairy Cattle [14] (Table 1), assuming a total daily feed intake of 25.1 kg of DM as an average of milk production of 28.7 kg d^{-1}. All animals were subjected to the same management and feeding conditions.

Immediately following parturition, the cows were randomly assigned to one of three groups, while at the same time using the number of lactations (2-6 lactations) as randomization criteria: BUT (n=12), receiving an aqueous solution of Butaphosphan (Butaphosphan diluted in water for injections); BUTSR (n=12), receiving a sustained-release formulation of Butaphosphan, and Control (CL; n=12), receiving saline solution (NaCl 0.9%). Each dose was 30 mL, independent of pharmaceutical-treated groups receiving 150 mg mL^{-1} of Butaphosphan, at each injection (BUT and BUTSR). The injection in each group was delivered subcutaneously in the neck on the day of parturition (day 0; within 12 h postpartum), and on days 3 and 7 after parturition (after morning milking). No animal was excluded during the duration of the experiment.

Table 1 Diet ingredients (%) of Holstein dairy cows receiving supplementation of Butaphosphan through different formulations during the recent postpartum period.

Ingredients, green matter	Animal/day/kg
Concentrated	13.00
Corn/ silage	32.50
Pre-dried ryegrass	6.00

Water	1.00
Total	52.50
Nutrients, dry matter	**Percentage (%)**
Dry Matter %	48.38
Ajusted Protein %	16.36
Sol Prot, % CP ratio	32.43
Degradable protein % of CP ratio	65.49
Undegradable protein % of CP ratio	34.51
Histidine g/kg	2.42
Methionine g/kg	2.55
Lysine g/kg	6.89
Phenylalanine g/kg	4.66
Amino Acids g/kg	55.20
Total digestible nutrients %	75.27
Rough Neutral detergent fiber %	26.50
Acid detergent fiber %	18.29
Neutral detergent fiber %	34.49

Collection and analysis. Blood samples were collected on 0, 3, 7 and 10 days after parturition, by puncture of the coccygeal vein, using a Vaccutainer system (BD diagnostics, SP, Brazil). Blood samples were collected in two tubes: one with sodium fluoride (4 mL vacuplast®, Zhejiang, China) to obtain plasma for the assessment of glucose levels; and the other with silica (clot activator) (10 mL vacuplast®, Shandong, China) to obtain serum for the remaining biochemical analyses.

Approximately 60 minutes after collection, the samples were centrifuged at 1800 g for 15 min. Plasma and serum were transferred to microcentrifuge tube, identified and cryopreserved at −80 ° C until the analyses were performed.

Analyses of urea, albumin (ALB), total plasma proteins (TPP), gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST), calcium, magnesium, phosphorus and glucose were performed using Labtest reagents (Labtest®, Lagoa Santa, MG, Brazil). Analysis of ß-hydroxybutyrate (BHB) was carried out with the commercial RANDOX Brasil Ltda (SP, Brasil) kit. All analyses were performed using an automatic biochemical analyzer from Labmax Plenno (Labtest®, MG, Brazil). Globulin concentrations were determined by subtracting the result obtained for ALB, from that of TPP [15]. Evaluation of paraoxanase 1 activity, was carried out using the protocol described by Browne *et al.,* [16].

Milk production was recorded at the time of milking, daily from 11 days postpartum (DPP) up to 60 DDP. Data relating to the daily production of milk was recorded through the software IDEAGRI® Windows (DeLaval, MG, Brazil).

Statistical analysis. All data were analyzed in the JMP 14 statistical program (SAS Institute Inc., Cary, USA, 2016), using PROC MIXED, to evaluate group, day and the interaction (group x day), using the ANOVA method with post-hoc Tukey-Krammer evaluation ($P<0.05$), and the covariance structure used was the one with the lowest value in the Bayesian information

criterion. All variables were analyzed for their normality using the Shapiro-Wilk method and showed normal distribution. P values <0.05 were considered significant.

3. Results

In vitro tests of the sustained-release formulation

The sustained-release formulation presented a gelation temperature of 36 ºC. The release of Butaphosphan occurred over 120 hours, with a considerable release during the first 24 hours, of 73 mg. Peak release was observed at 48 hours (92 mg; Figure 1).

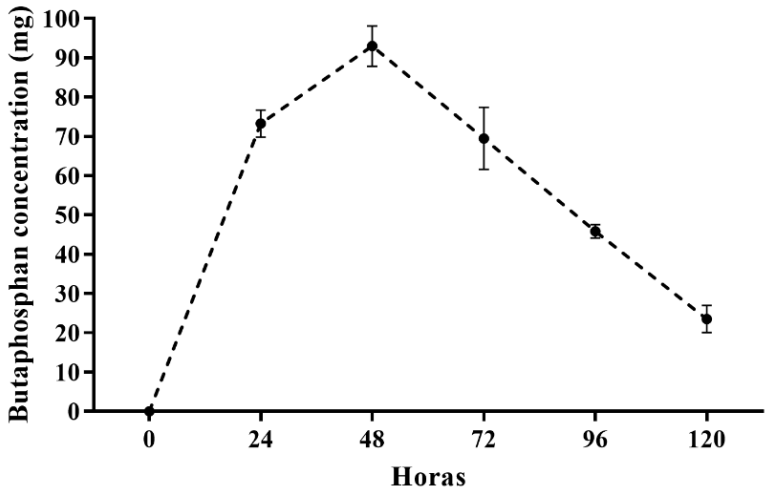

Figure 1 *In vitro* release of Butaphosphan (750 mg) from a thermosensitive formulation over 120 hours.

In vivo tests of the sustained-release formulation

The animals received a single application of a sustained-release formulation at a concentration of 10 mg/kg of Butafosfan. It took 2 hours to reach the maximum concentration (Tmax) of the active ingredient (µg L-1) after injection, with release occurring over 24 hours. The maximum concentration (Cmax) was 13,600.00 µg L-1 and the area under the curve (AUC) was 4,120,091.60 µg L-1.

Study with production cows

It was observed that cows from the BUTSR group had a higher milk production by 3.33 kg day^{-1} (43.41 ± 0.38 kg) with respect to those in the CL group (40.08 ± 0.39 kg), and by 1.81 kg day^{-1} compared to the BUT group (41.60 ± 0.37 kg), up to 60 DPP (P=0.001). No difference is observed between group*day (P=0.99).

The blood calcium concentration was higher in animals from the BUTSR (8.87 ± 0.29 mg/dL) and BUT (9.17 ± 0.29 mg/dL) groups compared to the CL group (7.82 ± 0.29 mg/dL) (P≤0.01; Figure 2a). The BUTSR (69.24 ± 2.43 mg/dL) and BUT (67.97 ± 2.36 mg/dL) groups had

a lower mean glucose value compared to the CL group (79.05 ± 2.34 mg/dL; P≤0.01; Figure 2d). Animals in the BUTSR group tended to show higher levels of ALB (2.39 ± 0.04 g/dL) and urea (30.68 ± 0.80 mg/dL) in relation to animals in the CL group (2.25 ± 0.04 g/dL; 27.91 ± 0.78 mg/dL, respectively; P=0.05), but did not differ from those subjected to the BUT treatment (2.36 ± 0.04 g/dL; 29.49 ± 0.82 mg/dL, respectively; P=0.05) (Figure 2b and c). Serum concentrations of other biochemical metabolites did not differ between groups (P>0.05, Table 2).

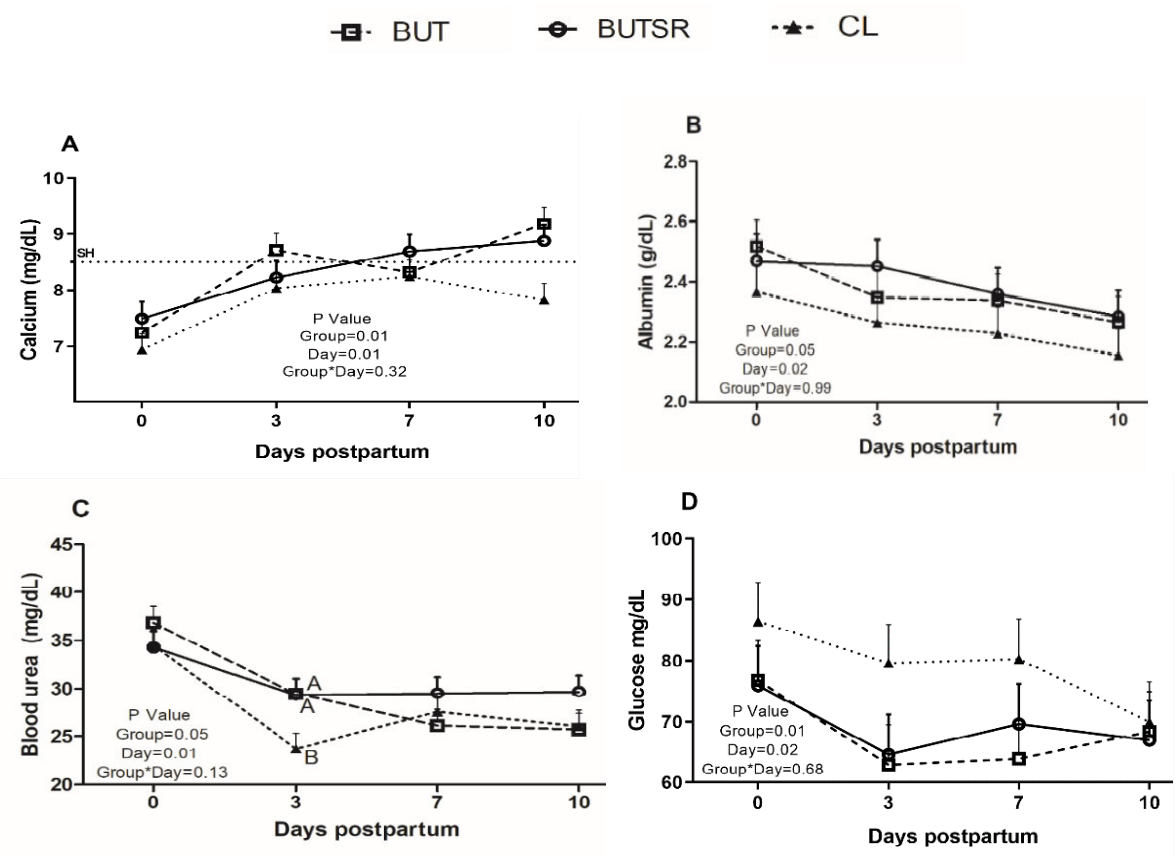

Figure 2 Blood concentrations of a) calcium (mg/dL), b) albumin (g/dL), c) urea (mg/dL) and d) glucose (mg/dL) in Holstein dairy cows receiving supplementation of Butaphosphan through different formulations on day 0 (parturition), 3 and 7 postpartum, subcutaneously.
BUTSR (n=12): animals receiving 30mL of Butaphosphan SR (150 mg/mL of Butaphosphan) through the platform of sustained-release; BUT (n=12): animal receiving 30 mL of Butaphosphan aqueous solution (150 mg/mL of Butaphosphan); CL (n=12): animals receiving 30 mL of saline (sc., NaCl 0,9 %). SH: subclinical hypocalcemia.

Table 2 Averages (± standard mean error) of biochemical parameters of Holstein dairy cows receiving supplementation of Butaphosphan via sustained-release form or aqueous solution, subcutaneously, during the recent postpartum period.

Analyzes [1]	Groups [2]			P value		
	BUTSR	BUT	CL	Group	Day[3]	Group*Day[4]
Phosphorus (mg/dL)	6.00±0.20	5.89±0.20	6.18±0.19	0.56	0.08	0.74
Magnesium (mg/dL)	1.98±0.07	2.10±0.07	2.03±0.06	0.49	0.05	0.96

Globulin (g/dL)	3.38±0.19	3.95±0.19	4.19±0.18	0.16	0.26	0.80
Total Proteins (g/dL)	6.07±0.18	6.32±0.18	6.45±0.17	0.32	0.54	0.79
BHB (mmol/L)	0.86±0.03	0.87±0.02	0.81±0.02	0.27	0.45	0.94
AST (U/L)	102.79±5.66	101.85±5.66	95.01±5.55	0.56	0.01	0.62
GGT (U/L)	33.56±2.20	31.97±2.27	30.76±2.21	0.66	0.41	0.92
PON1 (U/mL)	99.49±4.05	94.43±4.05	87.39±3.97	0.10	0.30	0.86

BHB = ß-hydroxybutyrate; AST = aspartate aminotransferase; GGT = gamma glutamyl transferase; PON1 = paraoxonase-1.
[1] Biochemical analyzes assessed on day of parturition, and 3, 7 and 10 days postpartum.
[2] Treatments consisted of three subcutaneous applications on the day of parturition (day 0), and 3 and 7 days postpartum. BUTSR (n=12): animals receiving 30 mL of Butaphosphan SR (150 mg/mL of Butaphosphan) through the sustained release platform; BUT (n=12): animals receiving 30 mL of Butaphosphan solution (150 mg/mL of Butaphosphan); CL (n=12): animals receiving 30 mL of saline (NaCl 0,9%).
[3] Day refers to the days on which blood collections were performed (day of parturition (0), 3 and 7 and 10 days postpartum).
*[4] Group*Day refers to the interaction of the group in relation to the days of blood collection. significant values at P<0.05.*

4. Discussion

Butaphosphan supplementation in dairy herds, especially in the postpartum period, is an alternative whereby to minimize the problems caused by energy deficit. However, supplementation protocols must be simplified with the aim at reducing costs and upkeep of animal welfare. Therefore, in the present study, a sustained-release formulation of Butaphosphan showed promise in *in vitro* tests, showing an adequate gelation temperature, as it changes from a liquid to a hydrogel state at temperatures ≥ 36 °C, taking into account the animals body temperature (usually 38 – 40 °C) [(17)].

It also showed prolonged release of the active principle for at least 120 hours. According to the results of the pharmacokinetic analysis the sustained-release formulation had a longer time to reach maximal plasma concentration (Tmax: 2 h), compared with the results of a study that analyzed the administration of a dose of Butaphosphan in aqueous solution intramuscularly and subcutaneously in pigs (10 mg kg^{-1} BW) and presented rapid absorption and elimination, with a mean Tmax of 0.31h [(7)]. These results are promising, taking into account the need for prolonged supplementation of the active ingredient in animals that have a high energy demand. Therefore, we decided to analyze the effectiveness of this supplementation *in vivo*.

The animals supplemented with BUTSR we demonstrated higher milk production compared to other groups (BUT and CL). And the animals supplemented with Butaphosphan (BUTSR and BUT group) exhibited higher milk production, lower glucose levels, and higher plasma calcium concentrations compared to the CL group, in this study. Notably, the most positive effect on milk production and sustained-release formulation of Butaphosphan was most likely exhibited due to prolonged supplementation.

In another study using Butaphosphan in combination with cyanocobalamin were administered around 6000 mg of Butaphosphan with the same number of injections as in our study, totaling 18.000 mg, obtaining an increase of only 1.9 kg day^{-1} of milk in comparison with the control group [(18)]. In the present study, animals in the BUTSR group showed an

increase in milk production compared to the other study groups, using a lower dose of Butaphosphan than the study of Kreipe et al., [18]. It is believed that, as it is a pharmaceutical form of prolonged release, the active principle has shown greater bioavailability compared to other pharmaceutical forms.

It is known that Butaphosphan supplementation may have favored ATP synthesis and phosphorylation of intermediates metabolic pathways such as gluconeogenesis and glycolysis, improving glucose synthesis and utilization, resulting in increased milk production, as has been suggested by other authors [2,8,18].

A decrease in glucose levels was observed in animals supplemented with butaphosphan, as evidenced by their higher milk production, with glucose known to be the main precursor of lactose [19,20]. Thus, the lower glucose concentrations recorded in the supplemented animals reflects its greater use in the production of lactose, which in turn, increased the entrance of water via the alveolar lumen of the mammary gland and increased the volume of milk produced [21].

In the study of Nuber et al., [8], the use of an aqueous solution of Butaphosphan increased concentrations of glucagon levels during early lactation. It was observed in the study Weiller et al., [22], Butaphosphan increased glucose and interfered with insulin signaling in calorie-restricted rats, with the authors suggesting that its action depends on the animal's energy status. This may have occurred in this study, in which a reduction in blood glucose was observed by possibly increasing the supply to the mammary gland and milk production.

High-production cows have greater requirements for minerals, mainly phosphorus and calcium, for the synthesis of colostrum and milk [23,24]. According to the Emam et al., [25] calcium needs can increase by up to 65% at the beginning of lactation. In the present study, all animals exhibited subclinical hypocalcemia (SH) on the day of parturition (≤8.50 mg dL^{-1}) [26]. However, the groups supplemented with organic phosphorus presented increased calcium levels, in contrast to the CL group which continued to exhibit SH throughout the experiment.

Possibly, phosphorus supplementation aided in bone demineralization, activation of which requires a parathyroid hormone to bind to its receptor in the bone tissue, activating the enzyme adenylate cyclase, which synthesizes from ATP the secondary messenger adenosine 3',5'-cyclic monophosphate (cyclic AMP) [27,28]. Therefore, increased availability of phosphorus may have accelerated the formation of this second messenger, causing it to increase plasma calcium levels faster than in the CL group.

Regarding the metabolites, ALB and urea, levels in the BUTSR group tended to increase in comparison with the CL group, but did not differ from the BUT group. As these two metabolites may indicate protein status [29,30], it is assumed that these animals increase in DMI, since Butaphosphan has been identified as an orexigenic compound [31].

Plasma inorganic phosphorus concentrations remained within the typical range of physiological values (1.29 - 2.58 mmol/L)[32], possibly because the Butaphosphan possesses a rapid metabolism and a short half-life of 116 min in dairy cows [33]. The new pharmaceutical

formulation of prolonged release of Butaphosphan, demonstrated its potential, as it allowed the treated animals to present greater milk production compared to the other groups. This probably occurred due to the increased bioavailability of the active ingredient in the body.

5. Conclusion

From this study, it was possible to conclude that the animals in the BUTSR group had greater milk production than the other groups, demonstrating that this pharmaceutical form has great potential for a future product and could be an alternative for the market. Furthermore, the BUTSR and BUT groups presented higher calcium levels and lower glucose levels compared to the CL group. More studies are needed to better understand the action of Butaphosphan on the metabolism of dairy cows in the recent postpartum period.

Declaration of Interest

All authors declare that they have no conflict of interest.

Author contributions

Conceptualization: Corrêa, M.N; Pereira, R.A. *Funding acquisition*: Corrêa, M.N; Pereira, R.A. *Project admnistration*: Bugoni, M; Machado, M.C; Pereira, R.A; Corrêa, M.N. *Validation*: Da Silva, T.C; Bilhalva, A.F. *Visualization*: Da Silva, T.C; Bilhalva, A.F. *Writing– review & editing*: Da Silva, T.C; Bilhalva, A.F; Feijó, J.O; Rabassa, V.R; Schmitt, E; Del Pino, F.A.B.

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