

Pro-inflammatory response markers and neutrophil activity index of mice treated with butaphosphan and challenged with bacterial endotoxin

Marcadores de resposta pró-inflamatória e índice de atividade de neutrófilos de camundongos tratados com butafosfano e desafiados com endotoxina bacteriana

Marcadores de respuesta proinflamatoria e índice de actividad de neutrófilos de ratones tratados con butafosfano y desafiados con endotoxina bacteriana

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Patrícia Mattei

ORCID: <https://orcid.org/0000-0002-3366-3383>
Universidade Federal de Pelotas, Brazil
E-mail: patymattei@gmail.com

Carolina Besspalhok Jacometo

ORCID: <https://orcid.org/0000-0001-5384-407X>
Universidade de La Salle, Colômbia
E-mail: cbjacometo@gmail.com

Rubens Alves Pereira

ORCID: <https://orcid.org/0000-0001-6313-8013>
Universidade Federal de Pelotas, Brazil
E-mail: rubens_ap@yahoo.com.br

Paula Montagner

ORCID: <https://orcid.org/0000-0003-0713-0185>
Universidade de Cruz Alta, Brazil
E-mail: pmontagner@unicruz.edu.br

Maria Amélia Agnes Weiller

ORCID: <https://orcid.org/0000-0001-5577-8686>
Instituto Federal de Ciência e Tecnologia do Rio Grande do Sul, Brazil
E-mail: mariaamelia.weiller86@gmail.com

Riteli dos Santos Teixeira

ORCID: <https://orcid.org/0000-0003-3739-6670>
Universidade Federal de Pelotas, Brazil
E-mail: rititeixeira@hotmail.com

Marcio Nunes Corrêa

ORCID: <https://orcid.org/0000-0003-0855-2750>
Universidade Federal de Pelotas, Brazil
E-mail: marcio.nunescorrea@gmail.com

Abstract

Butaphosphan, an organic source of phosphorus, potentiates humoral and cellular immune responses, however, it is still not clear how this occurs. We evaluate the effect of subcutaneous administration of injectable butaphosphan on pro-inflammatory markers and neutrophil activity index of mice challenged with bacterial lipopolysaccharide. Forty-two mice were randomly divided into three groups: Butaphosphan 50 (BUT50, n=14), that received injections of 50 mg/kg of butaphosphan; Butaphosphan 200 (BUT200, n=14), that received injections of 200 mg/kg of butaphosphan and Control (CTL, n=15), that received placebo. Subcutaneous injections were performed twice daily, for eight consecutive days. At the 8th day, half of animals per group were randomly selected to be challenged with bacterial lipopolysaccharide. Two hours after the challenge animals were euthanized, blood and spleen collected to analyze hematological parameters, circulating levels of interleukin 6 (IL-6), 1 β (IL-1 β) and myeloperoxidase (MPO) tissue activity. Red blood cell count was higher in butaphosphan treated groups. White blood cell count was lower in BUT200/LPS, as consequence of the decreased neutrophil count. Defense cells and IL-6 were elevated in BUT200 group. The use of butaphosphan ameliorate inflammatory markers, both in challenged and not challenged animals.

Keywords: Organic phosphorus; Cellular immunity; Lipopolysaccharide; Neutrophil; IL-6; IL-1 β .

Resumo

O butafosfano, fonte orgânica de fósforo, potencializa as respostas imunes humoral e celular, porém ainda não está claro como isso ocorre. Avaliamos o efeito da administração subcutânea de butafosfano injetável sobre marcadores pró-inflamatórios e índice de atividade de neutrófilos de camundongos desafiados com lipopolissacarídeo bacteriano.

Quarenta e dois camundongos foram divididos aleatoriamente em três grupos: Butaphosphan 50 (BUT50, n=14), que recebeu injeções de 50 mg/kg de butaphosphan; Butaphosphan 200 (BUT200, n=14), que recebeu injeções de 200 mg/kg de butaphosphan e Controle (CTL, n=15), que recebeu placebo. As injeções subcutâneas foram realizadas duas vezes ao dia, por oito dias consecutivos. No 8º dia, metade dos animais por grupo foram selecionados aleatoriamente para serem desafiados com lipopolissacarídeo bacteriano. Duas horas após o desafio os animais foram eutanasiados, sangue e baço coletados para análise de parâmetros hematológicos, níveis circulantes de interleucina 6 (IL-6), 1 β (IL-1 β) e atividade tecidual de mieloperoxidase (MPO). A contagem de glóbulos vermelhos foi maior nos grupos tratados com butafosfan. A contagem de leucócitos foi menor em BUT200/LPS, como consequência da diminuição da contagem de neutrófilos. As células de defesa e IL-6 estavam elevadas no grupo BUT200. O uso de butafosfano melhora marcadores inflamatórios, tanto em animais desafiados como não desafiados.

Palavras-chave: Fósforo orgânico; Imunidade celular; Lipopolissacarídeo; Neutrófilo; IL-6; IL-1 β .

Resumen

El butafosfano, una fuente orgánica de fósforo, potencia las respuestas inmunes humorales y celulares, sin embargo, aún no está claro cómo ocurre esto. Evaluamos el efecto de la administración subcutánea de butafosfano inyectable sobre los marcadores proinflamatorios y el índice de actividad de neutrófilos de ratones desafiados con lipopolisacárido bacteriano. Cuarenta y dos ratones se dividieron aleatoriamente en tres grupos: Butafosfano 50 (BUT50, n=14), que recibieron inyecciones de 50 mg/kg de butafosfano; Butafosfano 200 (BUT200, n=14), que recibió inyecciones de 200 mg/kg de butafosfano y Control (CTL, n=15), que recibió placebo. Las inyecciones subcutáneas se realizaron dos veces al día, durante ocho días consecutivos. En el día 8, la mitad de los animales por grupo fueron seleccionados al azar para ser desafiados con lipopolisacárido bacteriano. Dos horas después de la exposición, los animales fueron sacrificados, se recolectó sangre y bazo para analizar parámetros hematológicos, niveles circulantes de interleucina 6 (IL-6), 1 β (IL-1 β) y actividad tisular mieloperoxidasa (MPO). El recuento de glóbulos rojos fue mayor en los grupos tratados con butafosfano. El recuento de glóbulos blancos fue menor en BUT200/LPS, como consecuencia de la disminución del recuento de neutrófilos. Las células de defensa y la IL-6 estaban elevadas en el grupo BUT200. El uso de butafosfano mejora los marcadores inflamatorios, tanto en animales expuestos como no expuestos.

Palabras clave: Fósforo orgánico; Inmunidad celular; Lipopolisacárido; Neutrófilo; IL-6; IL-1 β .

1. Introduction

Phosphorus (P) is a macromineral involved in several essential functions in all living organisms. A major part (80%) of P is stored in bones and teeth (Takeda et al., 2012), and the other 20% play different roles, such as component of nucleic acids, phospholipids of biological membranes and buffers systems (Grunberg 2014, Cunningham, 2002). However, the most important function of P is implicated on its tight relationship with energy metabolism. It is a constituent of the energy storage molecule, the adenosine triphosphate (ATP), which is the main cellular fuel (Berg et al., 2006, Rollin et al., 2010). Furthermore, several metabolic pathways are modulated by phosphorylation and depend on P availability.

As the other cells of the organism, immune cells have high P dependence to entirely fulfill their functions. Since the 70's decade, scientists have been studied the relationships between P and immune response, initially demonstrated in dogs, where phosphate depletion led to a decrease in ATP levels of leukocytes, impairing the phagocytic capacity of these cells (Craddock et al., 1974). As P is required for DNA and protein synthesis, it is first used by cell division process, causing a competitive disadvantage in non-dividing cells, as the leukocytes, which can be a plausible mechanism. This speculation finds support in a study when dysfunctions in red cells, platelets, brain cells and leukocytes, all non-dividing cells, have been described in hypophosphatemic subjects (Yawata et al., 1974). Also a similar effect was observed in rats, specifically in polymorphonuclear cells (PMNLs) (Kiersztejn et al., 1991), where the impairment of phagocytosis was associated to lower phosphorus levels. Previously, it was postulated that hypophosphatemia would induce to a chronic increase of intracellular calcium (Ca) that could cause impairment of mitochondrial oxidation, production of ATP and cellular toxicity in pancreatic islets (Zhou et al., 1991) and brain synaptosomes (Massry et al., 1991). However, when a Ca channels blocker was used, the levels of ATP were not normalized, indicating that the low content of ATP in PMNLs is not a unique consequence of Ca

increase, but also due to P deficiency (Kiersztejn., 1992) Taken together, these studies point out to the importance of P availability to maintain the normal function of immune cells.

In weaning piglets, the increased oral dietary availability of phosphorus increased linearly the blastogenic response of lymphocyte in response to mitogen stimulation; however, when challenged with lipopolysaccharide, there was not interaction between P and cellular immune response, and there was a decrease on antibody response (Kegley et al., 2001). Mullarky and colleagues (Mullarky., 2009) demonstrated that different levels of oral dietary phosphorus (low, medium and high) in the diet of lactating dairy cows did not affect lymphocytes proliferation neither phagocytic activity of neutrophil. However, in our knowledge, most of published results used dietary phosphorus/phosphates. Therefore, the mechanisms by which organic sources of phosphorus in an injectable via influence the immune status are still not clear.

Butaphosphan is a molecule derived of phosphonic acid (1-butylamine-1-methyl phosphonic acid) and constitutes an organic source of phosphorus, which can be used to improve the production of cellular energy (Berg et al., 2002, Furll et al., 2010). In our knowledge, there is only one study linking injectable butaphosphan and immune system, which demonstrated that mice treated with intravenous injections had increased macrophages phagocytosis index and humoral and cellular responses ameliorate face to immunization, with the increased CD4 and CD8 lymphocytes populations (Há et al., 2005). However, it is still not clear how the butaphosphan acts as an immune stimulator at cellular levels in the innate response. In this sense, our hypothesis was that immune cells could have their functions enhanced by an extra supply of an injectable organic source phosphorus. Thus, we aimed to investigate if butaphosphan potentiates neutrophil activity, through the evaluation of pro-inflammatory cytokines and a marker of neutrophil activity in mice challenged with bacterial lipopolysaccharide (LPS).

2. Materials and Methods

2.1 Animals and experimental procedures

The experimental protocol was approved by the Animal Welfare Commission of Federal University of Pelotas (Rio Grande do Sul State, Brazil), under the number 6936, and all procedures were conducted according to the guidelines of laboratory animal use in research.

Female C57BL/6 mice, 28 weeks old, were obtained from Central Vivarium/UFPel. Mice were housed in groups of 7 animals and disposed in air circulating shelves, with controlled temperature and humidity (22-25°C and 60-70%, respectively), with 12:12h light-dark cycling (lights from 7 a.m. to 7 p.m.) The animals had free access to water and pelleted diet, and were weighed daily, with a digital electronical balance.

Mice were randomly divided into three groups: Butaphosphan 50 (BUT50, n=14), that received subcutaneous injections of 50 mg/kg of butaphosphan; Butaphosphan 200 (BUT200, n=14), that received subcutaneous injections of 200 mg/kg of butaphosphan and Control (CTL, n=15), that received saline subcutaneous injection. Injections were performed, twice daily, with a 12 hours interval, for eight consecutive days (adapted from Ha et al., 2005). Butaphosphan was dissolved in water for injection.

2.2 Endotoxin challenge

On the last experimental day (Day 8), 30 minutes after the last injection, half of the animals of each group were randomly selected to be challenged with LPS. Mice were injected intraperitoneally with 500 ng/kg of endotoxin derived from *Escherichia coli* (0111:B4, Sigma Aldrich®, St Louis, MO, USA). Therefore, the final six groups were: Control (CTL, n=8), Control + LPS (CTL/LPS, n=7), Butaphosphan 50 (BUT50, n=7), Butaphosphan 50 + LPS (BUT50/LPS, n=7), Butaphosphan

200 (BUT200, n=7) and Butaphosphan 200 + LPS (BUT200/LPS). Animals were monitored on rectal temperature at time 0 (immediately before the challenge), 60 and 120 minutes after the challenge.

2.3 Tissue and blood harvesting

Two hours after the LPS challenge, animals were anaesthetized via inhalation with Halotane (Crisália, Brasil), and then euthanized by decapitation. Blood was collected directly of cervical region in EDTA-KF tubes. An aliquot of whole blood was used to perform hemogram. The remaining blood was centrifuged at 3000xg for 15 minutes at 4°C, plasma was isolated and stored at -80°C for further analysis. In addition, spleens were harvested, snap-frozen on liquid nitrogen and stored in cryogenic tubes at -80°C until further analysis.

2.4 Hematological Analysis

Whole blood was used to perform red blood cell count (RBC) and white blood cell count (WBC), by impedance reaction, using a semi-automatic cell counter (Celm CC-530 - Celm, São Caetano do Sul, SP, Brazil). An aliquot was used to perform the differential count of leukocyte, through blood smears, using glass slides (Glass Slides to Microscopy Exacta - Perfecta, São Paulo-SP) and stained with Panoptic Dye (Laborclin, Paraná, Brazil).

2.5 Blood cytokines

Plasma samples were appropriately diluted and evaluated using a mouse IL6 and IL1 β ELISA kits (Sigma Aldrich®, St. Louis, MO, USA). Briefly, 100 μ L of sample and standards were added to each well, allowed to incubate for 2.5 hours, and then washed. A detection antibody specific to each cytokine was then added and allowed to incubate for 1 hour before washing. HRP-Streptavidin was used to detect the presence of bound antibodies, incubated for 45 minutes, and then washed. Colorimetric TMB Reagent was added to each well, incubate for 30 minutes and washed. At last, Stop Solution as added to each well and the plate was read at 450 nm immediately, in a plate reader (Thermo Plate Reader, Brazil). The absorbance readings were then used to calculate the total concentration of cytokine based on the standard curve.

2.6 Myeloperoxidase assay

The analyze of myeloperoxidase activity was performed following the protocol of Pulli et al., (Pulli et al., 2013), with modifications. According to the study of Pulli and colleagues, the neutrophil content of spleen is similar to the bone marrow. Thus, in brief, half of spleens were washed three times with PBS and incubated for 2 hours in an extraction buffer (0,32M sucrose [Synth, São Paulo, Brazil], 1mM CaCl₂ [Vetec Química Fina, Rio de Janeiro, Brazil], 10U/mL Heparin [Blau Farmacêutica, São Paulo, Brazil] in Hanks Balanced Salt Solution (Life Technologies, São Paulo, Brazil). After incubation, samples were centrifuged at 1000xg for 10 minutes, and the supernatant underwent protein precipitation by slowly mixing with 4 parts ice-cold acetone (Synth, São Paulo, Brazil). The acetone-protein mixture was then incubated for 1 hour at -20°C, and proteins were precipitated by centrifugation at 3500xg for 15 minutes at 4°C. The supernatant was discarded, and the protein pellet was air-dried and resuspended in PBS. Then, 10 μ L of prepared sample were combined with 80 μ L 0,75mM H₂O₂ (Synth, São Paulo, Brazil) and 110 μ L of tetramethylbenzidine (TMB) solution (2,9mM TMB [Sigma Aldrich, St Louis, MO, USA] in 14,5% DMSO [Synth, São Paulo, Brazil] and 150mM sodium phosphate buffer at 5,4pH), placed in a reaction plate (96-well) and incubated at 37°C for 5 minutes. The reaction was stopped by adding 50 μ L 2M H₂SO₄ (Synth, São Paulo, Brazil), and absorption was measured at 450 nm to obtain MPO activity.

2.7 Statistical Analysis

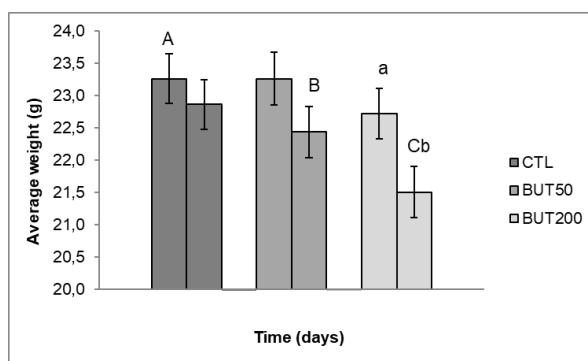
The results are presented as mean \pm standard error of mean (SEM). Data were analyzed with PROC MIXED procedure of SAS 9.3 (SAS Institute Inc., Cary, NC). Fixed effects in the model were butaphosphan treatment, the challenge with endotoxin and their interaction. Random effect was animal within the treatment. Hematological analyzes, cytokines and myeloperoxidase concentrations were log₂-scale transformed if needed to comply with normal distribution of residuals and back-transformed. Least squares means separation was performed using the PDIF statement. Statistical significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

3. Results

3.1 Weight

Initially animals had similar body weight ($P > 0.05$), however after eight days of treatment with butaphosphan, animals from BUT200 group had a lower body weight compared to BUT50 (22.08 g vs. 22.54 g; $P = 0.025$) and CTL (22.08 g vs. 23.10 g; $P < 0.001$). BUT50 animals also presented a lower body weight related to CTL (22.54 g vs. 23.10 g; $P = 0.003$; Figure 1). BUT200 mice demonstrated a reduction on body weight during the experiment ($P = 0.024$).

Figure 1 Average body weight of mice treated with different doses of butaphosphan throughout the experiment. Uppercase letter indicates differences between treatments at the 8th experimental day. Lowercase letter indicates differences between the first and last day of the experiment within the treatments.



Source: Authors.

3.2 Hematological analysis

All the results regarding hematological counts are presented on Table 1. There was a treatment effect on RBC count, with increased erythrocytes on groups BUT200 and BUT50 compared to CTL ($P < 0.001$ and $P = 0.015$, respectively). There was no challenge or treatment challenge interaction effect on RBC count.

There was a treatment \times LPS interaction effect on WBC count and a trend was observed for higher WBC on challenged animals (LPS) compared with the ones that were not challenged (No LPS) ($P = 0.094$). When challenged, the higher dose of butaphosphan reduced the leukocytes count (BUT200/LPS vs. CTL/LPS; $P = 0.007$ and BUT200/LPS vs. BUT50/LPS; $P = 0.005$), however there was no difference between CTL/LPS and BUT50/LPS groups. When comparing the treatments without the LPS challenge, the treatment with butaphosphan, independently from the dose, increased the WBC count ($P < 0.05$). Interestingly, when animals were challenged, CTL group increased WBC (CTL vs. CTL/LPS; $P < 0.001$), BUT50 response were affected by LPS challenge (BUT50 vs. BUT50/LPS; $P > 0.05$) and BUT200 group reduced the WBC count when challenged with LPS (BUT200 vs. BUT200/LPS; $P = 0.035$).

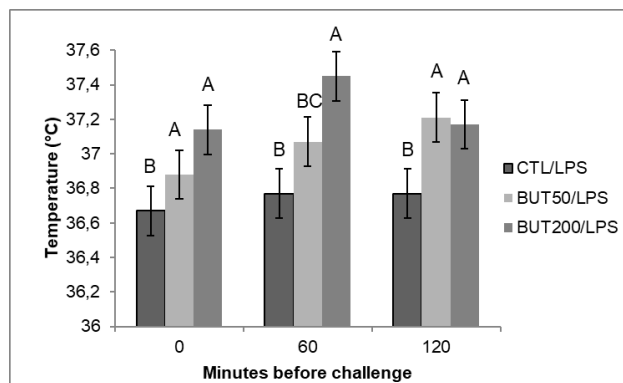
Neutrophil count also had a treatment by LPS interaction and LPS effect. The treatment with the higher dose of butaphosphan, when challenged with LPS (BUT200/LPS), reduced almost 50% the neutrophil count when compared with BUT50/LPS and CTL/LPS ($P < 0.001$). However, an opposite response was observed when animals were not challenged with LPS, where the BUT200 groups had higher neutrophil count than BUT50 and CTL groups ($P < 0.01$). The challenged with LPS increased the neutrophil count in the CTL and BUT50 groups ($P < 0.001$ for both), however, when animals were treated with the higher dose of butaphosphan, there was no effect of LPS challenge for neutrophil count.

For lymphocyte count there was also a treatment by LPS interaction and LPS effect. When animals were not challenged with LPS, lymphocyte count increased when treated with butaphosphan, independently from the dose, when compared to control ($P < 0.05$). However, when challenged with LPS, BUT200/LPS tended to reduce the lymphocyte count compared to CTL ($P = 0.081$). The challenge with LPS reduced the lymphocyte count on the groups treated with butaphosphan (BUT200 and BUT50; $P < 0.001$ for both), while CTL were not affected by the challenge.

3.3 Body temperature post-LPS challenge

There was a difference between body temperature of the animals before the LPS challenge, where BUT200/LPS group demonstrated higher body temperature related to CTL/LPS group ($P = 0.019$). After the challenge, this pattern was sustained, and at 120 minutes was observed a higher temperature also in BUT50/LPS group, related to the CTL/LPS ($P = 0.028$). No significant differences were observed between the initial and the final body temperature in all treatments ($P > 0.05$).

Figure 2 Body temperature after challenge with bacterial endotoxin in mice treated with different doses of butaphosphan. Time 0 indicates the basal temperature and capital letters indicate differences between treatments at the same time



Source: Authors.

3.4 Cytokine's concentration

IL-6 concentration was affected by treatment, LPS challenge and their interaction, while IL-1B concentration remained unchanged (Figure 2). The higher dose of butaphosphan (BUT200) increased circulating IL-6 compared to BUT50 and CTL ($P < 0.001$ for both) when animals were not challenged with LPS. When challenged with LPS, the treatment with butaphosphan did not affect IL-6 concentrations ($P > 0.05$). However, the challenge with LPS increased the IL-6 concentrations in all groups ($P < 0.001$).

3.5 Myeloperoxidase Activity

Overall, effects of treatment, LPS challenge and their interaction were not observed for myeloperoxidase activity ($P > 0.05$; Table 3). However, there was a trend of increased myeloperoxidase activity in BUT200/LPS group compared to BUT200 ($P = 0.069$).

4. Discussion

Studies carried out by our research group with cattle and sheep demonstrated the benefits of butaphosphane in modulating energy and lipid status. Individuals treated with butaphosphane during periods of intense immunological challenge, as the days around the parturition, demonstrated improved lipid and energy metabolism, resulting in a lower incidence of metabolic disorders and ameliorating productive and reproductive indexes, reflecting a general enhanced health status (Pereira et al., 2013, Pereira et al., 2013, Lima, 2014, Tabeleão et al., 2016, Pizoni et al., 2022). However, in this studies, as in others (Rollin et al., 2010, Furll et al., 2010, Temizel et al., 2015), the butaphosphane was used in association with cyanocobalamin (vitamin B12), an enzymatic co-factor of methylmalonyl – CoA mutase, that convert propionate to succinil CoA, an essential step to take part into acid citric cycle, to be used on gluconeogenesis (Kennedy et al., 1990). Besides that, a major part of studies relating P and immune response involves dietary P supplementation, which differs at several points in pharmacokinetics from injectable P. Then, as far as we know, our study is the first that aims to clarify the effects of butaphosphane as an immune stimulator and the relations of organic injectable phosphorus with the immune function in mice.

Mice treated with butaphosphane had a lower body weight compared to control mice, with greater weight loss in the group with the higher dose of butaphosphane. Similar results were observed by other authors with inorganic phosphorus, with a lower body weight gain and lower food intake with diets containing higher phosphorus (Karlen, 1989, Katsumata et al., 2004, Tani et al., 2007). In contrast, when the inorganic phosphorus was provided after a deprivation of phosphorus in rats, the body weight was increased immediately, with a similar effect on food intake (Landsman et al., 2001). Thus, both deprivation and supplementation of dietetic phosphorus exert an effect on body weight and on appetite (Landsman et al., 2005). Another hypothesis for the decrease of body weight could be found in the closely relation of phosphorus and calcium metabolism. Both calcium (Ca) and P homeostasis are orchestrated by osseous mobilization, absorption and re-absorption from intestine and renal excretion into urine, and involves several endocrine factors, as 1,25-dihydroxyvitamin D3 and parathyroid hormone (Berndt et al., 2009, Talmage, et al., 2008, González et al., 2019).

A study with swine demonstrated that animals treated with high levels of dietary P compared to low dietary levels, controlling the Ca:P ratio did not present different rates of bone resorption, and the hormones involved in the counterbalancing mechanisms worked to minimize urinary losses and increase enteral absorption and maximization of dietary renal loss. In contrast, the increase of dietetic phosphorus without an adequate calcium balance, as in our study, induced to hormonal changes, which is equivalent to a mild hyperparathyroidism, with decrease of 1,25-dihydroxyvitamin D3 (Portale et al., 1987) and consequent increase of bone reabsorption and reducing bone mass (Takeda et al., 2012, Shah et al., 1967), which can explain the greatest weight loss in the group with the higher dose of butaphosphane.

The increase of red blood cell count in the groups treated with butaphosphane observed in our study was previously reported by others that used the association of butaphosphane and cyanocobalamin in dogs (Deniz et al., 2009) and horses (Coppo & Gapel, 2018). However, this effect was strongly associated with the cyanocobalamin, essential to the normal maturation and development of red globes (Reece, 2007). Our study is the first that demonstrates that organic injectable phosphorus can enhance the production of red cells by itself. Also, an interesting pattern was observed in the not challenged groups treated with butaphosphane, with a more pronounced increase on neutrophil and lymphocyte count. These results can be

supported by the closely relationship between skeletal and immune system. Bone and hematopoietic/immune cells are derived from the same origin, interacting on bone marrow and therefore use common signaling pathways (Takayanagi, 2007), including the RANKL (receptor activator of NF- κ B ligand), which plays different roles in immune system, e.g. the antigen presentation by T cells, the necessity for the normal development of B cells and immunomodulatory and immunosuppressive effects (Guerrini & Takayanagi, 2014). Oster and colleagues (2016) demonstrated that swine receiving high amounts of dietary P exhibited an increase on abundance of RANK signaling mRNA, and in other pathways involved with inflammation responsiveness to P and related to the recognition pattern, as described previously (Camalier et al., 2013). Therefore, as in our study there was no balance between Ca: P and the effect on immune cells was could be more pronounced, with a positive increase in the number of cells derived from the bone marrow in the groups that received injectable organic phosphorus.

Neutrophil constitute the front line if innate immune system defenses, being the first cell to response against an infectious process induced by bacteria and fungi (Segal, 2005, Júnior et al., 2018), and its function is to fight against invasive pathogens not only by their own activity, but also stimulating the production of key-regulator of inflammation process, as the cytokines (Ganeshan & Chawla, 2014). IL-1 β is produced mainly by macrophages, monocytes, dendritic cells and neutrophils, and among its function it can mention the induction of adhesion molecules, that promotes the infiltration of inflammatory cells to the extravascular space, towards the injured tissue and also the increase on number of neutrophil on circulation (Dinarello, 2009), while the IL-6 is also activated by IL-1 β and it is a key molecule for the transition from innate into adaptive immunity, through the induction of neutrophil apoptosis (Kaplanski et al., 2003). After the endotoxin challenge, the number of neutrophils of BUT200 group was drastically reduced when compared to CTL and BUT50 groups; however, when we compared the number of neutrophils of this group before and after the challenge, it remained unchanged. In addition, there was no effect of butaphosphan treatment on the concentration of IL-6 and IL-1 β before the challenge. Taken together, these findings show that besides the lower number of neutrophil on BUT200 group after the challenge, they produced the same concentration of cytokines of the other groups, pointing out that animals treated with the major dose of injectable organic P was benefited from the greater supply of this nutrient, producing the same amount of IL-6 and IL-1 β . This mechanism could be used to resolve the inflammation more efficiently.

It is important to highlight that before the endotoxin challenge, BUT200 group demonstrated a higher concentration of IL-6, in addition to a higher neutrophil count. Despite that, this increase does not constitute an acute inflammatory response, as could be observed after challenge with endotoxin, in which the concentrations of the cytokine exceed 3,000 pg/ml. This result confirm that the endotoxin challenge was effective, since IL-6 is a classical pro-inflammatory cytokine and its concentration is increased during acute-phase immune response (Hirano et al., 1990), reaching similar concentrations as previously reported using the same dose of the endotoxin (Rollin et al., 2009).

The systemic administration of LPS in healthy laboratory animals triggers to an acute phase response, that can include fever (Dantzer, 2004) or an hypothermic response, as some studies have demonstrated (Queen et al., 2016, Skelly et al., 2013). This variation could be explained by the differences in the dose of LPS utilized in each study. Intermediary doses of LPS generally cause fever (Schneiders et al., 2015, Morimoto et al., 1988, Romanovsky et al., 1996) in other hand, highest doses of LPS can cause an hypothermic effect (Queen et al., 2016, Skelly et al., 2013). In our study we do not observed any increase on body temperature during the experiment, inside the treatments, that could be explained by the lower dose utilized in the endotoxin challenge, as seen by (Copeland et al., 2005). Then, the dose was sufficient to initiate an acute response, as confirmed by the elevation on IL-6 concentrations, but not to increase the body temperature.

Among the mechanisms used by neutrophils to fight against infections is the myeloperoxidase (MPO) production. This enzyme have the unique propriety of oxidizing the ion chloride and use the hydrogen peroxide, formed on oxidative burst,

to generate hypochlorous acid, during the process of phagocytosis (Kothari et al., 2010), and it is also necessary to the formation of neutrophil extracellular traps (NETs). NETs are formed by chromatin and nuclear proteins associated to enzymes from neutrophil granules, including MPO, with the aim of neutralize and kill bacteria extracellularly (Brinkmann et al., 2004), an efficient way to solve the infection. In our study, we did not observe significant differences on MPO splenic activity. However, the LPS challenge induced to a biological increase on MPO activity on BUT200 animals. It is important to emphasize that in our study, the technique used to measure the MPO activity extracts the extracellular fraction of this enzyme, which is found on NETs and it is essential to the formation of these structures (Metzler et al., 2010). Besides that, the lower number of neutrophil associated with the greater activity of MPO in the group that received the highest dose of injectable organic phosphorus point out to an enhanced neutrophil activity, which could result in a faster resolution of inflammation.

5. Conclusion

In conclusion, animals treated with an organic injectable source of phosphorus showed an increase on red and white blood cell count, specifically in neutrophil and lymphocyte, demonstrating a positive effect on hematopoiesis. Also, the animals treated with the highest dose of butaphosphan maintained the number of neutrophil unchanged after endotoxin challenge, associated with a similar concentration of IL-6 and IL-1B in respect to the other groups and a numerical increase in MPO activity, point out to an enhanced neutrophil function. It is plausible that the extra supply of phosphorus has been used by these cells to resolve the inflammation more efficiently.

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Núcleo de Pesquisa, Ensino e Extensão em Pecuária (NUPEEC), Faculdade de Veterinária, Universidade Federal de Pelotas.

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