CURRALEIRO PÉ DURO CATTLE BREED (*Bos taurus*) DIFFER IN PHAGOCYTIC NEUTROPHIL FUNCTION RELATED TO CROSSBREED CATTLE (*Bos taurus* X *Bos indicus*)

BOVINOS DA RAÇA CURRALEIRO PÉ DUTO (*Bos taurus*) APRESENTAM FUNÇÃO FAGOCÍTICA DE NEUTRÓFILOS DIFERENCIADA EM RELAÇÃO A BOVINOS CRUZADOS (*Bos taurus* X *Bos indicus*)

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Palavras chave: Imunidade inata; Fagocitose; Metabolismo oxidative; Raça bovina local.

ABSTRACT

This work aimed to evaluate hematological parameters and phagocytic neutrophil functions of naturally selected Curraleiro Pé Duro cattle breed (*Bos taurus*) and crossbreed cattle (*Bos taurus* X *Bos indicus*). Two groups of adult cows: Natural= Curraleiro Pé Duro cattle breed (n=10) and Selected = ¾ Nelore X Simental crossbreed animals (n=10) were evaluated. Blood was sampled to make hemogram, *in vitro* evaluation of neutrophil phagocytosis and basal Nitroblue Tetrazolium - NBT reduction (without any stimulus). The results showed no difference in hematological parameters between these groups. Natural group had greater phagocytic index of neutrophils through pathogen-associated molecular patterns receptors (PAMPs) while in Selected group this same index was greater using opsonin receptors. The reduction of NBT was lower for Natural group than Selected group. In conclusion, we suggest that both animal groups showed differences in neutrophil phagocytic functions in relation to pathway activation and Curraleiro Pé Duro cattle breed produced more oxygen radicals (superoxide, O_2^-) than the other tested group.

RESUMO

Este trabalho teve como objetivo avaliar os parâmetros hematológicos e funções fagocíticas de neutrófilos de animais Curraleiro Pé Duro (*Bos taurus*) e bovinos mestiços (*Bos taurus* X *Bos indicus*). Foram utilizados dois grupos de vacas adultas: Natural = Curraleiro Pé (n = 10) e Selecionado = animais mestiços da raça Nelore X ¾ Simental. O sangue foi amostrado para realizar hemograma, avaliação *in vitro* de fagocitose por neutrófilos e redução basal *Nitroblue Tetrazólium* - NBT (sem qualquer estímulo). Os resultados não mostraram diferença de parâmetros hematológicos entre esses grupos. O grupo Natural teve maior índice fagocitário pelos neutrófilos pelos receptores para padrões moleculares associado a patógenos (PAMPs), enquanto no grupo Selecionado, este mesmo índice foi maior pelos receptores para opsoninas. A redução do NBT foi menor para os animais do grupo Natural do que o grupo Seleccionado. Como conclusão sugere-se que ambos grupos apresentaram diferenças nas funções fagocíticas de neutrófilos no que se refere à via de ativação e animais Curraleiro Pé Duro produziram mais radicais de oxigênio (superóxido, O2-) do que os animais do outro grupo testado.

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INTRODUCTION

Most domesticated livestock species, including bovines, were introduced to Brazil by Portuguese settlers soon after its discovery. During almost five centuries, these cattle breeds have been naturally selected, they survived and adapted to unfavorable environmental and poor nutritional conditions. Curraleiro Pé Duro cattle is a naturalized cattle breed that lives in Brazilian savanas and semi-arid regions, submitted to high temperatures and several months without rain (Carvalho & Girão, 1999; Mariante & Egito, 2002). This breed is known by its resistance to diseases and few studies have evaluated the lymphocyte profile and compared the specific and nonspecific immune system responses of Curraleiro Pé Duro with Nelore animals challenged with BCG vaccine (Lobo, 2009. Moraes et al, 2009, Maggioli et al, 2013).

Because this cattle breed is at risk of extinction, its inclusion in livestock production systems is essential to survival and expansion of this animals. Taking into account that the possibility of checking parameters and functional markers of immune system performance can be useful to evaluate well-being, health and performance of these animals (Benatti, 2013) and then to justify the use of this breed in different production systems.

The innate immune system is responsible to identify the pathogen and defend the host at the time while specific adaptive responses are being constructed, its effector mechanisms help to contain the infection and increase the host possibilities to survive. Phagocytes play a key role in the first line defense against several infections. Phagocytosis and respiratory burst are the two most important functions and they are essentials for the elimination of invading microorganisms. The process of ingestion and killing of microorganisms is complex and requires a coordinated effort of several cellular activities. Phagocytosis of particles is mediated through several receptors on the cell surface, after ingestion of the pathogen, enzymes within the cell are activated and killing of the pathogen is enhanced by reactive oxygen species produced in the process of respiratory burst (Underhill & Ozinsky, 2002a,b)

The present study evaluated the *in vitro* phagocytic neutrophil functions of naturally select cattle breed and crossbreed cattle in order to contribute to a better understanding of the action of innate immune system involved in first line defense against pathogens.

MATERIAL AND METHODS

Study groups

Blood samples were obtained from 25 adult female healthy calves, aged 3.5 to 15 years old, the animals were not pregnant and all were submitted to same environmental and husbandry conditions. Two groups of cows: Natural= Curraleiro cattle breed (n=10) and Selected = ¾ Nelore X Simental crossbreed animals (n=10) were evaluated.

Hemogram evaluation

Blood sample was obtained by jugular venopuncture, collected in tubes with EDTA. The hemogram was undertaken by automated method ABCvet (*Animal Blood Counte* – Horiba ABX – São Paulo) and the leukocyte differential counting was in blood smears, stained by Rosenfeld method. For differential cell counts, smears were prepared, air-dried, fixed with Rosenfeld stain (Rosenfeld, 1947) and quantified by direct microscopic observation.

Phagocytosis evaluation.

Phagocytosis of *Saccharomyces cerevisiae* was adapted from the technique previously described by Muniz-Junqueira et al. (2003) by human. Briefly, samples of 40 μl per area of whole peripheral blood obtained by means of jugular venopuncture and individually placed on clean glass slides containing 8 marked areas of 7 mm diameter each, in duplicate preparations, incubated in a wet chamber for 45 min at 37°C. The slides were then rinsed with 0.15 M phosphate-buffered saline (PBS), pH 7.2, at 37°C, to remove non-adherent cells. Adhered cells (1266 ± 857 cells; 67 ± 13% neutrophils) were incubated for 30 min with a suspension of $2.2x10^5$ *S. cerevisiae* in 20 μl Hanks-triz solution (Sigma, St Louis, MO, USA), pH 7.2, with or without 10% serum from the individual animal donor, in a wet chamber at 37°C, to allow phagocytosis. Slides were rinsed with 0.15M PBS, at 37°C, to get rid of non-phagocytosed *S. cerevisiae* and the final washing were made with 30% serum in Hanks-Tris. The slides were fixed with absolute methanol and stained with 10% Giemsa

solution. The number of *S. cerevisiae* phagocytosed by 100 neutrophils, was individually assessed, in individual preparations, by optic microscopy. Microscopic fields distributed throughout the slide were randomly selected and all phagocytes in each particular field were examined. The phagocytic index (PI) was calculated as the average number of attached plus ingested *S. cerevisiae* per phagocytosing phagocyte multiplied by the percentage of these cells engaged in phagocytosis. Baking yeast (*S. cerevisiae*) was prepared according to the technique of Lachmann & Hobart (1978). In short, 50 g of fresh live yeast (Fleischmann, Brazil) were suspended in 220 ml of PBS, pH 7.2, autoclaved at 120°C for 30 min, washed in PBS until obtaining a clear supernatant and the sediment was suspended in 28 ml of a 0.1M 2-mercaptoethanol solution in PBS. After 2 hours incubation with stirring, yeasts were washed again and suspended in 55 ml of 0.02 M iodoacetamide in PBS. After extra 2 hours incubation at room temperature with stirring they were washed 3 times, and suspended in 220 ml of PBS, pH 7.2. Yeasts were again autoclaved, washed and suspended in 110 ml of veronal buffered saline, pH 7.2, containing sodium azide, and stored at 4°C until used. For each experiment, yeast suspensions were washed in PBS, quantified, suspended in Hanks-Tris solution, and sensitized or not with 10% fresh bovine serum at 37°C for 30 min.

Nitroblue tetrazolium slide test

Nitroblue tetrazolium (NBT) test was adapted from the technique described by Campbell & Douglas (1997). Briefly, phagocytes were adhered on slide as above described. After 30 min incubation, slides were washed, and phagocytes were incubated with 0.05% NBT solution in Hanks-Tris (Sigma, St Louis, MO, USA) for 20 min at 37°C in a humidified chamber. The slide was then washed, fixed with methanol and stained with a solution of 1.4% safranin and 28.6% glycerol in distillate water. The percent phagocytes with NBT reduced in the cytoplasm was observed by optic microscopy.

Statistical analysis

Hemogram values were compared with reference parameters described by Paula Neto (2004) to Curraleiro Pé Duro cattle breed and Costa (1994) to crossbreed cattle to verify alterations. Data were previously tested employing the Bartlett's test for equal variances and the Kolmogorov-Smirnov test for the normality of their distribution before comparative analysis. The analysis was performed by the t test to compare non-related normal samples. The Mann-Whitney test was used to compare unrelated non-normal samples. Differences with a two-tail value of $p<0.05$ were considered statistically significant. The *Prism 4*® software package (GraphPad, USA) was employed for statistical tests and graphical representation of the results.

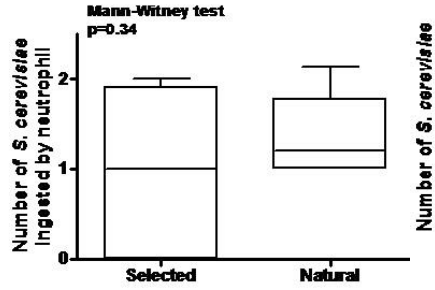
RESULTS

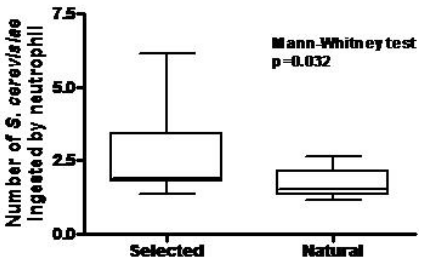
The results of hemogram in Natural group was inside reference parameters described by Paula Neto (2004) while the Selected group presented band neutrophil (253,93 ± 131,71) and eosinophil (1.829,73 ± 1.204,76) values increased when reference parameters to Nelore cattle breed were verified (Costa, 1994).

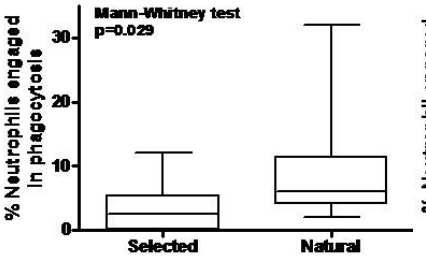
The median of the phagocytic index of neutrophils through pathogen-associated molecular pattern receptors (PAMPs) of Natural group (PI=7) was 1,46 times higher than that genetically Selected group (PI=4.8) ($p = 0.04$, t test) (Figure 1, bottom). The increased phagocytic index was due to the increase of the number of neutrophils engaged in phagocytosis ($p=0.029$), Mann-Whitney test) (Figure 1, middle), while there was no difference in the number of phagocytosed *Saccharomyces cerevisiae* by neutrophil ($p=0.34$, Mann-Whitney test) (Figure 1, top).

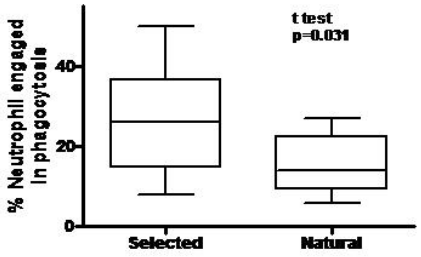
Phagocytosis through opsonin receptors showed oposite results. The median of phagocytic index of Neutrophils from Natural group neutrophils was significantly 2.56 times lower (PI=23) ($p=0.038$, t test) than that genetically Selected group (PI=59) (Figure 2 bottom). This decrease was due to lower quantitative involvement of neutrophils in phagocytosis ($p=0.031$, t test, Figure 2 middle) and due to the decrease of the number of phagocytosed *Saccharomyces cerevisiae* by neutrophil ($p=0.032$, Mann-Whitney test) (Figure 2, top).

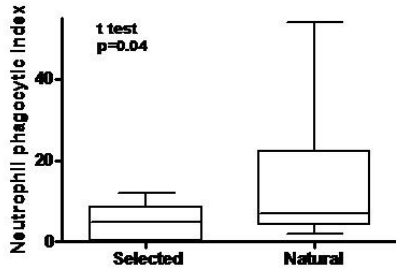
The capacity to generate oxygen radicals by phagocytes evaluated by the percent reduction of NBT was also increased in the Curraleiro Pé Duro animals, as illustrated in Figure 3. The median of percent reduction of NBT was significantly lower for crossbreed cattle breed (44%) than Curraleiro Pé Duro phagocytes (64%); $p=0.02$, t test.

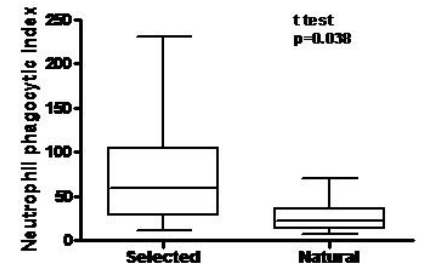












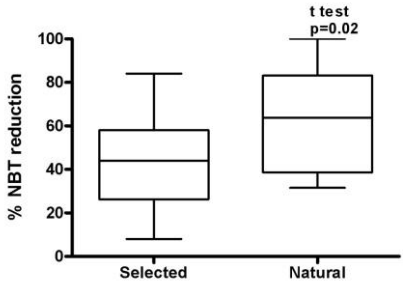


Figure 1 Figure 2 Figure 3

Figure 1. Phagocytosis of nonsensitized *S. cerevisiae* yeasts by neutrophils from peripheral blood of Selected group (3/4 Nelore X Simental crossbreed cattle) and Natural group (Curraleiro Pé Duro cattle breed). *[Fagocitose de leveduras não sensibilizadas de S. cerevisiae por neutrófilos do sangue periférico do grupo Selecionado (animais cruzados 3/4 Nelore X Simental) e grupo Natural (raça Curraleiro Pé Duro)]*.

*The data are expressed as medians (solid line in each box), quartiles (the tops and bottoms of each box), and minimum and maximum values (bars). Top- average number of S. cerevisiae yeasts ingested by phagocytosing neutrophils; Middle- proportion of neutrophils engaged in phagocytosis; Botton- phagocytic index.

Figure 2. Phagocytosis of sensitized *S. Cerevisiae* yeasts by neutrophils from peripheral blood of Selected group (3/4 Nelore X Simental crossbreed cattle) and Natural group (Curraleiro Pé Duro cattle breed). *[Fagocitose de leveduras sensibilizadas de S. cerevisiae por neutrófilos do sangue periférico do grupo Selecionado (animais cruzados 3/4 Nelore X Simental) e grupo Natural (raça Curraleiro Pé Duro)]*.

*The data are expressed as medians (solid line in each box), quartiles (the tops and bottoms of each box), and minimum and maximum values (bars). Top- average number of S. cerevisiae yeasts ingested by phagocytosing neutrophils; Middle- proportion of neutrophils engaged in phagocytosis; Botton- phagocytic index.

Figure 3. Percent reduction of nitroblue tetrazolium – NBT in Selected group (3/4 Nelore X Simental crossbreed cattle) and Natural group (Curraleiro Pé duro cattle breed). *[Porcentagem de redução do nitroblue tetrazolium – NBT no grupo Selecionado (animais cruzados 3/4 Nelore X Simental) e grupo Natural (raça Curraleiro Pé Duro)]*.

*The data are expressed as medians (solid line in each box), quartiles (the tops and bottoms of each box), and minimum and maximum values (bars).

DISCUSSION

The increase of band neutrophils in bovine is associate with acute stress, these cells remain in peripheral blood during six to 24 hours and them they decrease gradually (Jain, 1993). Coppo et al. (2000) cited that eosinophil increases in bovine was related to catecolamins release during handling, containment or blood

collection in crossbreed calves, this process was defined as sympathic alarm and can explain the results in Selected group, that presented a poor temperament during blood collection.

Galapero et al (2015) reported that despite little information about the phagocytosis index in ruminants, this would be a good parameter for assessing the defense capability of the animal against external aggressions including stress factors.

Both studied groups were capable to affect the phagocytosis but Curaleiro cattle used mainly the pathway of activation through pathogen-associated molecular pattern receptors (PAMPs) while Selected group obtains a higher phagocytosis index through opsonin receptors. The phagocytosis index obtained in this study were lower than those described by Dewangan et al (2014) that worked with a Pakistan cattle breed. However, it may be related to the use of *S. aureus* as antigen to promote phagocytosis.

The PAMPs receptors bind phagocytes and pathogen, transduce cellular signals derived from these molecular patterns and alert the host to the presence of a danger to the host. Their purpose is to sense foreign structural components and to organize quickly, appropriate defense response (Janeway Jr & Medzhitov, 2002; Werling & Coffey, 2007). On the other hand, the opsonin-dependent mechanism of phagocytosis requires serum components (IgG and C3) that function as a bridge between the microorganisms and the specific receptors on the phagocyte surface (Ofek et al., 1995).

The nonopsonic phagocytosis may have developed even earlier, because they do not require complement. The requirement for antibodies to function as opsonins may have developed later to cope with microbial mutations that either rendered the microbes unable to activate complement or that resulted in constituents not recognized by phagocytes. It is not clear what factors or which phagocyte receptors are more important, because nonopsonic phagocytosis and opsonophagocytosis appear to be connected (Ofek et al., 1995).

Basal NBT reduction found in this work was higher than results obtained by Poli & Mantelli (1974), that cited median of 1,35% in forty-five bovines with three years old, and Valinoti et al. (1988) that found 1% of spontaneous NBT reduction in seventeen Holstein-Friesian cows. On the other hand, these results were similar to those described by Dewagan et al (2014). The discrepancies of results can be justified by individual variance, laboratorial differences, anticoagulant and possibility of interference in neutrophil membrane permeability and spontaneous activities of enzymatic systems involved in NBT restoration (Gerasimov & Ignatov, 2004).

In bovine neutrophils, the respiratory burst does not occur in its fullest extent following phagocytosis and oxygen-dependent microbicidal activities of bovine neutrophils may not be fully operative to all pathogens (Silva & Jain, 1988).

We showed in this work that there are different way and intensity to express innate immune response in these cattle breed and this is in agreement with studies carried with other cattle breeds (Semerdjiev, et al, 2006). The methodology used in this experiment proved to be practical and adaptable under field conditions, and may be useful in studies related to the animal welfare in different production systems. However, more investigation must be carried through to clarify how much is this important for the resistance and productive performance of these animals.

CONCLUSION

In conclusion, our findings contribute to broaden the understanding of the phagocytic neutrophil functions in these cattle breeds. Our data showed that crossbreed cattle was sensible to stress factors during blood sampling.

Curraleiro cattle breed neutrophils had best phagocytic index through pathogen-associated molecular pattern receptors (PAMPr) while in crossbreed cattle it occurred when opsonins receptor was activated and Curraleiro cattle breed (*Bos taurus*) produced more oxygen radicals (superoxide, O^-) than genetically selected crossbreed cattle (*Bos taurus* X *Bos indicus*) during phagocytosis process.

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