

## Effects of *in vitro* selenium supplementation on blood and milk neutrophils from dairy cows<sup>1</sup>

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**ABSTRACT.**- Souza F.N., Blagitz M.G., Latorre A.O., Mori C.S., Sucupira M.C.A. & Della Libera A.M.M.P. 2012. **Effects of *in vitro* selenium supplementation on blood and milk neutrophils from dairy cows.** *Pesquisa Veterinária Brasileira* 32(2):174-178. Departamento de Clínica Médica e Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Prof. Dr. Orlando Marques de Paiva 87, Cidade Universitária, São Paulo, SP 05508-270, Brazil. E-mail: [nogueirasouza@yahoo.com.br](mailto:nogueirasouza@yahoo.com.br)

The study was designed to assess the effects of *in vitro* selenium addition on intracellular hydrogen peroxide production by neutrophils from the milk and blood of dairy cows. Blood from 10 dairy cows and 20 milk samples from five dairy cows were incubated with 0 mg (control) or 10 $\mu$ M of sodium selenite. Then, milk and blood neutrophils were submitted for evaluation of intracellular hydrogen peroxide production by flow cytometry using 2',7'-dichlorofluorescein diacetate as a probe. The selenium status of the animals was evaluated by determination of the blood glutathione peroxidase activity. The results of the present work showed that *in vitro* selenium supplementation leads to an enhancement in intracellular hydrogen peroxide production, which indicates an improvement in the bactericidal effects of blood and milk neutrophils even in cows with a selenium-adequate status. Thus, the present study showed that *in vitro* Se supplementation leads to an enhancement in intracellular hydrogen peroxide production, indicating an improvement in the bactericidal effects of blood and milk neutrophils in cows with Se-adequate status.

INDEX TERMS: Antioxidants, bovine, polymorphonuclear leukocytes, mastitis.

**RESUMO.**- [Efeito da suplementação *in vitro* de selênio sobre neutrófilos do leite e sanguíneos em vacas leiteiras.] O presente estudo avaliou o efeito da suplementação *in vitro* de selênio sobre a produção intracelular de peróxido de hidrogênio (H<sub>2</sub>O<sub>2</sub>) por leucócitos polimorfonucleares do leite e do sangue em bovinos. Assim, 10 e 20 amostras de sangue e leite, respectivamente, foram incubadas com 0 mg (controle) ou 10 $\mu$ M de selenito de sódio.

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A determinação da produção intracelular de peróxido de hidrogênio se deu por citometria de fluxo através da utilização do 2',7' diclorodihidrofluoresceína diacetato como sonda. A mensuração do conteúdo de selênio foi avaliada pela atividade da glutathione peroxidase eritrocitária. Os leucócitos polimorfonucleares tanto sanguíneos quanto do leite apresentaram significativo aumento na produção intracelular de H<sub>2</sub>O<sub>2</sub> com a suplementação *in vitro* de selênio. Desta forma, o presente estudo apontou para aumento da produção intracelular de H<sub>2</sub>O<sub>2</sub>, indicando aumento da capacidade microbicida dos leucócitos polimorfonucleares sanguíneos e lácteos mesmo em animais com níveis adequados de selênio.

TERMOS DE INDEXAÇÃO: Antioxidantes, bovinos, leucócitos polimorfonucleares, mastite.

### INTRODUCTION

In spite of improvements in management practices, mastitis remains a worldwide problem for the dairy industry and producers. Shortly after entry of the invading patho-

gen, the resident leukocytes together with epithelial cells initiate the inflammatory response necessary to eliminate the invading bacteria (Paape et al. 2002, 2003, Rainard & Riollot 2003). These cells release chemoattractants for the rapid recruitment of polymorphonuclear neutrophil leukocytes (PMNL) to the site of infection. Neutrophils are essential for innate host defense against invading microorganisms and eliminate pathogens by a process known as phagocytosis. During phagocytosis, PMNL produce reactive oxygen species, including superoxide, hydrogen peroxide and hypochlorous acid, and release granule compounds into pathogen-containing vacuoles to kill the invading pathogen (Paape et al. 2002, 2003, Rainard & Riollot 2003, Kobayashi et al. 2003, Mehrzad et al. 2005).

In the last few years, in an attempt to reduce the impact of mastitis and decrease the use of antimicrobials on dairy farms, there have been numerous efforts to try to exploit the immune capacity of the bovine mammary gland to stimulate the animal's natural defense mechanisms (Salman et al. 2009). In addition, many studies point to a reduction in the incidence of mastitis with selenium (Se) supplementation, an idea reinforced by the negative correlation between somatic cell count (SCC) and the Se status of the animals (Hogan et al. 1993, Paschoal et al. 2003, Kruze et al. 2007, Sánchez et al. 2007, Salman et al. 2009, Cortinas et al. 2010). Most of the studies that investigated the effect of Se on immune function only compared animals with a low Se status to those with an adequate Se status. Only a few studies have tried to investigate the mechanisms by which Se acts. There are still many gaps in our understanding of the interactions of Se with the immune response of the bovine mammary gland (Salman et al. 2009). Additionally, some recent reports have demonstrated that levels of Se higher than those regarded as adequate can lead to an increase of the natural defense mechanisms of the bovine mammary gland (Salman et al. 2009).

The aim of the present study was to evaluate the effect of *in vitro* selenium supplementation on intracellular hydrogen peroxide production by blood and milk bovine neutrophils in dairy cows with adequate selenium status.

## MATERIALS AND METHODS

The cows used in the present study were clinically healthy and in the middle of lactation. They came from a commercial Holstein dairy herd that was free of brucellosis and tuberculosis and that was located in the microregion of Pirassununga in the São Paulo State, Brazil. Heparinized blood from 10 Holstein cows was collected to assess glutathione peroxidase (GSH-Px) activity and intracellular hydrogen peroxide production. Additionally, fore-milk samples (at least 25mL) were collected from five cows (20 samples) to determine hydrogen peroxide production. The milk was then diluted 1:1 with phosphate-buffered saline (PBS). The milk and blood samples were kept at 4°C for approximately 4 hours until they could be analyzed. The separation of milk cells was performed as described by Koess & Hamann (2008). Briefly, after centrifugation of the milk samples at 1000g for 15 min, the cream layer and supernatant were discarded. The cell pellet at the bottom of the container was then washed once with 50mL of PBS and further centrifuged at 400g for 10 min. Afterwards, the cells were taken up in 1000µL of nutrition media (RPMI 1640, Sigma

Aldrich, USA) with 10% fetal bovine serum (Cultilab, Brazil). The milk cells were diluted with the nutrition media with 10% of fetal bovine serum to a concentration of  $2 \times 10^6$  viable cells/mL. Cell count was determined by counting in a Neubauer chamber, cell viability was evaluated by trypan blue exclusion.

Intracellular hydrogen peroxide ( $H_2O_2$ ) production was assessed as described by Hasui et al. (1989). Briefly, 100µL of whole blood or  $2 \times 10^5$  viable milk cells from each quarter were incubated at 37°C for 30 minutes with 200µL of 0.3µM of 2',7'-dichlorofluorescein-diacetate (DCFH-DA) (Sigma Aldrich, USA) in the nutrition media without sodium selenite (control) or containing sodium selenite ( $Na_2O_3Se$ ) (Sigma Aldrich, USA) at a final concentration of 10µM of  $Na_2O_3Se$  (treatment).

After centrifugation, the erythrocytes from the blood samples were removed by hypotonic lysis. Finally, the samples were centrifuged and resuspended in 1000µL of PBS and analyzed by flow cytometry.

The intracellular 2',7'-dichlorofluorescein (DCH) fluorescence of PMNL was determined by flow cytometry (Becton Dickinson Immunocytometry System™, San Diego, EUA) using 488nm excitation. The DCFH-DA, which is a cell-permeable nonfluorescent probe, is converted by hydrogen peroxide to DCF in a dose-dependent manner and turns highly fluorescent. The green fluorescence from DCF was detected at 500-530nm (FL-1 channel). The PMNL population was identified based on their cell size and granularity characteristics (Mehrzad et al. 2001, Rivas et al. 2001, Kampen et al. 2004, Sladek & Rysanek 2010), as shown in Figure 1. The quantification of hydrogen peroxide production was estimated by the geometric mean DCF fluorescence/cell.

The percentage of PMNL that produced hydrogen peroxide was equal to the number of fluorescent PMNL divided by the total PMNL count multiplied by 100. At least 20,000 cells were examined in each sample. The data was read using the Flow Jo Tree Star software (TreeStar Inc., Ashland, OR, USA).

The erythrocyte GSH-Px activity was assessed by the method of Paglia & Valentine (1967) using a commercial kit (RANSEL® Laboratories, Randox, Crumlin, UK). The values of GSH-Px were regarded as deficient, marginal or adequate when the GSH-Px activity was lower than 60 U/g of hemoglobin (Hb), between 60 and 130 or higher than 130 U/g Hb, respectively (Ceballos & Wittwer 1996, Ceballos et al. 1999).

Gaussian distribution was confirmed with the Kolmogorov and Smirnov test. Differences between the control and 10µM of sodium selenite PMNL were calculated using a paired t-test. Data were analyzed using the GraphPad Prisma 5.0 software (GraphPad Software, Inc., San Diego, CA, USA). Results are reported as means  $\pm$ SD. A value of  $P < 0.05$  was considered significant.

## RESULTS

The mean value for erythrocyte GSH-Px activity of the 10 animals used to evaluate blood intracellular hydrogen peroxide production was 287.06 U/g Hb ( $\pm 27.65$ ). The mean value for erythrocyte GSH-Px activity of the five animals used to evaluate intracellular hydrogen peroxide in the milk samples was 226.70 U/g Hg ( $\pm 30.75$ ).

The *in vitro* intracellular hydrogen peroxide production by both blood and milk neutrophils was improved by sodium selenite addition. The geometric mean DCF fluorescence/cell, which measure intracellular hydrogen production, was 32.59 ( $\pm 13.22$ ) and 362.18 ( $\pm 127.82$ ) for the milk and blood control neutrophils and 43.16 ( $\pm 17.34$ ) ( $P = 0.0004$ ) and 881.39 ( $\pm 515.90$ ) ( $P = 0.0083$ ) with sodium selenite addition, respectively (Table 1). The

percentages of neutrophils that produced hydrogen peroxide were 99.60% ( $\pm 0.42$ ) and 99.65% ( $\pm 0.71$ ) ( $P=0.73$ ) for blood and 90.64% ( $\pm 10.61$ ) and 97.83% ( $\pm 4.15$ ) ( $P=0.0045$ ) for milk in samples without and with Se supplementation, correspondingly (Table 1). The increases in intracellular hydrogen peroxide production with sodium selenite addition were 143.36% and 32.42% for blood and milk neutrophils, respectively. Additionally, the mean intracellular hydrogen peroxide production by blood neutrophils was significantly higher ( $P=0.0001$ ) compared with that in milk neutrophils.

**Table 1. The geometric mean 2',7'-dichlorofluorescein (DCF) fluorescence/neutrophil and the percentage of neutrophils that produced hydrogen peroxide by blood and milk samples with and without sodium selenite (10  $\mu$ M) addition**

Sodium selenite	Blood		Milk	
	0 $\mu$ M	10 $\mu$ M	0 $\mu$ M	10 $\mu$ M
Percentage of neutrophils that produced hydrogen peroxide	99.60% ( $\pm 0.42$ ) <sup>a</sup>	99.65% ( $\pm 0.71$ ) <sup>a</sup>	90.64% ( $\pm 10.61$ ) <sup>b</sup>	97.83% ( $\pm 4.15$ ) <sup>a</sup>
Geometric mean DCF fluorescence/neutrophil	362.18 ( $\pm 127.82$ ) <sup>a</sup>	881.39 ( $\pm 515.90$ ) <sup>b</sup>	32.59 ( $\pm 13.22$ ) <sup>c</sup>	43.16 ( $\pm 17.34$ ) <sup>d</sup>

Data are presented as mean  $\pm$ SD (Blood = 10, Milk = 20).

<sup>a,b,c,d</sup> Means within the same row followed by different letters differ at  $P \leq 0.01$ .

## DISCUSSION

The present study is, to our knowledge, the first report demonstrating the ability of Se to increase the microbicidal capacity of both blood and milk PMNL in cows with a Se-adequate status. Other surveys have also pointed to an increased microbicidal activity in animals supplemented with Se (Grasso et al. 1990, Hogan et al. 1993, Wuryastuti et al. 1993, Salman et al. 2009). However, all of these studies compared only the improvement of microbicidal activity in cows with a low Se status to that of those with an adequate Se status. For instance, Erskine et al. (1989) showed lower milk production, higher SCC and a higher duration of infection in animals with a low dietary Se content (0.04mg/kg dry matter) compared to those who were fed with the same basal diet plus 2.0mg Se/day.

Silvestre et al. (2007) showed an improvement in blood neutrophils phagocytosis and killing activities in dairy cows supplemented with Se-yeast (an organic source of Se) compared with those given an inorganic source of Se. It should be noted that cattle supplemented with Se-yeast had an increase in serum and milk Se concentrations in comparison with those given sodium selenite (Weiss et al. 2005, Ceballos et al. 2009, Cortinas et al. 2010) and also lower SCC and new cases of subclinical mastitis (Cortinas et al. 2010). In face of, the improvement in the microbicidal activity of milk and blood PMNL even in dairy cows with a Se-adequate status, as encountered here, is probably one reason that explain the lower incidence of mastitis and also lower SCC described by others studies. However, Weiss et al. (2005) indicated that the source of Se treatment did not affect bacterial killing or the percentage of blood neutrophils that phagocytized bacteria.

Most recent surveys have confirmed that Se levels higher than those considered adequate can potentially maximize

the natural defense mechanisms, especially the humoral immune response (McKenzie et al. 1998, Rayman 2000, Alvarado et al. 2006, Salman et al. 2009). For instance, some of the most recent reports on trials with high supplemental doses of Se from both organic and inorganic sources have described potential benefits in terms of disease resistance and immunoglobulin (Ig) production (Pavalata et al. 2004, Guyot et al. 2007, Salman et al. 2009).

Congruently, Ndiweni & Finch (1995) demonstrated that the *in vitro* supplementation of mammary gland macrophages with sodium selenite significantly enhanced the production of factors that were chemotactic to PMNL such as leukotriene B4 (LTB4). The same authors (1996) showed that *in vitro* Se supplementation (10  $\mu$ M) in PMNL from the blood of Se-adequate cows had a greater stimulatory effect on their random migration. During mastitis, active macrophages have been shown to produce prostaglandins, which might in turn inhibit the functions of neutrophils. Supplementation with Se may therefore reduce prostaglandin production and relieve the antilactogenic and immunosuppressive effects of these compounds (Ndiweni & Finch 1995, Rayman 2000). With this in mind, some reports have proposed that dietary Se recommendations can be increased to 0.6mg kg<sup>-1</sup> dry matter (DM) to enhance the benefits of Se while remaining far below toxic levels (Salman et al. 2009).

The biological effects of Se are mainly due to its incorporation into selenoproteins, such as GSH-Px. Because of this, GSH-Px activity has been used as a biomarker to assess body Se status (Ceballos et al. 1999). Indeed, this seleno-dependent enzyme is a component of cellular antioxidant defense mechanisms that removes potentially damaging lipid hydro-peroxides and hydrogen peroxides and protects the immune cells from oxidative stress-induced damage (Salman et al. 2009). In fact, it has been demonstrated that the GSH-Px activity of granulocytes in goats was enhanced after only 15 minutes of *in vitro* Se incubation (Aziz et al. 1984). Since the granulocytes in our experiments were incubated for 30 minutes with Se under comparable conditions, their GSH-Px activity was likely also enhanced.

Thus, adequate levels of Se are pivotal to ensure optimal immune function and protection against oxidative stress leading to cell death (McKenzie et al. 1998, Rayman 2000). Asfour et al. (2006) demonstrated that the sodium selenite administration to humans with non-Hodgkin's lymphoma and treated by chemotherapy resulted in a significant reduction in neutrophils apoptosis compared to those who were not supplemented with sodium selenite. The viable neutrophils (nonapoptotic) markedly improved the overall post therapy neutropenic phase and decreased significantly possible life threatening infections. Additionally, Piepers et al. (2009) described a relationship between higher serum Se concentrations and lower blood polymorphonuclear leukocyte apoptosis rates in heifers. This is interesting because apoptosis in bovine PMNL implies an impaired phagocytic and oxidative burst activity (Van Oostveldt et al. 2002, Merhzad et al. 2004). Singh et al. (2008) showed an increase in different innate immune factors that occur after the increases in antioxidant factors during the involution of the bovine mammary gland.

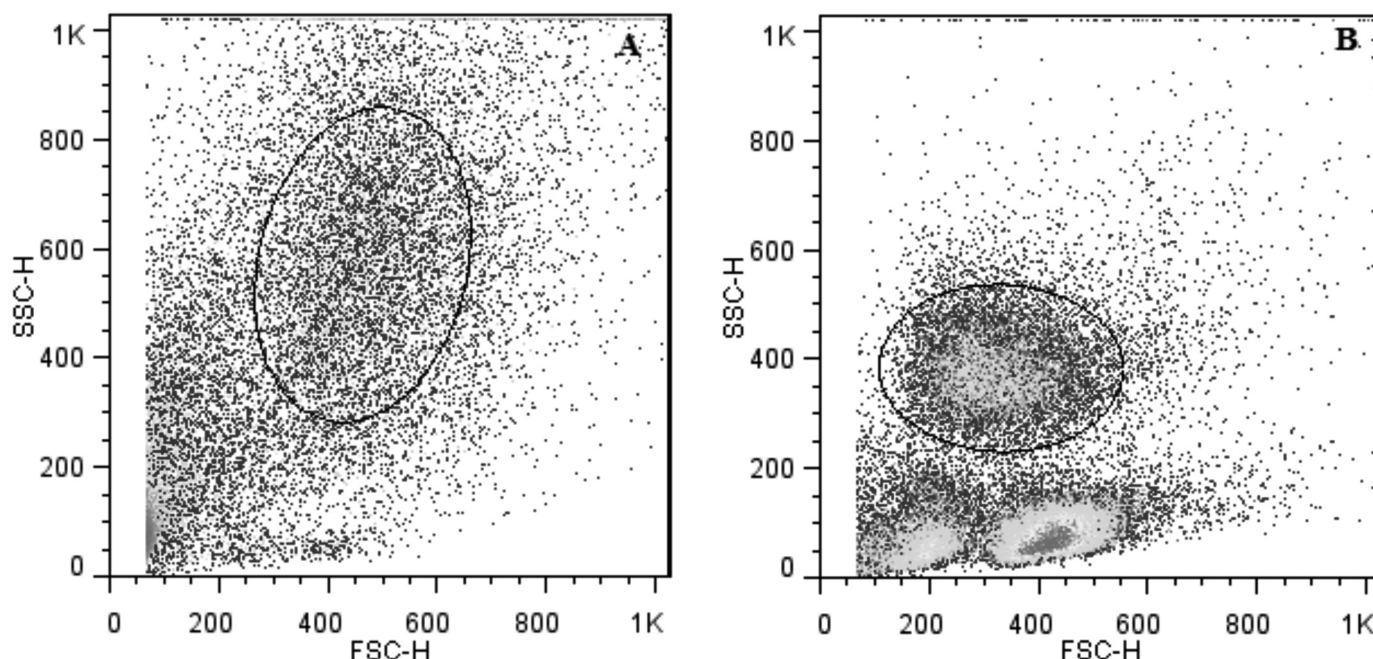


Fig.1. Dot plots showing the cell size (forward scatter [FSC-H]) and cell granularity (side scatter [SSC-H]) of cells isolated from blood and milk. The polymorphonuclear leukocytes are shown in the gate. (A) Dot plot representing cells isolated from a milk sample. (B) Dot plot representing cells isolated from a blood sample.

Besides this, the mean intracellular hydrogen peroxide production by blood neutrophils was significantly higher compared with that in milk neutrophils. It is widely accepted that this pronounced reduction in bactericidal activity by milk-resident PMNL may be due to the ingestion of milk fat globules and casein by PMNL, causing a loss of cytoplasmic granules, which are associated with a reduction in bactericidal activity (Mehrzaad et al. 2001, Paape et al. 2002, Mehrzaad et al. 2009).

## CONCLUSION

The results show that *in vitro* selenium supplementation leads to an enhancement in intracellular hydrogen peroxide production, indicating an improvement in the bactericidal effects of blood and milk neutrophils in dairy cows with selenium-adequate status. Given this knowledge, it follows that selenium supplementation may lead to an enhancement in the immune capacity of the mammary glands even in non-deficient dairy cows.

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