Selenium and vitamin E enriched diet increases NK cell cytotoxicity in cattle[1]

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ABSTRACT.- Latorre A.O., Greghi G.F., Netto A.S., Fukumasu H., Balieiro J.C., Côrrea L.B. & Zanetti M.A. 2014. **Selenium and vitamin E enriched diet increases NK cell cytotoxicity in cattle.** *Pesquisa Veterinária Brasileira 34(11):1141-1145.* Departamento de Zootecnia, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Av. Duque de Caxias Norte 255, Pirassununga, SP 13630-000**,** Brazil. E-mail: giselegreghi@usp.br

A number of studies has shown that antioxidants, fatty acids and trace minerals may modulate different immune cell activities, and that their deficiency may be associated with diseases and impaired immune responses. In innate immunity, natural killer (NK) cells have a central role, killing virally infected and cancerous cells, and also secreting cytokines that shape adaptive immune responses. Thus, the aim of this study was to evaluate the effect of enriched diets in selenium plus vitamin E and/or canola oil on complete blood count and on NK cell cytotoxicity from blood lymphocytes of Nellore bulls. Bulls that received selenium plus vitamin E had (*P*=0.0091) higher NK cell cytotoxicity than control bulls. This result positively correlated with serum selenium levels. To the best of our knowledge, this is the first study that showed immunostimulatory effects of selenium plus vitamin E on NK cell cytotoxicity of Nellore bulls.

INDEX TERMS: Selenium, vitamin E, NK cells, cytotoxicity, antioxidant, fatty acid, immunostimulation, Nellore bull, trace mineral.

RESUMO.- [Dieta enriquecida com selênio e vitamina E aumenta a citotoxicidade de células NK em bovinos.] Vários estudos demonstraram que antioxidantes, ácidos graxos e minerais podem modular a atividade de diferentes células do sistema imunológico e que as suas carências podem estar associadas a doenças e a respostas imunes comprometidas. Na imunidade inata, os linfócitos natural killer (NK) têm um papel central matando células infectadas por vírus e células cancerígenas, ao mesmo tempo em que também secretam citocinas que modulam as respostas imunes adaptativas. Assim, o objetivo deste estudo foi avaliar o efeito de dietas enriquecidas em selênio e vitamina E e/ou óleo de canola no hemograma e na citotoxicidade das células NK do sangue de bovinos da raça Nelore. Os animais que receberam selênio e vitamina E tiveram (P = 0,0091) maior citotoxicidade das células NK do que os animais do grupo controle. Este resultado foi positivamente correlacionado com os níveis de selênio no sangue. Para o melhor do nosso conhecimento, este é o primeiro estudo que mostrou efeitos imunoestimulatórios do selênio e vitamina E sobre a citotoxicidade das células NK de bovinos Nelore.

TERMOS DE INDEXAÇÃO: Selênio, vitamina E, citotoxicidade, células NK, antioxidantes, ácidos graxos, imunoestimulação, bovinos Nelore, micromineral.

INTRODUCTION

Nowadays it is unquestionable how nutrition can impact immune responses. A number of studies have shown that antioxidants, fatty acids and trace minerals can modulate different immune cells activities and their deficiency can be associated with diseases and impaired immune responses (Calder & Kew 2002). As example, low dietary uptake of se-

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lenium and vitamin E is associated with a well-recognized disease causing sudden death in cattle, the Nutritional Degenerative Myopathy (Cawley & Bradley 1978). In addition, the deficiency of selenium and vitamin E has been linked to reduced ability of blood and milk neutrophils to kill yeast and bacteria in cattle (Spears 2000). Vitamin E is an important antioxidant that has been shown to improve oxidative burst and chemotactic responsiveness of neutrophils in periparturient cows, but without effects on macrophages and lymphocytes (Spears & Weiss 2008). Conversely, selenium that is an essential trace element and an integral component of a variety of selenoproteins has been shown to influence cell-mediated immunity, lymphocyte proliferation and neutrophils activities in cows (Spears & Weiss 2008).

Supplementation with fatty acids has also been highlighted as immunomodulatory, but much controversy still remains since the proportion and the type of polyunsaturated fatty acids of dietary influence their effects on immune cells (Yaqoob 1998). In fact, the fatty acid composition of immune cells is altered according to the fatty acid composition of the diet and this modifies the capacity of those cells to produce eicosanoids, such as prostaglandin E2, which are involved in immunoregulation (Calder et al. 2002). For instance, pigs fed a fish oil diet (rich in eicosapentaenoic and docosahexaenoic acids) presented a significantly reduced natural killer cell activity compared with those pigs fed a canola oil diet (rich in oleic acid) (Thies et al. 1999).

Natural killer (NK) cells are lymphocytes that have a central role in innate immunity, killing virally infected and cancerous cells, and also secreting cytokines that shape adaptive immune responses (Narni-Mancinelli et al. 2013). Taking into account the immunomodulatory effects of antioxidants, fatty acids and trace minerals, it is possible that bulls fed highly enriched diets become less susceptible to infections. Thus, the aim of the current study was to evaluate the effects of diets enriched in selenium and vitamin E and/or canola oil on complete blood count and on NK cell cytotoxicity from blood lymphocytes of Nellore bulls. To the best of our knowledge, this is the first study that showed immunostimulatory effects of selenium plus vitamin E, above the minimum requirements, on NK cell cytotoxicity of Nellore bulls.

MATERIALS AND METHODS

Animal ethics statement and study design. The animals used in this experiment were cared for by acceptable practices as summarized in the Guide for the Care and Use of Agricultural Animals Research and Teaching (FASS 2010), and the protocol was reviewed and approved by the University of São Paulo, College of Animal Science and Food Engineering (USP/FZEA), Ethics Committee for the Use of Animals in Experiments. The Nellore bulls with approximately 2 years originated from Sítio Cachoeirinha, Bairro Barrocão, Pirassununga, SP, Brazil. Body weights at arrival ranged from 272 to 408 kg (326.04±29.87 kg, mean ±SD). There were 48 Nellore bulls randomly distributed into four groups that received the following diets for 12 weeks: control (Co) - basal diet; selenium + vitamin E (SE) - 2.5mg Se and 500IU vitamin E/kg dry matter (DM); selenium + vitamin E + canola (SEC) - 2.5mg Se, 500IU vitamin E and 3% canola oil/kg DM; Canola (C) - 3% canola oil/kg DM. The total diet composition is presented at Table 1. As source of selenium was used Sel-Plex® (Alltech) that is an organic form of selenium yeast manufactured. Individual intakes were measured using Calan Gate© feeders (American Calan, Northwood, NH) throughout the supplementation period. The body weights of the bulls were also measured once a month during the supplementation period

Serum selenium and vitamin E level. To assess the effect of Sel-Plex® supplementation on serum selenium status, all bulls were bled at 0 time (baseline) and once each month for 12 week until study termination. Blood samples were collected by puncture of *v. jugularis* into heparin-containing test tubes (BD Vacutainer®) at weeks 0, 4, 8 and 12 of diet supplementation. The serum selenium concentration was determined by the method proposed by Olson et al. (1975) at the Laboratory of Minerals (FZEA/USP).The serum vitamin E concentration was determined at week 12 by HPLC using the kit Vitamins A and E in serum/plasma – HPLC (ChromSystems, Gräfelfing - Germany) according to the manufacturer's protocol.

Complete blood count and leukocyte differential count. Blood samples were collected from each animal by puncture of *v. jugularis* into heparin-containing test tubes (BD Vacutainer®) at week 10 of diet supplementation. The results of the blood cells count were obtained using a hematology automated analyzer (ABX ABC VET, Horiba ABX Diagnostic®, Montpellier, France). The leukocyte differential count was made by manual microscopic scan of Giemsa-stained blood smear.

Evaluation of NK cell cytotoxicity. To evaluate the blood NK cytotoxicity (effector cells), it was used blood samples collected at week 10 of diet supplementation. First we separated blood mononuclear cells from each animal using Ficoll-Paque density gradient media (GE Healthcare) following the manufacturer's instructions. As target cells, we used the BL3-1 cell line (ATCC® CRL-2306™) a *Bos taurus* B-lymphosarcoma that exhibit no expression of MHC class I and consequently is a target for NK assays. Next, we labeled BL3-1 cells with 1 µl CFSE (5mM) (Molecular Probes™) in PBS for 20 minutes at 37°C. Effector and target

Table 1. Ingredients (DM basis) and chemical composition (% of DM) of the basal diet

Ingredients	% of DM
Dry corn grain	48.12
Soybean meal 46%	13.60
Pelleted citrus pulp	18.20
Corn silage	30.00
Urea	0.80
Mineral mix	1.00
Ammonium sulfate	0.05
Potassium chrolide	0.20
Rumensin	0.03
Nutrients	
Crude protein %	14.60
Rumen-degradable protein %	9.90
TDN % *	74.50

* Stated by the formula of Weiss et al. (1992). DM = dry matter.

cells were adjusted in 10% SFB RPMI medium (Gibco) and co-incubated at 100:1 ratio in triplicate and maintained at humidified stove at 37°C and 5% CO_2. After 3 h incubation, 40 µl of propidium iodide (PI) [12.5µg/ml] (Sigma) was added to identify the dead target cells (BL3-1 $CFSE^+PI^+$) by flow cytometry. The spontaneous death rate was determined by incubating BL3-1 cells alone in complete RPMI medium. As positive control, it was used Phorbol 12-myristate 13-acetate (PMA) [2.5 µg/ml] (Sigma) plus ionomycin [0.5 µg/ml] (Sigma). Overall, 5,000 target cells were collected by flow cytometry (BD FACSAria). The data were analyzed using FlowJo 7.6.4® software. The level of NK cell cytotoxicity was expressed as percentage of dead target cells (BL3-1 $CFSE^+PI^+$).

Statistical analyses. The data were analyzed in GraphPad Prism 5.00® software (GraphPad Soft-ware, Inc., San Diego, CA). Serum selenium concentrations were analyzed using Two-way ANOVA as repeated measures (mixed model) followed by Bonferroni post-test. The other data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons or Student's t-tests to compare two groups. Non-parametric data were compared using the Kruskal-Wallis test followed by Dunn's test, and percent data from two groups were compared using the Mann-Whitney test. Data are expressed as the mean ± SD, or as the median with range, and differences were considered statistically significant at $P<0.05$.

RESULTS

Effect of selenium + vitamin E and/or canola oil supplementation to Nellore bulls on weight gain and on serum selenium and vitamin E levels

Body weights were measured at the beginning of the treatment period (baseline), and at 4, 8 and 12 week (end of the supplementation period) and were similar among groups (data not shown). Diet supplemented with Sel-Plex® was effective at increasing serum selenium levels in bulls and the time required to reach plateau was 8 weeks for bulls supplemented with selenium + vitamin E (SE group) whereas those supplemented also with canola oil (SEC group) did not reach a plateau until the end of the supplementation period (*P* treatment, *P* time and *P* treatment x P time: all $P<0.0001$, two-way ANOVA; $P<0.001$ for the Co vs. SE groups and for the Co vs. SEC at 4, 8 and 12 week, Bonferroni's post-hoc test; Fig.1). The vitamin E level instead was slightly higher in bulls from SEC group than SE group when compared with Co group at the end of supplementation period ($P<0.0001$, Kruskal-Wallis test; $P<0.05$ for the Co vs. SE groups and $P<0.01$ for the Co vs. SEC groups, Dunn's post-hoc test; Fig.2).

Effect of selenium + vitamin E and/or canola oil supplementation to Nellore bulls on complete blood and leukocyte differential counts at the week 10 of feeding period

Bulls that were supplemented with canola oil (C group) displayed lower mean corpuscular hemoglobin (MCH) ($P=0.0463$, one-way ANOVA; $P<0.05$ for the Co vs. C groups, Tukey's post-hoc test) and mean corpuscular hemoglobin

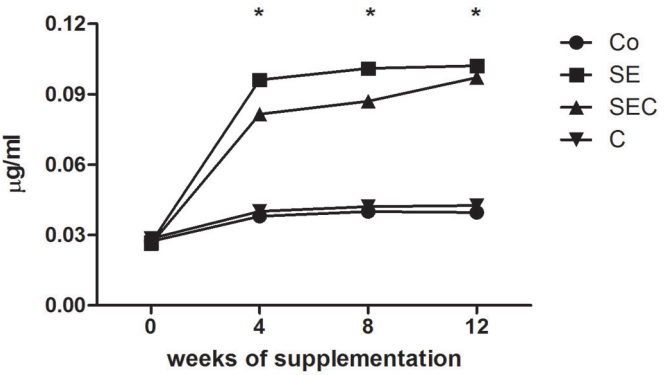

Fig.1. Comparison of serum selenium levels (median) in Nellore bulls consuming a basal diet (Co), or selenium (2.5mg) + vitamin E (500IU) (SE), or selenium (2.5mg) + vitamin E (500IU) + 3% canola oil (SEC), or 3% canola oil (C) (n=12 bulls per group). *$P<0.001$ Bonferroni's post-hoc test.

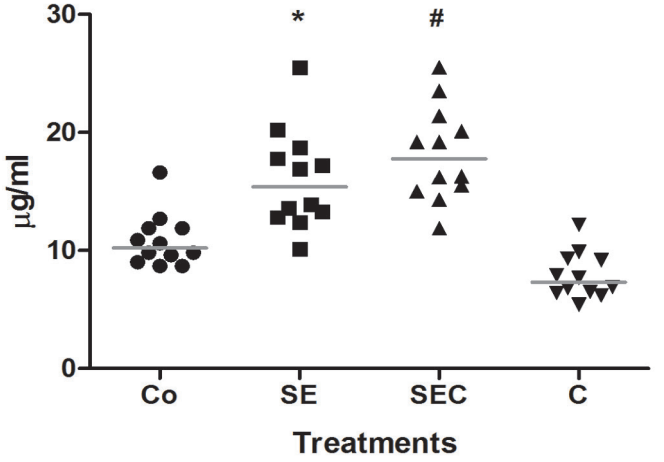

Fig.2. Comparison of serum vitamin E levels (median) in Nellore bulls at the end of 12 weeks consuming basal diet (Co), or selenium (2.5mg) + vitamin E (500IU) (SE), or selenium (2.5mg) + vitamin E (500IU) + 3% canola oil (SEC), or 3% canola oil (C) (n=12 bulls per group). *P<0.05, #P<0.01 Dunn's post-hoc test.

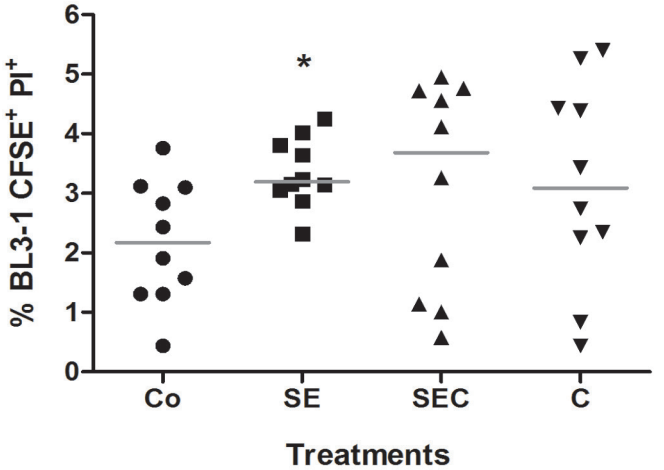

Fig.3. Blood NK cytotoxicity (median) in Nellore bulls at the end of 10 weeks consuming basal diet (Co), or selenium (2.5mg) + vitamin E (500IU) (SE), or selenium (2.5mg) + vitamin E (500IU) + 3% canola oil (SEC), or 3% canola oil (C) (n=10 bulls per group). *P(two-tailed)=0.0091, Mann Whitney test for the Co vs. SE groups.

Table 2. Complete blood and leukocyte differential counts of the Nellore bulls that received a basal diet (Co) or an enriched diet containing 2.5 mg selenium + 500IU vitamin E (SE), 2.5 mg selenium + 500IU vitamin E + 3% canola oil (SEC) or 3% canola oil (C)/kg dry matter for 10 weeks

		Co	SE	SEC	C
RBC	(106/mm3)	9.37±1.24	9.33±1.20	9.47±1.07	9.85±1.10
Hematocrit	(%)	39.4(31.8-8.2)	39.5(31.2-6.1)	40.3(31.5-7.1)	39.8(35.5-8.3)
Hemoglobin	(g/dl)	13.11±1.37	12.68±1.27	12.88±1.45	12.88±1.15
MCV	(fm3)	43.42±2.19	41.92±2.91	42.67±2.06	41.42±2.23
MCH	(pg)	14.05±0.79	13.67±0.93	13.63±0.60	13.12±0.76•
MCHC	(g/dl)	32.42±0.71	32.63±0.75	31.98±0.52	31.60±0.67•
Platelet	(103/mm3)	345.6±84.7	409.2±122.4	339.6±118.6	391.7±97.3
WBC	(103/mm3)	9.74±1.86	10.51±2.28	11.19±2.64	11.09±1.86
Neutrophils	(103/mm3)	3.23±1.00	3.17±1.12	3.58±1.71	3.94±1.09
	(%)	30.0(24.0-9.0)	33.0(16.0-0.0)	31.5(19.0-5.0)	36.0(26.0-8.0)
Monocytes	(103/mm3)	0.32±0.21	0.13±0.17	0.38±0.18	0.46±0.20
	(%)	3.0(0-6.0)	0.5(0-4.0)	3.5(0-5.0)	4.0(1.0-6.0)
Lymphocytes	(103/mm3)	5.87±1.31	6.86±1.49	6.88±1.23	6.18±1.08
	(%)	61.0(47.0-2.0)	64.5(53.0-9.0)	61.5(49.0-6.0)	57.5(43.0-5.0)
Eosinophils	(103/mm3)	0.32±0.36	0.41±0.28	0.29±0.27	0.44±0.33
	(%)	1.5(0-9.0)	4.5(1.0-6.0)	3.0(0-9.0)	3.5(1.0-9.0)
Basophils	(103/mm3)	0.0±0.0	0.0±0.0	0.04±0.08	0.06±0.09
	(%)	0(0-0)	0(0-0)	0(0-2.0)	0(0-2.0)

Data are presented as mean ±SD or median and range values (N = 12 bulls/group). RBC = red blood cells; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; WBC = white blood cells. •p<0.05 vs. Co; Tukey's post-test.

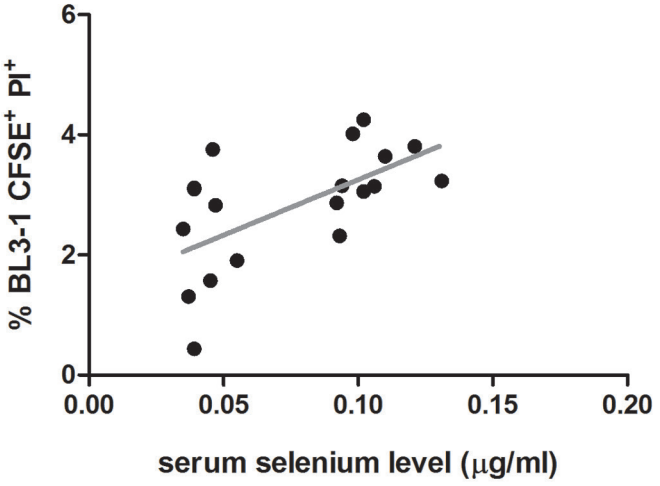

Fig.4. Correlation between serum selenium levels and observed blood NK cytotoxicity (%BL3-1 CFSE+ PI+) in Nellore bulls consuming basal diet (Co) or selenium (2.5 mg) + vitamin E (500IU) (SE) for 10 weeks (n=10 bulls per group). Spearman r=0.6928, P(two-tailed)=0.007.

concentration (MCHC) (P=0.0021, one-way ANOVA; P<0.05 for the Co vs. C groups, Tukey's post-hoc test) when compared to the bulls from the Co group (Table 2). No differences in the differential leukocyte counts were observed (Table 2).

Effect of selenium + vitamin E and/or canola oil supplementation to Nellore bulls on NK cell cytotoxicity from blood lymphocytes at the week 10 of feeding period

Blood NK cell cytotoxicity from bulls consuming an enrichment diet in selenium plus vitamin E was increased when compared with bulls consuming a basal diet (P(two-tailed)=0.0091, Mann Whitney test for the Co vs. SE groups, Fig.3). Moreover, the intensity of NK cytotoxicity, measured as percentage of dead target cells (% BL3-1 $CFSE^+$ PI^+), was positively correlated with selenium levels when considered Co and SE groups (Spearman r=0.6928, P(two-tailed)=0.007, Figure 4).

DISCUSSION AND CONCLUSIONS

Here in we sought to evaluate the effect of enriched diets in selenium plus vitamin E and/or canola oil on innate immunity of Nellore bulls, mainly on NK cells. To the best of our knowledge, this is the first study that showed immunostimulatory effects of selenium plus vitamin E, above the minimum requirements, on NK cell cytotoxicity of Nellore bulls.

Selenium is an essential trace element and an integral component of a variety of selenoproteins being considered critical in maintaining optimal immune function (Arthur et al. 2003). Despite that at certain levels selenium can be toxic and to avoid it the maximum tolerable concentrations for beef cattle was stated as 2 mg/kg DM by the National Research Council (2005). In the present study however, bulls were supplemented with Sel-Plex® at 2.5mg/kg DM and did not present any sign of selenium toxicity. Conversely, this concentration of selenium supplementation showed to be effective to increase serum selenium levels and to improve NK cell cytotoxicity since the concentration of this mineral was positively correlated with NK cell activity.

Selenium supplementation *in vivo* was already demonstrated to increase NK cell activities as cytotoxicity and IFNγ production in human and mice; however, in cows it was only demonstrated to increase neutrophil migration and respiratory burst and lymphocyte proliferation as reviewed by McKenzie (1998). In mice, the increase of NK cytotoxicity

was related to the ability of the nutrient to enhance the expression of intermediated affinity interleukin-2 receptors/cell (Kiremidjian-Schumacher et al. 1996). It is plausible to assume that the same mechanism followed in the blood NK cells from bulls fed an enriched diet in selenium.

Vitamin E is an important antioxidant that is essential for prevention of various diseases and protection of the integrity of tissues. Furthermore, it was implicated in enhancement of the defense system against mastitis generally associated with selenium status as well (McDowell et al. 1996). In human, high-dose of vitamin E was showed to enhance NK cell function in cancer patients, which was related to the induction of NKG2D receptor expression (Hanson et al. 2007). Taking into account this effect on expression of NK cytotoxicity receptor, we could hypothesized that both selenium and vitamin E supplementation added to increased blood NK cell cytotoxicity of Nellore bulls observed in the present study.

Nevertheless, when we added canola oil in the diet the effect of selenium plus vitamin E on NK cell cytotoxicity was not maintained in all animals. It is known that the fatty acid composition of immune cells is altered according to the fatty acid composition of the diet and this modifies immunoregulation (Calder et al. 2002). Canola oil is rich in oleic acid and human NK cells supplemented with this fatty acid showed any alteration in cytotoxicity despite significant alterations in phospholipid fatty acids content of the membranes (Rice et al. 1981). Furthermore, canola oil was showed to alter hematological indices in piglets at 10 day of age as lower platelet counts and lower mean corpuscular hemoglobin concentration (MCHC) (Innis & Dyer 1999). In the same manner, Nellore bulls supplemented with 3% canola oil presented slightly lower MCHC and MCH. Considering these effects of canola oil, we can conclude that it has no beneficial effect on NK cell cytotoxicity of Nellore bulls.

In summary, we showed that high enriched diet in selenium and vitamin E improves blood NK cell cytotoxicity in Nellore bulls. As NK cells have a central role in innate immunity, killing virally infected and cancerous cells, and also secreting cytokines that shape adaptive immune responses, we can suppose that Nellore bulls fed highly enriched diets in selenium plus vitamin E become less susceptible to infections.

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