ORIGINAL ARTICLE

The influence of bovine neutrophils on *in vitro* phagocytosis and killing of *Staphylococcus aureus* in heifers supplemented with selenium and vitamin E

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Abstract

Suwanpanya, N., Wongpratoom, W., Wanapat, M., Aiumlamai, S., Wittayakun S. and Wachirapakorn,C.

The influence of bovine neutrophils on *in vitro* phagocytosis and killing of *Staphylococcus aureus* in heifers supplemented with selenium and vitamin E

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An experiment was performed to determine the influence of bovine nutrophils on *in vitro* phagocytosis and killing of *Staphylococcus aureus* after selenium (Se, selenium yeast) and vitamin E (vit E) was supplemented in heifers. Twelve healthycrossbred 75% Holstein-Friesian x Sahiwal heifers were divided into 4 groups in a 2x2 factorial arrangement in CRD. Heifers were supplemented organic selenium (selenium yeast) and vitamin E powder. The treatments were as follows; treatment 1) 3 mg Se 2,000 IU vit E /hd/d (3Se2E), treatment 2) 3 mg Se 4,000 IUvit E/hd/d (3Se 4E), treatment 3) 6 mg Se 2,000 IU vit E/hd/d (6Se2E), and treatment 4) 6 mg Se 4,000 IU vit E/hd/d (6Se 4E). The experiment comprised 3 periods: pre-

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supplementation (8 days), supplementation (8 days) and post-supplementation (8 days) periods. All heifers were offered concentrate (15% CP) at 4 kg/hd/d and rice straw hay *ad libitum*. Blood neutrophils were isolated from each heifer. Phagocytosis was determined by direct ingested count and killing of *S. aureus* by NBT reducting test. Phagocytosis and killing of *S. aureus* had a greater non-specific immune response during the supplementation period than in the pre-supplementation period in all treatments (P<0.05). Supplementation of 3Se4E resulted in a greater number of white blood cells (54,900 cells/cu.mm) and neutrophils (11,398 cells/cu.mm.) and improved phagocytosis (85%) and killing of *S. aureus* (47%) as compared to the pre-supplementation period.

Key words : neutrophils, *in vitro*, phagocytosis, killing, selenium, vitamin E, heifers

บทคัดย่อ

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อิทธิพลนิวโทรฟิลของโค โดยวิธี *in vitro* การกินและฆ่าเชื้อ *Staphylococcus aureus* ในโคสาวที่เสริมซีลีเนียมและวิตามินอี

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การศึกษาอิทธิพลนิวโทรฟิลของโคในการทำหน้าที่นอกร่างกายจากการเก็บกินและฆ่าเชื้อ *Staphylococcus aureus* หลังการเสริมซีลีเนียมยีสต์ และวิตามินอี ในโคสาวลูกผสมเลือด 75% โฮลสไตน์ฟรีเชียน x 25% ซาฮิวาล จำนวน 12 ตัว จัดการทดลองแบบ 2x2 factorial experiment in CRD โดยกลุ่มทดลองที่ 1 ได้รับซีลีเนียม 3 มก. กับวิตามินอี 2,000 หน่วยสากล (ไอยู)/ตัว/วัน กลุ่มทดลองที่ 2 ได้รับซีลีเนียม 3 มก. กับวิตามินอี 4,000 หน่วยสากล (ไอยู)/ตัว/วัน กลุ่มทดลองที่ 3 ได้รับซีลีเนียม 6 มก. กับวิตามินอี 2,000 หน่วยสากล (ไอยู)/ตัว/วัน กลุ่มทดลองที่ 4 ได้รับซีลีเนียม 6 มก. กับวิตามินอี 4,000 หน่วยสากล (ไอยู)/ตัว/วัน การทดลองแบ่งออกเป็น 3 ช่วง โดยช่วงแรกก่อนเสริมเป็นเวลา 8 วัน ช่วงเสริม 8 วัน และช่วงหลังเสริม 8 วัน โดยโคได้รับระดับโปรตีน 15 % วันละ 4 กก./ตัว/วัน และฟางข้าวไม่จำกัดโดยเก็บเลือดโคทุกตัวแล้วแยกนิวโทรฟิล ศึกษาความสามารถของนิวโทรฟิลโดยการเก็บกินเชื้อและการฆ่าเชื้อ *S. aureus* โดยวิธีตรวจสอบด้วยสาร NBT พบว่าการเสริมซีลีเนียมและไวตามินอีทำให้เพิ่มความสามารถในการตอบสนองต่อระบบภูมิต้านทานต่อสิ่งแปลกปลอมของร่างกายที่มีอยู่แล้วโดยธรรมชาติ พบว่าการเสริมซีลีเนียม 3 มก. กับวิตามินอี 4,000 หน่วยสากล (ไอยู)/ตัว/วัน เพิ่มจำนวนเม็ดเลือดขาว (54,900 เซลล์/ลบ.มม.) นิวโทรฟิล (11,398 เซลล์/ลบ.มม.) เพิ่มการกิน(85%) และการฆ่าเชื้อ *S. aureus* (47%) เมื่อเปรียบเทียบกับก่อนเสริม

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Mastitis is the most common disease in dairy cows. The incidence and prevalence of subclinical mastitis in dairy farm is quite high and subclinical mastitis is still an important problem in Thailand (Aiumlamai *et al.*, 2000: Boonyayatra and Chaisri., 2004). Scientific experiments have established that Se and vitamin E can influence the function of certain immune cells, improve reproductive and mammary gland health in adult dairy cows (Smith *et al.*, 1999). Neutrophils are the cells most involved with non-specific immunity (Barrio *et al.*, 2000). Phagocytosis and intracellular killing by bovine polymorphonuclear neutrophil (PMN) are important host defense mechanisms against mastitis

caused by *Staphylococcus aureus* (Smith *et al.*, 1997). Selenium and vitamin E appear to enhance host defenses against infection by improving phagocytic cell function (Hogan *et al.*, 1990). Selenium is located in the cytosol of the cells as an important component of the enzyme glutathione peroxidase (GSH-Px). GSH-Px converts hydrogen peroxide to water and lipid hydroperoxides to the corresponding alcohol. Vitamin E inhibits auto-oxidation of polyunsaturated fatty acids in neutrophil membranes. Vitamin E is localized in cellular membranes in close proximity to the mixed function oxidase enzymes that initiate the production of free radicals (Baker and Cohen, 1983). Both nutrients are involved in the cellular antioxidant system that protect phagocytic cells and surrounding tissues from oxidative attack by free radicals produced by the respiratory burst of neutrophils and macrophages during phagocytosis (Smith *et al.*, 1999). National Research Council (2001) established requirement for dietary selenium is 0.3 mg/kg DM and vitamin E is 545 IU/d for dairy heifers whereas Weiss *et al.* (1999) who reported during the last 2 weeks prepartum cows were fed a diet that provided 4,000 IU/day of supplemental vitamin E. National Research Council (1987) reported that ruminants can tolerate an intake of about 40,000 IU/day of supplemental vitamin E for several months without adverse effects. No positive results were found when high levels of vitamin E were fed to peripartum cows. However, data are limited on the effects of selenium and vitamin E on function of bovine neutrophils of dairy cattle in the tropical area. Therefore, the objective of the study was to determine the influence of bovine nutrophils on *in vitro* phagocytosis and killing of *Staphylococcus aureus* after selenium and vitamin E was supplemented in heifers.

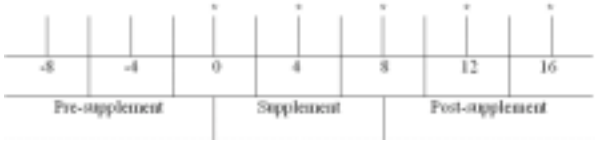
Materials and Methods

The heifers were subjected to a 2x2 factorial arrangement in completely randomized design (CRD). Twelve healthy crossbred 75% Holstein-Friesian x Sahiwal heifers (210±10 kg of BW) were randomly allotted to receive one of 4 treatments. The treatment combinations were treatment 1) 3 mg Se and 2,000 IU vit E /d (3Se2E); treatment 2) 3 mg Se and 4,000 IU vit E /d (3Se4E); treatment 3) 6 mg Se and 2,000 IU vit E /d (6Se2E); and treatment 4) 6 mg Se and 4,000 IU vit E /d (6Se4E). Heifers were offered 4 kg/d of concentrate (Table 1) and rice straw hay *ad libitum* throughout the experimental period. Heifers were individually housed with freely access to clean water at all times. The experiment was devided into 3 periods; pre-supplementation (8 days), supplementation (8 days) and post-supplementation (8

Table 1. Ingredients and chemical composition of feeds fed to heifers

Ingredients	Concentrate %	
Cassava chip	46.6	
Rice bran	14.8	
Corn meal	15.3	
Palm oil meal	17.4	
Dicalcium phosphate	1.1	
Sea salt	0.4	
Urea	2.7	
Lime stone	1.2	
Trace mineral premix[1]	0.5	
Chemical composition	**Concentrate**	**Rice straw hay**
DM %	88.3	93.6
Ash %	10.9	14.2
CP % 15.2	3.0	
NDF % 24.5	78.5	
ADF % 11.0	52.8	

[1] **Per kg; mineral premix provided copper 2,000 mg, magnesium 12,000 mg, zinc 12,000 mg, iron 7,750 mg, iodine 100 mg, cobalt 100 mg, phosphorus 2,500 mg and sodium chloride 28,000 mg.**
DM = dry matter, CP = crude protein, NDF = neutral detergent fiber, ADF = acid detergent fiber



* Blood collection

Figure 1. Layout of experiment and times of blood collections

days) (Figure 1). During supplementation period, heifers were supplemented with organic selenium (selenium yeast) which contains selenium in the form of seleno-methionine powder and vitamin E powder (dl-alpha-tocopheryl acetate) (standard USP conversion factors; 1 mg of dl-alpha-tocopheryl acetate = 1.49 IU of vitamin E) were used to calculate total vitamin E content of the diets (2,000 IU = 1.34 g, 4,000 IU = 2.68 g) sprinked on top of the concentrate. Blood samples were collected from jugular vein into heparinzed vacutainer tubes (10 ml) on d 0 (assigned as pre-supplementation) d 4 and d 8 (supplementation) and d 12 and d 16 (post-supplementation). Blood samples were processed within 2 hours after collection. White blood cell (WBC) counts were determined using a hemocytometer. Polymorphonuclear neutrophils (PMN) were isolated and studied for ability of neutrophils on *in vitro* phagocytosis and killing of S. aureus using modified methods of Rainard (1986); Gilmore (1986); Hogan *et al.* (1990); Burvenich (2000).

Phagocytosis and killing of *S. aureus* assay

Isolation of polymorphonuclear neutrophils (PMN)

At the time of blood collection, all cows were healthy with no manifestation of clinical signs. Three ml of blood were mixed with 4 ml of 4% dextran T500 left in a rack for 45 min. The leukocyte rich supernatant was collected and then centrifuged at 1000 x g for 45 min. Supernatant was removed. The pellet PMN was washed with Hank's balanced salt solution (HBSS, pH 7.2) and centrifuged at 10 x g for 15 minutes and then washed the PMN pellet for the second time with HBSS. The PMN pellet was counted in hemocytometer and adjusted to a final concentration of 5 x 106 cells/ml with HBSS and resuspended PMN pellet in HBSS and placed on ice.

Viability of polymorphonuclear neutrophils

The viability of isolated PMN was checked at the beginning of the experiments on phagocytosis and killing by dye exclusion of trypan blue. Procedures were as followed: a stock solution of 1 percent trypan blue in distilled water was diluted 1 : 5 with saline. To a sample of 10 µl of PMN suspension, an equal volume of the 0.05 percent trypan blue solution was added, and the cells were examined within 2 min. in a microscope country chamber. One hundred cells were counted under 40 objective lens 10 x eye pieces lens. Cells excluding dye were viable while dead cells were light to dark blue. The proportion of cells that had taken up the dye was determined by exploring at least 100 cells [(number of live cells (number of live + dead cells x100)]. The isolated cells were PMN with viabilities of 96-98%.

Preparation of bacteria

S. aureus was used for testing ability of neutrophil on phagocytosis and percentage of killing of *S. aureus*. Isolates were obtained from the Pathobiology Laboratory, Department of Pathobiology, Faculty of Veterinary Medicine, Khon Kaen University, Thailand. Cultures were prepared from 20-30 µl of frozen stocks (containing 15% glycerin at -70^{o}C) by inoculating on a brain heart infusion (BHI) agar plate and incubated overnight at 37^{o}C. A working bacterial suspension was prepared by inoculating 4-5 similar colonies isolates from an agar plate added to BHI broth and incubated at 37^{o}C on a gyratory shaker at 100 rpm for 2 hours (log phase growth). The bacteria were washed twice with HBSS and finally resuspended at $1x10^{8}$ colony forming units per ml (CFU/ml). Bacterial concentrations were quantified using measurement of absorbance at 450 nm (0.32 corresponds to $1x10^{8}$) with spectrophotometer and then diluted to approximately $10x10^{6}$ CFU/ml.

Sources of opsonins

A pool of normal bovine serum was obtained from 10 healthy dairy heifers (no manifestation of clinical signs). Whole blood was collected in 10 ml syringes and centrifuged at 300 x g for 15 min. Serum was removed and heated at 56^{o}C for 30 min. to inactivate complement and placed on ice. Bacteria was opsonized with 100 µl of 10% HIA (heat inactivated) serum and 900 µl of diluted

bacteria for 20 min. at 20°C and placed on ice.

Phagocytosis assay

Suspensions of neutrophils 500 µl and opsonized bacteria 120 µl were added into sterile tubes and incubated for 60 min. at 37°C at 100 rpm on a gyratory shaker. The precipitate was smeared and stained with Wright's solution before being examined under the microscope with a 10x oil immersion objective. At least 200 phagocytic cells were counted and mean number of ingested bacteria/cell was obtained from 3 counts.

Killing of *S. aureus* assay

The nitroblue tetrazolium (NBT) reduction test was used to identify the dead bacteria that were killed by neutrophils. The NBT solution was prepared with an equal volume of 0.2% NBT dissolved in phosphate buffered saline (PBS). Suspension of neutrophils 500 µl, opsonized bacteria 120 µl and 50 µl of NBT were added in sterile tubes and incubated for 60 min. at 37°C. Tubes were centrifuged at 100 rpm on a gyratory shaker before smeared on slides with Wright's stain and examined under the microscope with a 10x oil immersion objective with dark granules due to NBT reduction abundant in the cytoplasm. Approximately 200 cells were assessed.

Statistic analysis

Data on the number of total white blood cells, differential white blood cells phagocytosis and killing of *S. aureus* were analyzed across time using the repeated measurement in completely randomized design (CRD), relationship between neutrophils and either phagocytosis or killing of *S. aureus* was tested with Pearson correlation coefficient by SAS (SAS, 1998).

Results

White blood cells and differential white blood cells

Supplementation of Se and vit E in dairy heifers showed a significant x increase in white blood cell counts in all treatments between pre-supplementation, supplementation and post-supplementation periods (SEM±4,565 P<0.05). However, white blood cells were not significantly different (P>0.05) among treatments as shown in the Table 2. Supplementation of 3Se4E increased white blood cells (54,900 cells/ml) during supplementation period and remained constant throughout the post-supplementation period (Figure 2). The high levels of Se and vit E (6Se4E) sharply increased the number of white blood cells within 4 days of supplementation but the numbers then declimed levels similar to levels similar to the other treatments. Differential white blood cell counts showed as significantly differences (P> 0.05) in neutrophils among treatments. However, supplementation of 3Se4E had highest neutrophils (11,398 cells/cu.mm) and followed by 6Se4E, 3Se2E and 6Se2E (6,502, 6,125 and 4,504 cells/ cu.mm, respectively). Absolute neutrophils of heifers supplemention by 3Se4E was higher increased (260.7%) than those of dairy heifers supplemented with 6Se4E, 3Se2E and 6se2E (219.7, 151.1 and 78.7%, respectively). The number of absolute eosinophils were not significantly different (P>0.05). The heifers supplemented by 3Se 4E had greater eosinophils during supplementation (3,569 cell/cu.mm). Absolute lymphocytes counts were higher than neutrophils on pre-supplementation and lower than neutrophils on supplementation. Lymphocytes were dramatically changed in 6Se2E and 6Se4E. Supplementation of 3Se2E had higher monocytes (1,653 cells/cu.mm).

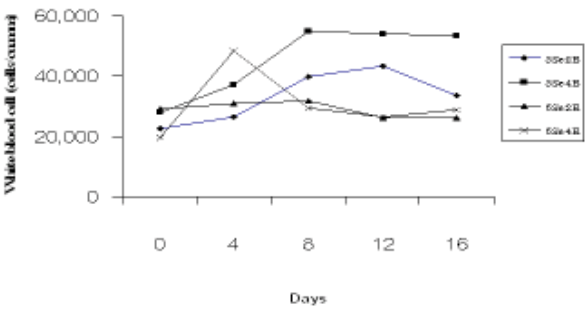


Figure 2. Effects of selenium and vitamin E supplementation on white blood cell counts (cells/cu.mm)

Table 2. Effects of selenium and vitamin E supplementation on total white blood cells and differential white blood cell

Treatments	WBCs (cells/cu.mm)	Differential white blood cells (cells/cu.mm) (%)			
		neutrophils	eosinophils	lymphocytes	monocytes
3Se2E					
Pre-supplementation	22,450 [b]	2,439	1,173	17,530	1,306
		(10.8)	(5.4)	(78.0)	(5.8)
Supplementation	39,666 [a]	6,125	2,484	29,402	1,653
		(15.5)	(6.5)	(74.0)	(4.0)
3Se4E					
Pre-supplementation	27,816 [b]	3,160 [b]	2,108	21,169	1,386
		(11.6)	(7.5)	(76.0)	(4.9)
Supplementation	54,900 [a]	11,398 a	3,569	38,332	1,599
		(20.8)	(6.5)	(69.8)	(2.9)
6Se2E					
Pre-supplementation	29,116	2,521	1,131	23,846	1,617
		(8.6)	(3.8)	(81.8)	(5.8)
Supplementation	31,650	4,504	1,519	24,237	1,387
		(14.2)	(4.7)	(76.5)	(4.3)
6Se4E					
Pre-supplementation	19,666 [b]	2,034 [b]	515	16,037 [b]	1,078
		(10.3)	(2.6)	(81.5)	(5.4)
Supplementation	29,533 [a]	6,502 a	1,194	20,807 [a]	1,029
		(22.0)	(4.0)	(70.4)	(3.4)
% increased[1]					
3Se2E	76.7	151.1	111.8	67.0	26.6
3Se4E	97.4	260.7	69.3	81.0	15.4
6Se2E	8.7	78.7	34.3	1.63	-14.2
6Se4E	50.2	219.7	131.8	29.7	-4.5

[1] Compared between pre-supplementation and supplementation period
[a] [b] Means within column differ (P<0.05)

Monocytes were slightly changed by supplementation of Se and vit E. In most cases, basophils were not found in any treatment during the experimental period.

Phagocytosis and killing of *S. aureus*

Phagocytosis is one of the most important host defense mechanisms against microorganisms. Supplementation of Se and vit E resulted in an increase percentage of phagocytosis and killing of *S. aureus* in all treatments (P<0.05). The greatest improvement of phagocytosis and killing of *S. aureus* was by supplementation of 3Se4E (85 and 47%, respectively) (Table 3). Phagocytosis in heifers received 3Se4E was significantly higher (P<0.05) than that in heifers received the other treatments. Phagocytosis and killing of *S. aureus* increased linearly during supplementation period and then decreased to post-supplementation period (P>0.05) (Figure 4 and 5).

Main effects of Se and vit E supplementation were shown in Table 4. Phagocytosis and killing of *S. aureus* were responded well at 3 mg Se/d and 4,000 IU vit E/d. Interactions between Se and vit E supplementation was not significant.

The correlation coefficient of number of

Table 3. Percentage of phagocytosis and killing of *S. aureus* by bovine neutrophils.

Measures	Pre-supplementation	Supplementation	SEM	% Improved
Phagocytosis				
3 Se 2 E	50.6	69.3^{x}	2.7	37.0
3 Se 4E	40.0	74.0^{y}	2.7	85.0
6 Se 2 E	44.6	61.0^{z}	2.7	37.0
6 Se 4 E	40.3	64.3^{z}	2.7	60.0
Mean	43.9a	67.1^{b}		
Killing of *S. aureus*				
3 Se 2 E	48.3	57.6	3.6	19.0
3 Se 4E	48.0	70.6	3.6	47.0
6 Se 2 E	44.3	62.3	3.6	41.0
6 Se 4 E	43.3	61.3	3.6	42.0
Mean	46.5a	62.5^{b}		

^{ab} Means within row differ (P<0.05)
^{xyz} Means within column differ (P<0.05)
SEM = Standard error of the mean

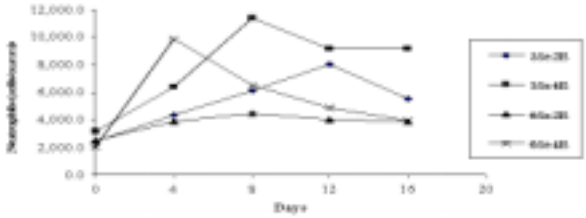


Figure 3. Effects of selenium and vitamin E supplementation on neutrophils (cells/cu.mm)

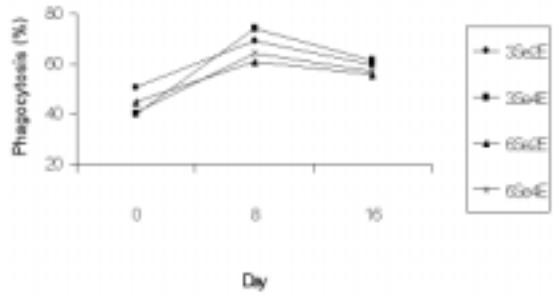


Figure 4. Effects of selenium and vitamin E supplementation on phagocytosis (%)

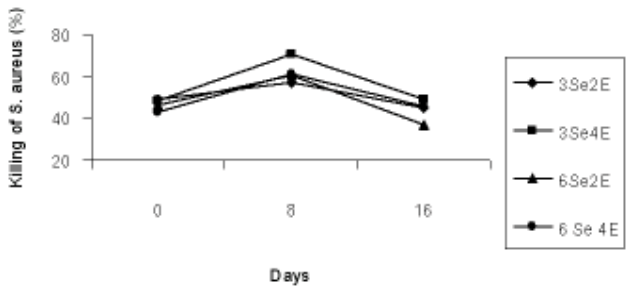


Figure 5. Effects of selenium and vitamin E supplementation on killing of *S. aureus* (%).

neutrophils and phagocytic activity was 0.60 (P< 0.05), while correlation coefficient of neutrophils and killing of *S. aureus* was slightly higher 0.63 (P<0.05).

Discussion

The high level of Se and vitamin E (6Se4E) increased the number of white blood cells and

Table 4. Main effect of percentage of phagocytosis by bovine neutrophils (%).

Measures	Pre-supplementation	Supplementation	SEM	% Improved
Phagocytosis				
Selenium/mg				
3	45.3	71.6a	3.24	58.0
6	42.5	62.6b	3.24	47.0
Vitamin E/IU				
2,000	47.6	65.1b	3.2	37.0
4,000	40.1	69.1a	3.2	72.0
Killing of *S. aureus*				
Selenium/mg				
3	48.1	64.1	4.3	33.0
6	43.8	61.8	4.3	41.0
Vitamin/IU				
2,000	46.3	60.0	4.3	30.0
4,000	45.6	66.0	4.3	45.0

[ab] Means within column differ (P<0.05)
SEM = Standard error of the mean

then sharply decreased to normal level from d 4-8, whereas the low level of Se and high level of vitamin E (3Se4E) had the opposite effect and maintained white blood cells through day 16. These observations indicate longer immune response improvement of 3Se4E than 6Se4E in healthy cows and no positive result was found in heifers. This in accordance with Roger *et al.* (2004), who reported that normal bovine WBC was 6.5-25.5 x 10^6 cells/ml. Smith *et al.* (1999) reported that WBC is a major component of the body's immune system when a cow has a bacterial infection. There are five major kinds of WBC: neutrophils (0.6-4.0 x 10^6/ml or 15-45%), eosinophils (0.0-2.4 x 10^6 / ml or 2-20%), lymphocytes (2.5-7.5 x 10^6 /ml or 45-75%), monocytes (0.03-0.84 x 10^6 /ml or 2-7%) and basophils (0.0-0.2 x 10^6 /L or 0-2%). Each type of cell plays a different role in protecting the body. The number of each one of these types of WBC indicates important information about the immune system. An increase or decrease in the numbers of the different types of WBC can help identify infection or to monitor the body's response to diseases treatment. Neutrophils are part of the innate immune system and play an important role in non-specific immunity as the first line of defense against infection. National Research Council (1983) reported chronic toxicity could occur when cattle are fed diets with 5 to 40 mg of Se/kg for period of several weeks or months. Acute toxicity can occur when cows fed 10-20 mg of Se/kg of body weight. In this study, heifers fed below the toxicity level as reported by National Research Council (1983). National Research Council (1987) suggests ruminants can tolerate intake of about 40,000 IU/day of supplemental vitamin E for several months without effects. Supplementation of 3Se4E increased white blood cells while increased neutrophils by 97.4% and 260.7%, respectively. These reasons are explained by Shank (2002) and Gibert *et al.* (1993) who reported that the number of white blood cells was mainly increased as a consequence of an increase in number of neutrophils. Hogan *et al.* (1990) reported that neutrophils were the cells most involved non-specific immunity. Most information concerning the bovine host defense deals with neutrophil function and neutrophils from cows supplemented with 0.3 mg of selenium during the dry period and first 30 days of lactation had a greater killing ability against *E. coli* and *S. aureus*.

Absolute eosinophils from heifers fed 3Se4E

had highest at 3,569 cells/cu.mm. Heifers might be infected by helminth. This also in accordance with Roitt *et al.* (1998) who reported that eosinophils usually have a bilobed nuclear and many cytoplasmic granules they comprise 2-5% of blood leucocytes in healthy, non-allergic individuals. Although it is not their primary function, they appear to be capable of phagocytosing and killing ingested microorganisms. Eosinophils are thought to play a specialized role in immunity to parasitic worms this mechanism.

In this study, lymphocytes changed in the opposite direction to neutrophils. As reported by Shank (2002), heifers injected with 50 mg/ml selenium and 50 mg/ml vitamin E showed an increased percentage of neutrophils while and decreased number lymphocytes. Smith *et al.* (1999) reported that lymphocytes and macrophages are the cells primarily involved with specific immunity. Specific immunity occurs when animals develop or acquire immunity to a specific pathogen once it is exposed to the pathogen. Antibodies specific to that pathogen are produced and the immune system memorizes the antigenic properties of the pathogen so that an immune response can be initiated quickly when the host is exposed to the pathogen again.

Dietary supplementation of 3Se4E improved phagocytic ability (85%) and killing of *S. aureus* (47%) by neutrophils, whereas 6Se 2E supplementation improved phagocytic ability (37%) and improved killing (41%), A similar finding reported by Hogan *et al.* (1990) and Hogan *et al.* (1992) that supplementation of 1,000 and 3,000 IU of vitamin E increased neutrophil function. Phagocytosis was not affected but ability to kill *S. aureus* and *E. coli* was improved in dairy cows. Grasso *et al.* (1990) reported that the ability of neutrophils to phagocytize bacteria was independent of Se. The response of neutrophils to Se and vit E supplementation was also found in dairy ewes. Neutrophils from cows fed 0.1 mg of supplemental selenium killed mastitis pathogens more effectively than did neutrophils from cows not fed supplemental selenium. Hidiroglou (1995) who administered 3 g of d-alpha-tocopheryl acetate in emulsions to two cows increased their milk d-alpha-tocopherol concentration by 50 and 54% 36 h after injection. In both cows, the d-alpha-tocopherol concentration returned to the base line 3 days after injection. This is in accordance with Erickson *et al.* (1963) who reported that either the injection of unemulsified d-alpha-tocopheryl acetate (1 to 3g) or 1.5 g emulsified d-alpha-tocopheryl acetate caused marked change. These researchers observed a peak of vit E in milk 2 days after administration (81% increased) and return to the baseline 3 days later. Phagocytosis and killing of *S. aureus* were improved by Se and vit E supplementation but these activities decreased after withdrawal of Se and vit E. This observation indicated that Se and vit E should continue to be supplemened for maintaining the immune response.

Conclusion

The results illustrated that Se plus vitamin E played important and synergistic roles in innate defense mechanisms. Supplementation of 3 mg Se and 4,000 IU vitamin E per head per day improved neutrophils. Daily supplementation of both Se and vitamin E may be beneficial to maintain the number of WBC, phagocytosis and killing capacity of neutrophils. Selenium and vitamin E contents were more closely related to neutrophil function.

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