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The Effect of Vitamin C on Blood Lymphocytes of American Mink (*Neovison Vison*): Variation with Sex and Genotype

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HIGHLIGHTS

- Vitamin C supplementation influences lymphocyte proliferation.
- Mutations associated with fur color affect the morphology of leukocytes.
- Morphometry of lymphocytes is method for assessing their development.

Abstract: Vitamin C (VC) is involved in many biological processes including immune response. This experiment was conducted to investigate the effects of supplemental VC on the development of mink lymphocytes in wild type (Standard genotype mink) and animal model of Chediak-Higashy (CH) syndrome (Sapphire genotype mink). Chediak-Higashy syndrome is genetically determined disease of human and animals affecting morphology and functions different cells including leukocytes. Using morphometry and cytochemistry methods, we reveal that VC supplementation (100 mg/animal/day) induces lymphocyte activation in wild type mink and CH-like mink. However, the effect of VC supplementation is sex-related and depends on the genotype of mink. In general, we obtained data that VC supplementation neutralizes genotype-related size differences among lymphocytes.

Keywords: lymphocytes; vitamin C; Chediak-Higashi syndrome; *Neovison vison*; morphometry.

INTRODUCTION

Vitamin C (VC), or ascorbic acid, is an important micronutrient playing a major role in many biological processes including protection against oxidative damage, regulation of protein and carbohydrate metabolism, biosynthesis of collagen, L-carnitine, steroid hormones, activation of digestive enzymes, etc. [1, 2]. VC is widely regarded as a positive regulator of immune function [3, 4]. It has been shown that VC supplementation

inhibits age-related thymic involution, influences neutrophil bactericidal ability, and regulates stem cell maturation and differentiation [5-7].

Lymphocytes are important immunological cells present in blood in large quantities and playing a key role in acquired immune response. There are studies regarding VC effects on lymphocyte differentiation [8, 9] (and its essential role in maintaining the natural killer (NK) cytotoxicity against cancer cells [10].

As discussed by some authors [11, 12], there is variation in genotype-related susceptibility to disease in mink. It is speculated that increased susceptibility in some genotypes (fur color type) of mink to infections may be one of the explanation for variation in mortality between farms. In a previous study, we found that sapphire puppies showed a delay in immune and hematopoietic system development compared to standard (brown wild type) puppies [13]. In addition, sapphire mink with Aleutian coat color allele are characterized by abnormal morphology and dysfunction of leukocytes. Similar clinical abnormalities were found in humans and other species, and are linked to the autosomal recessive Chediak-Higashi syndrome (CHS).

VC supplementation (1 g/d) has been shown to significantly improve leukocyte chemotaxis in patients with CHS [14, 15]. In another study, high VC doses (20 mg/mouse) prolonged the survival of CHS mice from lethal infection with *Candida albicans* [16]. A majority of VC effects in CHS were demonstrated for innate immunity; however, role of VC in adoptive immunity in CHS is less clear. The fact that lymphocytes accumulate much higher, up to 80-fold, concentrations of vitamin C compared to those in serum also suggests a certain role of vitamin C in these cells [17].

To clarify the effect of VC on various immune functions in CHS species, further studies will be needed. We speculated that dietary intake of VC can affect lymphocyte parameters, improving the state of mink weakened by CH-like syndrome.

Lymphocyte parameters can be assessed by the cell morphometry method. This method provides valuable information on clinically important properties of blood cells [18]. Digital microscopy and computerized image analysis is useful along with proven flow cytometry and molecular techniques. Previous studies have shown that lymphocyte morphometry can be widely used for clinical diagnosis and prognosis regarding immune responses [19, 20].

In this study, the impact of VC supplementation on blood lymphocyte parameters in mink was characterized, taking into account mink genotype, sex and hereditary pathology.

MATERIAL AND METHODS

Animals and sampling

Six-month-old American mink (*Neovison vison*) of the Standard dark-brown genotype and the Sapphire genotype were used. The mink were housed in conventional outdoor facilities and exposed to ambient temperatures and light conditions at fur farm “Pryazhinskoe” Ltd. (Republic of Karelia, North European Russia (geographical coordinates 66°41'33''N, 33°37'12''E)). The experiment was performed in November in environmental conditions typical for the North-West of Russia during this period. Average temperature in November is -0.3°C. During the study period, the animals were checked daily for any clinical signs of diseases. This included physical examination of each animal, presence of normal physical activity, normal food and water intake, and absence of signs of discomfort during the observation period.

All the mink were fed a paste-like diet containing processed fish products and grains, and given free access to water. Mink with approximately the same weight were randomly divided into four groups: control (4♂♂, 4♀♀) and experimental (4♂♂, 4♀♀) mink of the Standard genotype, and control (4♂♂, 4♀♀) and experimental (4♂♂, 4♀♀) mink of the Sapphire genotype. Experimental mink were fed a diet containing VC (100 mg/animal/day) for 20 days. Therapeutic effect high dose of VC was demonstrated in previous studies with CHS-mouse (20 mg/day) and CHS-patient (500 mg/day) and use in therapy in present time [15, 16, 21]. The VC dose of 100 mg/mink/day was chosen based on the successful experience with mega doses.

Blood and was taken in the morning after night fasting from the caudal vein. All the experiments were conducted according to EU guidelines on the use of animals for biochemical research (86/609/EU).

All investigations were performed in accordance with the “Guide for the Care and Use of Laboratory Animals” published by the US National Institute of Health (NIH publication No. 85–23, 1996). It conforms to the principles outlined in the Helsinki Declaration. The Ethics Committee of the Institute of Biology, Karelian Research Centre approved all animal care procedures prior to initiation of the experiment (permit number: 2010-09-14).

The research was performed using the equipment of the Core Facility of the Karelian Research Centre of the Russian Academy of Sciences.

Light microscopy and lymphocyte morphometry

Peripheral blood smears were prepared on glass slides after sampling following a standard procedure. The blood smears were stained with May-Grünwald and Romanowski stains (MiniMed, Russia) and examined under a light microscope (Axioscop 40, Zeiss, Germany). Digital images of single cells were captured by Pixera 150ES camera (Pixera Corporation, Los Gatos, CA, USA). Single lymphocytes in the field were identified and selected for imaging. Measurements from at least 50 cells were taken for analysis in each animal. Computerized image analysis was performed by using image analysis software VideoTest 4.0 (Video Test Inc, Russia). Total cell areas were calculated in μm^2.

Cytochemistry

To determine the alpha naphthyl acetate esterase (ANAE) activity, blood smears were immersed in an incubation solution (phosphate buffer, pH 7.8 0.1M) containing the enzyme substrate, 0.25 ‰ alpha naphthyl acetate (dissolved in acetone), and fast blue BB salt [22]. The staining period was 30 minutes. Blood smears were counterstained with nuclear dye hematoxylin. The ratios of ANAE positive lymphocytes in each smear were determined by counting 100 lymphocytes assigned as negative or positive according to stain uptake.

Statistical analysis

Differences between groups (control and experimental) were tested with nonparametric Mann-Whitney U-test. Nonparametric tests were used because of the small sample size. The data are represented as mean ± SEM. Differences at the $p<0.05$ level were considered statistically significant. Type III MANOVA testing of total cell area of lymphocytes and ANAE-positive lymphocyte ratio was done to assess the effects of vitamin C, genotype, sex, and their interactions. The statistical analysis was performed using MS Excel (Microsoft Corp., Inc., USA) and Stat Graphics Version 5.0 (Statistical Graphics Corp., USA).

RESULTS

The morphology of peripheral blood lymphocytes in mink is typical for most mammalian species. Usually, lymphocytes in mink are roundish cells, with basophilic cytoplasm and apparently without granulations; their total cell area can vary from 39.7 μm^2 to 144.5 μm^2. There are small, medium and large wide-cytoplasmic lymphocytes in blood mink. Three out of ten sapphire mink were found to have abnormal lymphocytes. The anomaly consisted in the presence of giant cytoplasmic granules, usually 1-2 per cell. Only a small part (5.3%) of sapphire mink lymphocytes contained abnormal cytoplasmic granules.

The morphometric lymphocyte parameters from all four groups are summarized in Table 1. Differences between genotypes appear not only in morphology, but also in size characteristics (Figure 1). MANOVA revealed that lymphocyte size in males and females depended on the combination of two factors: genotype and group (Table 1).

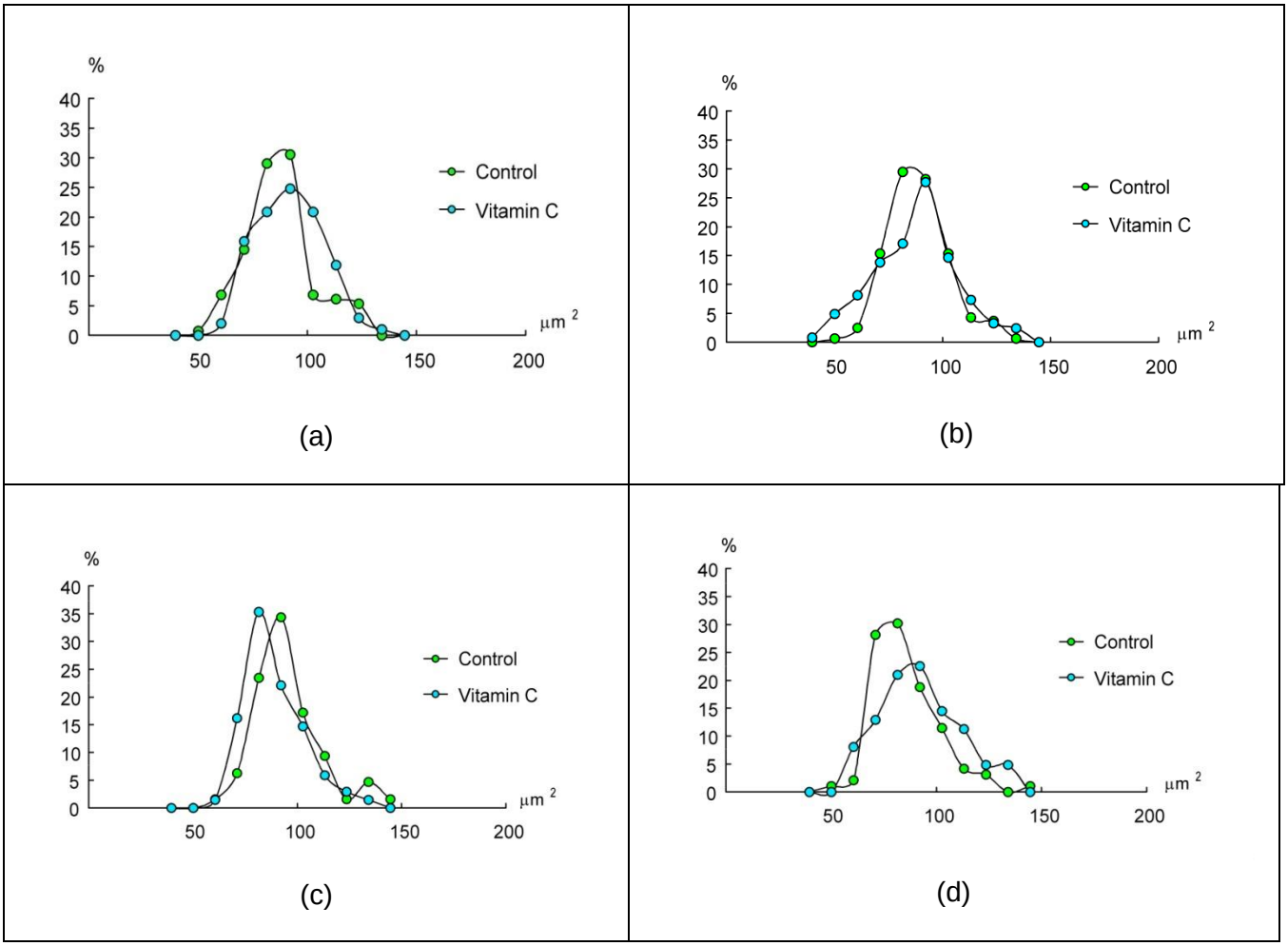

Figure 1. Histograms of the distribution of lymphocyte area in mink: standard female (a) and male (b); sapphire female (c) and male (d).

Table 1. Total cell area of lymphocytes (μm²) in standard and sapphire mink.

Standard		Sapphire		Factors	η^2 (%)	p
Control	**Vitamin C**	**Control**	**Vitamin C**			
Female						
82.17±1.32	87.13±1.47**	89.02±2.11◊◊	83.27±1.81*	AB	3.00	0.00015
Male						
83.67±1.09	82.36±1.69	80.27±1.63◊	86.49±2.43*	AB	1.00	0.0255

Numbers are mean ± SEM; * significant difference from control group, ◊ from standard mink, * p <0.05,** p<0.01, ◊p <0.05, ◊◊ p < 0.01. A – group (control / vitamin C), B – genotype, η^2 – factorial influence, p values – significant level

The results for ANAE-positive lymphocyte ratios in mink are shown in Figure 2. Vitamin C supplementation significantly increased the number of positive cells (by 20.79% in standard mink and by 43.15% in sapphire mink), while neither genotype, nor sex, nor their combined effect affected the ANAE-profile (Table 2).

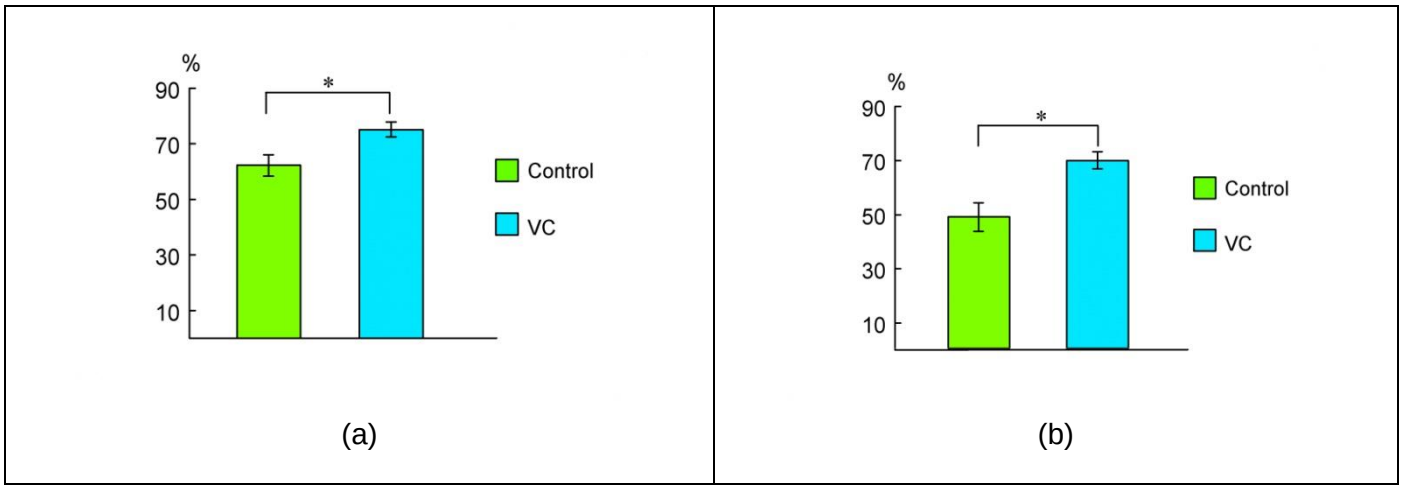

Figure 2. Effect of vitamin C on ANAE-positive lymphocyte ratios in standard (a) and sapphire (b) mink, * – significant difference from the control, p <0.05.

Table 2. Results of MANOVA testing for ANAE-positive lymphocyte ratios of sapphire and standard minks from control and VC groups.

Factors	η^2 (%)	p
A	3	0.005
B	10	0.076
C	6	0.174
Interactions		
AB	0.20	0.79
AC	0.55	0.66
BC	0.13	0.83
ABC	2.56	0.35

A - group (control / vitamin C), B – genotype, C – sex, η^2 – factorial influence, p values – significant level.

DISCUSSION

Analysis of size parameters of lymphocytes allows characterizing their subset ratio. Previously, it has been shown that an increase in the diameter, perimeter, and area of lymphocytes indicates lymphocyte activation, whereas the absence of changes in some inflammation cases is considered as impairment of immune response [20]. Our results demonstrate that the effect of VC on lymphocyte size is different in sapphire and standard mink, and depends on sex (Table 1). Following VC supplementation, the lymphocyte size in standard females and sapphire males increased significantly ($p<0.05$).

This study has demonstrated that both male and female mink have significant genotype-related differences in lymphocyte size ($p<0.05$). The lymphocytes of sapphire females were larger when compared to standard females; the lymphocytes of sapphire males were smaller than those of standard males. VC supplementation induced a considerable alteration of lymphocyte size (Figure 1) and eliminated genotype-related differences.

We observed no effect of VC supplementation on lymphocyte size in standard mink males. In a previous study, VC supplementation induced an increase in WBC counts and absolute number of monocytes and neutrophils in sapphire mink, whereas leukocytes in standard mink were not susceptible to VC [23]. The alteration of WBC level and differential leukocyte counts in sapphire mink can be attributed to the role of VC in stimulating cell proliferation [6]. Despite the fact that VC supplementation did not influence lymphocyte counts in sapphire or standard mink [22], there are numerous evidences that VC enhances lymphocyte proliferation and function in human and different animal species [24, 25].

According to several sources [22, 26], ANAE-positive reaction in lymphocytes is a T cell marker. In our study, ANAE-positive lymphocyte ratio increased in both genotypes under VC supplementation (Figure 1). This result points to an active role of VC in T cell development in mink. Moreover, the effect of VC on ANAE-

positive lymphocyte ratio encompasses females and males of both genotypes (Table 2). VC speeds up the process of T lymphocyte maturation. In an experiment with guinea pigs, T-cell numbers decreased in animals fed a VC-free diet, and increased in VC-supplemented animals (25 and 250 mg/animal/day) [8]. There are no unequivocal results yet on how VC influences the proliferation and function of B lymphocytes [25].

Although sapphire mink have traits of human CHS, there are some differences in hematology. Mink do not have neutropenia, as was described for CHS patients. Abnormal granules were found in all types of leukocytes in mink. Peripheral blood examination in humans with CHS revealed abnormal granules in neutrophils, monocytes and lymphocytes [21]. In both mink and humans, abnormal granulogenesis in lymphocytes leads to formation of abnormal granules and apparently impairs the cytotoxic activity of NK and cytotoxic T lymphocytes. In CHS patients, NK cells were affected more severely than cytotoxic T lymphocytes, both in terms of granule morphology and cytotoxic function. Where NK cells generally contained a single giant granule, cytotoxic T cells predominantly contained several smaller granules [27].

Another feature of CHS progression in humans is uncontrolled T cell activation (also called the ‘‘accelerated phase’’). Spontaneously or, more commonly, after a viral infection, CHS patients experience an accumulation of benign, activated T cells in several organs [28]. At the same time, the lymphoproliferative syndrome had not been described in either the mink or the murine homologue of human CHS [29]. Available reports on VC effects on the development and functions of lymphocytes in CHS are limited. Weening and coauthors [15] did not find any effect of ascorbate (500 mg/day) on the observed defect in antibody-dependent lymphocytotoxicity in CHSpatients. Our study demonstrates that the morphological defect in lymphocytes is not very pronounced. This fact makes it difficult to speculate about the role of VC in abnormal granulogenesis in mink. Nevertheless, alterations of size characteristics and ANAE ratio are evidence that VC induces lymphocyte activation in mink.

VC can have different effects on lymphocytes in different species. Previously, Gallin and coauthors [16] found that ascorbate (20mg/day) had a positive effect in murine homologue of CHS, however CHS patients treated with VC (6g/day) might not respond in a similar way. There are conflicting data on the effect of vitamin C on the production of antibodies by B lymphocytes. High-dose VC supplementation increased the immunoglobulin level in healthy volunteers, guinea pigs, and chickens [30-34], whereas in other animal models (dog, mouse) VC supplementation had no effect on antigen-induced immunoglobulin levels after immunization [35, 36]. Our data reveal that VC effect is possibly subject to a combination of sex and genotype (Table 1).

Apparently, the effect of VC supplementation is dose-dependent. Both animal and human studies show that physiological VC concentrations have a beneficial effect on T cell proliferation, whereas supraphysiological concentrations are toxic for T cells [14]. Anderson and coauthors [14] reported that an increased mitogen-activated proliferation was noted in connection with a lower vitamin C concentration, whereas a higher concentration had an inhibitory effect.

Thus, findings on the effects of VC supplementation should be extrapolated to different species with care. The study has contributed new data on the response to VC supplementation in mink. Normally, mink are fed a diet supplemented with VC at 20-30 mg/animal daily; this dose can be increased to 50 mg/animal during pregnancy and lactation. We argue that a high dose of VC (100 mg/animal/day) can activate T-cell immunity. Therefore, 100 mg dietary intake of VC can be recommended for animals weakened by pathology or age-related thymic involution. The effect is dependent on sex and genotype of mink.

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