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Research Paper

EFFECTS OF VITAMIN A OVERDOSE ON THE IMMUNE SYSTEM IN RATS

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Of all the body's systems, the immune system responds more sensitively to subtle changes in the nutritional status. One nutrient that possesses known and potential effects on health and the immune response is vitamin A. This study investigates the effects of vitamin A administration above dietary requirements on some cells of the immune system in healthy male rats. A 105 male Wister rats were divided equally into three groups (control and two experimental). Test groups were orally administered with vitamin A at two different concentrations (8,000 and 15,000 IU/kg body weight) for 21 days, while the control group was not administered with vitamin A. Blood samples were collected from all rats and assayed for the different cells and antibodies. Compared to their respective controls, total white blood cells and neutrophil counts were significantly increased, while the basophil and total lymphocyte counts were decreased after low dose vitamin A administration. Vitamin A did not affect NK, T, and B lymphocyte cell counts; eosinophils, platelets, and monocytes counts; IgA and IgG concentrations; or hemoglobin and vitamin A concentrations. Therefore, vitamin A affected the immune system in specific ways and these results may be extrapolated to modulate the immune response in humans.

Key words: Vitamin A overdose, Rat Immune System, White blood cells, Lymphocytes, Neutrophils

INTRODUCTION

Vitamin A, a fat-soluble vitamin, is absorbed in the intestines with the help of lipids, making it difficult to dispose of excess amounts of the vitamin. Thus, increased consumption of vitamin A may easily lead to its accumulation in the body and subsequent overdose or toxicity, otherwise known as hypervitaminosis A. Due to the fact that vitamin A deficiency is one of the world's major malnutrition problems (Cunningham-Rundles *et al.*, 2005), many countries supplement foods with vitamin A and many people regularly consume vitamin A supplements, sometimes at doses surpassing the recommended amounts, to avoid a deficiency and to reap the benefits of the vitamin. It is established that vitamin A plays important roles in vision, growth, reproduction, hematopoiesis, and immunity (Biesalski, 1989; Levin and Leonard, 2004), and it is protective against infections, heart disease and some types

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of cancer (Koo *et al*., 2006, Love and Gudas, 1994).

It is well known that the immune system and its components are highly sensitive to both excess and deficiency of many nutrients and that a balanced diet strengthens the immune system and keeps immune components at optimal amounts and activities (Kelley, 2001). Vitamin A is strongly linked to the production, maturation, and activity of immune cells and the immune system (Villamor and Fawzi, 2005; Kang *et al*., 2009). It is important for the proper functioning of both the innate and acquired immune systems (Yang *et al*., 2009), although its effects are not fully understood (Cunningham-Rundles and Ho Lin, 1998). It is known that vitamin A is essential for the integrity of epithelial surfaces (Von Lintig *et al.,* 2005) and maturation of the immune system (Ross and Gerald, 2007). Supplementation with vitamin A has been shown to be of value in maintaining the intestinal integrity, although there is no consensus on its effects on mucosal surfaces and associated anti-infective or inflammatory markers (Fitch and Neville, 2002).

There are some animal and human studies on the effects of different levels of vitamin A supplementation on health and the immune system. Studies using very high doses of vitamin A are rare and different amounts and modes of administration are used (Villamor and Fawzi, 2005), making comparisons of findings and generalized conclusions difficult and sometimes not feasible. Thus, the main aim of the research work here was to study the effects of oral administration of very high doses of vitamin A on the innate and acquired (humoral and cellular) immune systems of healthy rats through the determination of the counts and concentrations of related cells and molecules. This may help shed a light on the effects that may be present in humans upon ingesting very high doses of vitamin A.

MATERIALS AND METHODS

Animals

A total of 105 young adult male Wister rats, aged about 9 weeks, with an initial body weight in the range of 170-250 g were used in this study. These rats were also used for histological studies (Mahassni and Al-Shaikh, in press) on some organs important in immunity and vitamin A storage and metabolism. All rats were supplied by and housed at King Fahd Medical Research Center, Jeddah, Saudi Arabia. The rats where housed at room temperature (25 ^{o}C), with a fixed 12 h light-dark cycle and free access to food and water. All rats received the same diet. This diet contained 8.44% moisture, 6% ash, 20% proteins, 4% fats, 59.55% soluble carbohydrates, 3.50% fibers, 1% calcium, 0.60% phosphorus, 20 International Units (IU)/g vitamin A, 2.20 IU/g vitamin D, and 70 IU/kg vitamin E (Grain Silos and Flour Mills Organization, Jeddah, Saudi Arabia).

The rats were randomly divided into two groups of 60 and 45. The 60 rats were used for all parameters except for the determination of lymphocyte subclasses (T, B, and NK cells) counts, which were determined using the group of 45 rates. The groups of 60 and 45 rats were each randomly divided into three equal groups. The control group rats were not manipulated in any way. The experimental groups received vitamin A daily for 21 days at two different concentrations. The low dose (LD) group rats received vitamin A at 8,000 IU/kg body weight, and

the high dose (HD) group rats received vitamin A at 15,000 IU/kg body weight. The weights of the rats were recorded at the beginning of the study and weekly thereafter.

Vitamin A Preparation and Administration

Vitamin A (retinol) was obtained in a powder form (Lanospharma Laboratories Company, China) with a purity of 99%. Each dose was prepared by dissolving 8,000 IU (2.4 mg) of the vitamin in 0.5 ml of pure virgin olive oil (cold pressed) for the low dose group, and 15,000 IU (4.5 mg) in 0.5 ml of olive oil for the high dose group. The vitamin was administered daily to the experimental rats by oral gavage, while only olive oil was administrated to the control groups in the same manner.

BLOOD COLLECTION

Blood was collected from all rats the day after administering the last (21st) dose of vitamin A for the experimental groups, and only olive oil for the control rats. Rats were anesthetized by ether and blood was collected, from the orbital sinus, into plain and EDTA vacutainer tubes.

LEVELS OF IMMUNOGLOBULINS IGG AND IGA

The serum concentrations of IgG and IgA were determined by using rat immunoglobulin ELISA kits **(**USCN LIFE Company, China) as per the manufacturer's instructions. Absorbances were read at 450 nm by using a microplate reader (Stat fax-2100, Awareness Company, Florida, USA).

COMPLETE BLOOD COUNTS

Total white blood cell counts and differential complete blood counts were carried using a Coulter Counter (Advia 120, Simens Company, Germany).

DETERMINATION OF LYMPHOCYTE SUBSETS CONCENTRATIONS

The whole blood counts of the lymphocyte subsets were determined using flow cytometry (FACScalibur Becton-Dickinson Company, Germany). Phycoerythrin (R-PE)-conjugated mouse anti-rat CD45 monoclonal antibody (Becton-Dickinson Company, USA) was used to determine the total concentration of lymphocytes. Fluorecin-isothio-cyanate (FITC)-conjugated mouse anti-rat CD3 monoclonal antibody (Becton-Dickinson Company, USA) was used to determine the concentration of T-lymphocytes. Phycoerythrin (R-PE)-conjugated mouse anti-rat CD161a monoclonal antibody (Becton-Dickinson Company, USA) was used to determine the concentration of natural killer cells.

QUANTITATIVE DETERMINATION OF SERUM VITAMIN A CONCENTRATIONS

The concentration of vitamin A in serum was determined for all samples using rat vitamin A ELISA kit (USCN LIFE Company, China) according to the manufacturer's instructions. Absorbances were measured at 450 nm by using a microplate reader (Syva Micro Trak, USA).

STATISTICAL METHODS

The data was analyzed by using the statistical program SPSS-V16 to obtain the mean ($\bar{x}$), standard deviation (± SD), and standard error of the mean (± SE) for all parameters.

RESULTS

Serum Vitamin A Levels

The serum vitamin A mean concentrations for the

groups are shown in Table 1, along with the Standard Errors (SE) and Standard Deviations (SD). The ANOVA one-way test shows that the serum vitamin A concentrations for the test groups (low and high doses) were not significantly different from the concentrations for the control group.

Serum IgG and IgA Concentrations

Table 1 shows the effect of the two doses of vitamin A on serum IgG and IgA concentrations. The ANOVA one-way test shows that the IgG and IgA mean concentrations for the test groups were not significantly different from those of the respective control groups.

Blood Platelet Counts and Hemoglobin Levels

The mean blood platelet counts and hemoglobin levels for all rats are shown in Table 1. The ANOVA one-way test shows that the platelet counts and hemoglobin levels for the test groups were not significantly different from those of the respective control groups.

Differential Complete Blood Count

The mean counts for the total leukocytes (WBC), neutrophils, basophils, eosinophils, monocytes, and lymphocytes for the control and the two doses of vitamin A groups are shown in

Table 1: Descriptive Statistics and Test of Significance for Vitamin A, Antibodies, and Hemoglobin Concentrations and Platelet Counts

	Groups	Max	Min	Mean	SE	SD	P
Vitamin A (μmol/L)	Control	3.12	0.02	0.93	0.19	0.88	0.14NS
	Low dose	4.89	0.1	1.63	0.34	1.38	
	High dose	4.78	0.2	1.06	0.23	1.01	
IgG (μg/ml)	Control	68.28	52.35	59.73	1.17	5.24	0.30NS
	Low dose	70.38	51.1	57.61	1.4	5.78	
	High dose	66.56	51.04	57.26	1.14	5.1	
IgA (μg/ml)	Control	4281	1554.33	2516.33	143.54	641.96	0.61NS
	Low dose	3767.66	1717.66	2697.27	120.27	495.89	
	High dose	3654.33	1734.33	2618.83	112.84	504.66	
Hemoglobin (g/dl)	Control	18.1	11.1	12.2	0.6	2.8	0.7NS
	Low dose	15.2	13.5	14.6	0.5	2.3	
	High dose	18.4	12.9	12.5	0.5	2.3	
Platelet counts	Control	530	102	283.15	23.12	103.37	0.45NS
	Low dose	571	150	320.41	38.67	159.44	
	High dose	547	130	271.68	20.55	89.58	

Note: The ANOVA one way test was used for the significance test; NS: Non-significant ($P > 0.05$); Max: Maximum, Min: Minimum, SE: Standard error, SD: Standard deviation.

Table 2. Using the ANOVA one-way test, the mean counts for the experimental groups for each type of cell were each compared to its respective control.

Table 2: Descriptive Statistics and Test of Significance for White Blood Cells Counts

Counts	Groups	Max	Min	Mean	SE	SD	P
WBC	Control	12.42	4.75	7.49	0.49	2.2	0.02S
	Low dose	29.1	2.56	10.74	1.5	6.21	
	High dose	12.3	4.09	7.33	0.65	2.86	
Neutrophil (%)	Control	78.1	32.6	53.7	3	13.8	0.01S
	Low dose	85.1	36.1	69.6	3.4	14	
	High dose	84.4	40.3	60.7	3.2	14.1	
Basophil (%)	Control	2.9	0.3	0.9	0.1	0.7	0.02S
	Low dose	1.2	0.2	0.5	0.1	0.3	
	High dose	2.4	0.3	0.7	0.1	0.5	
Eosinophil (%)	Control	7.9	0.8	3.28	0.43	1.96	0.11NS
	Low dose	9.6	0.1	1.83	0.59	2.46	
	High dose	7.8	0.4	2.32	0.46	2.02	
Monocyte (%)	Control	7.8	2.5	4.9	0.3	1.5	0.7NS
	Low dose	12.8	2.2	4.9	0.6	2.6	
	High dose	10.7	1.2	5.4	0.5	2.3	
Lymphocyte (%)	Control	58.8	15.3	37.1	2.8	12.7	0.01S
	Low dose	57.6	13.1	23.2	3.5	14.6	
	High dose	50.6	20.2	30.7	2.9	12.5	
B cell	Control	93.9	43.53	45.42	3.7	14.34	0.38NS
	Low dose	95.19	43.14	48.47	3.86	14.89	
	High dose	57.22	0.16	40.94	3.68	13.3	
T cell	Control	30.2	0.3	21.12	1.93	7.48	0.49NS
	Low dose	30.7	0.15	19.12	2.05	7.97	
	High dose	24.35	0.04	17.76	1.86	6.73	
NK cell	Control	38.38	31.02	34.38	0.61	2.31	0.08NS
	Low dose	40.39	27.73	33.38	0.87	3.28	
	High dose	45.12	29.17	36.43	1.29	4.49	

Note: The ANOVA one way test was used for the significance test; S: Significant ($P < 0.05$); NS: Non-significant ($P > 0.05$); Max: Maximum, Min: Minimum, SE: Standard error, SD: Standard deviation.

The ANOVA one-way test shows that there were significant differences between the mean counts for the vitamin A groups and the respective control counts for the total WBC, neutrophil, basophil, and lymphocyte counts. Using multiple comparisons testing (Table 3), significant increases in the WBC and neutrophil cell counts were obtained for the low dose groups in comparison to the respective controls, while the high dose groups showed no differences. As for the basophil and total lymphocyte counts, using the multiple comparisons testing (Table 3), the low dose groups showed significant decreases while the high dose groups showed no changes when compared to the respective control groups. On the other hand, using the ANOVA one-way test, there were no significant effects of vitamin A administration on the mean counts of eosinophils and monocytes compared to the mean counts of the respective controls.

LYMPHOCYTE SUBCLASSES COUNTS

The mean B, T, and NK cell counts are shown in Table 2. Using the ANOVA one-way test, there were no significant effects of oral administration of either dose of vitamin A on B, T, or NK lymphocyte mean counts compared to the respective control groups.

DISCUSSION

Vitamin A has been reported (Bellovino *et al*., 2003; Cunningham-Rundles *et al*., 2005) to play a crucial role in the mechanisms that govern proliferation, differentiation, and the proper functioning of the immune system and its components. The present study investigated the effects of daily oral administration of two very high doses of vitamin A (8,000 IU/kg and 15,000 IU/kg body weight) for 21 days on cells and molecules involved in innate immunity (total WBC, neutrophils, basophils, eosinophils, monocytes, and natural killer cells) and the humoral (B lymphocytes, and IgG and IgA antibodies) and cellular (T lymphocytes) adaptive immune systems in healthy young male rats. Additionally,

Table 3: Multiple Comparisons Tests Between the Mean WBC, Neutrophil, Basophil and Lymphocyte Counts for the Vitamin a Groups and the Control Groups

Counts	Group	Group	Mean Difference	SE	P	95% Confidence Interval	
						Lower Bound	Upper Bound
WBC (%)	Control	LD	-3.25	1.33	0.05^{S}	-6.54	0.03
		HD	0.16	1.29	1.00^{NS}	-3.03	3.35
Neutrophil (%)	Control	LD	- 15.9	4.6	0.00^{S}	- 27.3	- 4.5
		HD	- 7.1	4.5	0.36^{NS}	- 18.2	4.0
Basophil (%)	Control	LD	0.5	0.2	0.02^{S}	0.1	0.9
		HD	0.2	0.2	0.68^{NS}	- 0.2	0.6
Lymphocyte (%)	Control	LD	13.9	4.4	0.01^{S}	3.1	24.7
		HD	6.4	4.2	0.42^{NS}	- 4.1	16.8

Note: The mean difference is significant at the 0.05 level; LD: Low dose, HD: High dose.

concentrations of serum vitamin A and hemoglobin, and platelet counts were also determined.

Excess vitamin A may lead to nausea, jaundice, irritability, anorexia, vomiting, blurry vision, headaches, hair loss, muscle and abdominal pain, weakness, drowsiness, and an altered mental state (Ramanathan *et al.*, 2009). In chronic cases symptoms may be, in addition to the previously mentioned symptoms, hair loss, dry skin, drying of the mucous membranes, fever, insomnia, fatigue, weight loss, bone fractures, anemia, and diarrhea (Reddy and Benjamin, 2003). Also in the case of chronic toxicity, increased susceptibility to infections has been observed (Chandra, 1993), which is due to a weakened immune system and may be evidence to the effect of vitamin overdose on the immune system.

The National Research Council (NRC, United States) has established the approximate daily requirements of vitamin A in rats to be 0.37-4 IU (Sharp and Regina, 1998), although there is no general agreement on this level. Therefore, regardless of the requirements followed, the doses used here would be considered very high doses. Nevertheless, no symptoms of hypervitaminosis A, such as behavioral inactivity, diarrhea, hair loss, skin scaling, or weight loss were observed in the rats administrated with vitamin A (Mahassni and Al-Shaikh, in press). This indicates that the used doses are safe for the duration of the experiment.

The oral gavage route of vitamin A administration was used since the aim of the study was to emulate the ingestion route of food and vitamins in humans and, additionally, it insures the ingestion of the full dose of the vitamin. More importantly, the introduction of the vitamin directly into the gastrointestinal tract of rats may affect cell mediated and humoral immunities, while the intraperitoneal route, which is the commonly used route of vitamin administration in animal studies, delivers the vitamin directly into the blood stream, which has the capability of inducing humoral immunity only.

The results of this study show no significant effect of oral administration of vitamin A on serum vitamin A levels. This agrees with the finding (Jeyakumar *et al.,* 2006) of unaltered serum retinol levels in male rats supplemented with high doses of vitamin A for 2 months, while disagrees with other studies (Mallia *et al.,* 1975; Cui *et al*., 2000) that found increased serum vitamin A concentrations in supplemented mice and rats.

In this study, the concentrations of IgG and IgA were determined since IgG is the major antibody in antibody-mediated immune responses, while IgA is the major antibody in mucosal secretions and surfaces, which are also affected by vitamin A, and it protects these surfaces from pathogens. The results show no significant effects of vitamin A on IgA and IgG concentrations. These results are contradictory with the findings in humans of increased serum levels of IgA and IgG and decreased IgG levels (Mora and Von Andrian, 2009) upon vitamin A supplementation. Studies in children supplemented with vitamin A at the time of immunization show contradictory results for the production of antibodies against the immunizing agent, with some showing no effect (Rosales and Kjolhede, 1994) and some showing increased antibody titers (Ross and Gerald, 2007; Coutsoudis *et al*., 1992).

Platelets have recently been considered as immune cells and to have important roles in both

innate and acquired immune responses and as first responders against pathogens (Cox *et al.,* 2011; Li *et al*., 2012). The findings of our study show that blood platelet counts are not affected by vitamin A administration. This result confirms the finding of Calzada *et al*. (1997) in healthy humans supplemented with β-carotene. Our results also show that there is no relationship between vitamin A administration and hemoglobin levels. This may rule out anemia, a symptom of vitamin A overdose, in the experimental rats. In a study by Roodenburg *et al*. (2000), vitamin A deficiency in rats did not lower blood hemoglobin levels after feeding them a vitamin A diet for 70 days.

The results of this study show that oral administration of vitamin A is associated with significant increases in the blood total WBC and neutrophils counts for the low dose rats compared with the control, which agrees with the findings of Seguin-Devaux *et al*. (2005) of increased WBC in supplemented rats. On the other hand, these results are not in agreement with the findings of Raoofi *et al.* (2009) of no significant effect on white blood cells and differential leukocyte counts in supplemented sheep and with the finding of Wood *et al.* (2000) of no changes in WBC differentials with supplementation in healthy adults.

Our results of increased neutrophil counts upon supplementation with vitamin A confirm the findings of other vitamin A supplementation studies on cultured neutrophils (Ribeiro *et al.,* 2003) and studies on animals (Higuchi and Nagahata, 2000; Seguin-Devaux *et al*., 2005), while they are contradictory to the decrease of neutrophils in rats found by Torii *et al.* (2004).

The data show that vitamin A supplementation is associated with significant decreases in mean basophil and lymphocyte counts in the blood of the low dose group compared to the respective controls. This result is not in agreement with the findings of Wood *et al.* (2000) of no changes due to β-carotene supplementation in healthy volunteers. Other vitamin A supplementation studies in humans have shown increased total lymphocyte levels, while some other studies are contradictory (Villamor and Fawzi, 2005). Our lymphocyte results are in disagreement with the findings of Seguin-Devaux *et al*. (2005) of increased counts of lymphocytes in rats. In another study (Coutsoudis *et al*., 1992) it was found that vitamin A supplementation in infants significantly increased the total lymphocyte counts.

The findings of this study show that the mean blood eosinophil and monocyte counts for both experimental groups are not affected by daily oral administration of vitamin A when compared to the control group. This is confirmed by the finding of Jiang *et al*. (2007) that there are no statistical differences in the means of the monocytes in human between the vitamin A deficient and normal groups. These results are not in agreement with the findings of Torii *et al.* (2004) where supplemented rats showed inhibited eosinophil counts.

The data from this study show that there is no significant effect of oral administration of vitamin A on T lymphocyte counts. This result confirms the results of Benn *et al.* (2000) and Watson *et al.* (1991) in humans, while it contradicts the findings of other researchers (Seguin-Devaux *et al.,* 2005; Villamor and Fawzi, 2005) that supplementation of vitamin A in rats changed (increased or decreased) the number of T

lymphocytes. In this study, there were no statistical differences in the means of the B lymphocyte and NK cell counts between the test groups and the control group. A study (Villamor and Fawzi, 2005) found that children with low serum retinol concentrations had a greater proportion of NK cells than those with higher retinol concentrations. We were not able to find other studies on the B and NK cell counts although studies on vitamin A deficiency show contradictory results with reduced NK cell counts in animals Zhao *et al.* (1994) and greater counts in children (Jason *et al.,* 2002).

CONCLUSION AND RECOMMENDATIONS

The findings of the present study conclude that administration of vitamin A much above dietary requirements in healthy rats has an enhancing effect on some immune system cells, while for other types of cells there is an inhibitory effect. There were significant increases, and therefore an enhancing effect of vitamin A, compared to controls, of the counts of neutrophil and total white blood cells for the low dose group. There was an inhibitory effect of vitamin A in the form of significant decreases of basophil and total lymphocyte cell counts for the low dose group compared to the respective controls. On the other hand, all other parameters showed no changes upon vitamin A administration compared to the control group.

Based on this study, it may be concluded that vitamin A offers some protection against infections by influencing the innate immune system in the body. This protective effect of vitamin A administration is mediated by significantly increased total white blood and neutrophil counts. On the other hand, vitamin A showed no significant effects on the measured parameters that relate to the adaptive immunity that include T and B lymphocyte cell counts in blood and the IgA and IgG concentrations in serum, with the exception of the significantly reduced lymphocyte counts.

A noteworthy observation is that all changes occurred only in the lower dose groups while none of the higher doses showed any decrease or increase. The reason for this could be that vitamin A modulates the immune system related cells and molecules only at a specific concentration range. It could be that vitamin A concentrations higher or lower do not affect the immune system since they are very low or very high concentrations or that a longer experimental period is required to be able to observe any changes to the immune system.

Studies of supplementation of very high doses of vitamin A in healthy rats are very few, thus it was not possible for us to compare all results of our study with similar studies. Vitamin A deficiency studies are much more prevalent in the literature. It is evident that there are many differences in the results in this study and previous studies by other researchers on the relationship between vitamin A and the immune system. This may be due to using different types, preparations and doses of vitamin A, different lengths of experimental period, different routes of administration or supplementation and different types and strains of experimental animals. This compounded the difficulty of comparing our results to those of other researchers and made it impossible to have an exact comparison. Additionally, there probably are many other factors and conditions that may contribute to the differences since the immune

system is sensitive to many factors in the human body and the environment.

It is recommended that further study be carried using larger sample sizes and different doses of vitamin A in healthy and infected or sick rats. Also, it is important to initiate similar studies in humans at different ages and different health states.

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