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Synergistic Effect of Zinc and Vitamin A on T Cell Functions[1]

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Objective To determine whether supplementation of zinc and vitamin A may improve the function of T cells in human PBMC. **Methods** T cells were separated and cultured *in vitro*, supplemented with either Zn or vitamin A alone, or both Zn and vitamin A (10^{-6} mol/L, 10^{-5} mol/L, 10^{-4} mol/L). After harvesting, cell proliferation, cell cycle, apoptosis, expression or function of cell-surface molecules, such as CD^{3+}, CD^{4+}, and CD^{8+} were detected. **Results** Higher proliferation level and lower apoptosis level were observed in cells supplemented with both Zn and vitamin A, showing the strongest effect ($P<0.05$). Zn-supplement increased the CD^{4+}/CD^{3+} cell percentage, and simultaneously decreased the CD^{8+}/CD^{3+} cell population. VA-supplement showed the opposite effect in comparison with Zn-supplement. **Conclusion** T-cell function can be improved, depending on the zinc and/or vitamin A supplemented.

Key words: Vitamin A supplementation; Zinc supplementation; T cell; Immunity

INTRODUCTION

Zinc interacts with vitamin A (VA)[1]. Zinc deficiency is accompanied with a reduction in circulating retinol concentrations in animals[2-3], and vitamin A supplementation alone fails to recover this specific condition. However, when animals are given either zinc supplements or zinc-containing diets, their serum retinol concentrations improve, suggesting that the low serum retinol concentrations are related to zinc deficiency[3-4].

Zinc and vitamin A are essential micronutrients for the immune system[5-6]. Even a mild form of their deficiency in different population groups, could suppress human immunity. T-helper (Th) cells (CD^{4+}) are affected by zinc and vitamin A deficiency, causing an imbalance between Th1 and Th2 cell functions. Altered secretion of typical Th1 and Th2 cytokines has been observed during zinc depletion. The Th2 cell products (IL-4, IL-6, and IL-10, remain unchanged during zinc deficiency, whereas the Th1 cell products: interferon (IFN)-*r* and IL-2, are decreased. Production of both IL-2 and IFN-*r* is corrected by zinc supplementation[7]. However, vitamin A deficiency increases IFN-r production, but decreases IL-4 and IL-5 production[8]. It is thus evident that zinc and vitamin A affect the immune system in different aspects, especially T-helper cells. So far, whether zinc and vitamin A exert synergistic effects on T-helper cell function remains unclear.

Data on the interaction between zinc and vitamin A in humans are limited and the results of such studies are inconclusive. In developing countries where protein-energy malnutrition is highly prevalent, children are usually deficient in multiple micronutrients, particularly in zinc and vitamin A. The present study aimed to investigate the effects of simultaneous supplementation of zinc and vitamin A on human T cell function *in vitro*.

MATERIALS AND METHODS

Reagent

All-*trans* RA, a major active retinoid metabolite in target cells purchased from Sigma-Aldrich (St. Louis, MO), was used to study the effect of vitamin A on T cell proliferation. All-*trans* RA is present in peripheral blood at a concentration of ~1nmol/L and can be directly taken up by cells *in vitro* and *in vivo*. Retinoids were diluted aseptically in DMSO (Sigma-Aldrich). The concentration of all-*trans* RA was confirmed by HPLC analysis as previously described[9]. Stock solutions at 1 or 10 mmol/L were

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stored at -70℃ and discarded after three freeze-thaw cycles. Retinol concentration was measured by the same method.

$ZnSO_4$ from Sigma was diluted in PBS. Zn^{2+} concentration was measured by atomic absorption spectrophotometry.

Cell Culture and Dosage Groups

T cells were isolated from peripheral blood obtained from healthy blood donors. Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation as previously described[10] and cultured at 5×10^6 cells/mL in RPMI1640 (Hyclone SH40012.10) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L glutamine and 125 U/mL penicillin at 37℃ in a humidified incubator with 5% CO_2.

All-*trans* RA and $ZnSO_4$ solutions were added into the culture medium to control the concentration of vitamin A at 10^{-6} mol/L (AN), 10^{-5} mol/L (A1), and Zn^{2+} at 10^{-6} mol/L (ZN), 10^{-5} mol/L (Z1), 10^{-4} mol/L (Z2). T cells were divided into ZNAN, ZNA1, Z1AN, Z1A1, Z2AN, Z2A1 groups by orthogonal analysis.

Standard Cell Proliferation Assay

T cells were cultured in 96-well plates after stimulation by PHA-P solution. About 48 h later, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) solution was added into each well. Cells were stimulated by PHP-P solution and cultured for 4 h. Cells were washed three times with PBS and harvested. DMSO was added into each well to dissolve formazan. Optical density (OD) at 490 nm was measured using a 96-well multi-scanner autoreader with the solubilization buffer serving as blank.

Flow Cytometric Analysis of Cell Cycle and Apoptosis

Cell cycle distribution and apoptosis were assessed by flow cytometric analysis, which was performed on activated T cells using differential staining of DNA and RNA with propidium iodide (PI). The cells were cultured and stimulated in different media for 24 h before harvesting, washed in cold PBS containing 0.1% fetal calf serum and fixed in cold 70% ethanol before the cells were stained with PI for 20 min at 20℃. The concentration in each medium was adjusted to about 10^6 cells/L. The cells were analyzed according to the manufacturer's instructions. Data were presented as ratio of G_1/G_2 of cell cycle and percentages of apoptosis.

Flow Cytometric Analysis of $CD^{4+}/CD^{8+}/CD^{3+}$ T Cells

The cells were cultured and stimulated for 48 h. The harvested cells were washed with PBS containing 2% FBS, re-suspended in 5×10^6 cells/mL, and incubated with FITC-conjugated anti-CD^{4+} Ab, anti-CD^{8+} Ab or anti-CD^{3+} Ab for 1 h at 20℃. The cells were subsequently washed three times, re-suspended in 0.5 mL PBS containing 2% FBS, and analyzed by FACS can flow cytometry according to the manufacture's instructions.

Statistical Analysis

The experimental data were expressed as $\bar{x} \pm s$ for 3 wells per group and statistically evaluated by analysis of variance (ANOVA) with the SPSS10.0 computer programs. One-way ANOVA, *t*-test and two-way ANOVA were used to determine the significant effects of Zn or vitamin A supplement. $P<0.05$ was considered statistically significant.

RESULTS

Effect on Cell Proliferation Assay

Proliferation is generally assessed by measuring the values of MTT, which is a method frequently used to estimate cell proliferation in lymphocytes[11]. A MTT-based colorimetric assay was used in our study to quantify cell proliferation. As shown in Table 1, the value of OD_{450} was increased with the dosage of zinc and vitamin A. The proliferation of Z1A1 group was significantly higher than that of other groups ($P<0.05$), especially than that of the control groups (ANZN group). By comparison, in the zinc supplemented groups, the OD_{450} of Z1AN group and Z1A1 group were higher than that of Z2AN group and Z2A1 group respectively.

TABLE 1

Results of MTT (OD_{450}) From Different Dosage Groups ($\bar{x} \pm s$)

Concentration of Vitamin A	The Concentration of Zinc		
	10^{-6} mol/L	10^{-5} mol/L	10^{-4} mol/L
10^{-6} mol/L	0.367±0.0231	0.756±0.0164*	0.598±0.0082
10^{-5} mol/L	0.546±0.0032	1.191±0.0030*	0.707±0.0040*

Note. $^*P<0.05$ *vs* control group (Vit A 10^{-6} +Zn 10^{-6}).

Effect on Cell Cycle and Apoptosis

The progression of T cell activation during the first 24 hours of culture was examined by evaluating the surface expression of activated markers after stimulation of PBMC with PHA-P. As shown in Table 2, the fraction of cells in the G_2 phase of the cell cycle increased steadily in the zinc and vitamin A supplement groups. The peak value was reached when zinc 10^{-5} mol/L zinc and 10^{-5} mol/L vitamin A were given in combination. The fraction of cells in G_2 remained at the level of the control group within 24 hours in the ANZN group, suggesting that supplementing zinc and vitamin A could activbate T cells.

Cells stained with propidium iodide (PI) were analyzed by flow cytometry after stimulation. The percentages of cells with fragmented DNA, sub-G1 population and pictures of cell apoptosis from FCM are shown in Fig. 1. The percentage of fragmented DNA was significantly decreased in the treatment group compare to the control group (ANZN), which coincided with the results of MTT. The apoptosis seemed to have increased if only vitamin A supplement was given. However, when both VA and Zn were given, the apoptosis declined again.

TABLE 2

Results of Cell Cycle and Apoptosis From FCM

Groups (mol/L)	G_1/G_2	Apoptosis (%)
Vit A 10^{-6} +Zn 10^{-6}	1.552	26.31
Vit A 10^{-6} +Zn 10^{-5}	2.951*	18.1*
Vit A 10^{-6} +Zn 10^{-4}	2.751*	17.8*
Vit A 10^{-5} +Zn 10^{-6}	1.151	33.4
Vit A 10^{-5} +Zn 10^{-5}	3.952*	14.3*
Vit A 10^{-5} +Zn 10^{-4}	2.356	14.2*

Note. $^*P<0.05$ *vs* control group (Vit A 10^{-6} +Zn 10^{-6}).

Effect on $CD^{4+}/CD^{8+}/CD^{3+}$ T Cells

CD expression of cells was detected by FCM (Figs. 2A and 2B). Zinc supplementation was associated with increased percentage of CD^{4+}/CD^{3+} T-cells in a dose-dependent manner. VA showed a significant effect on the percentage of CD^{8+}/CD^{3+} and CD^{4+}/CD^{3+} T-cells, which provided a different picture in contrast to the effect of Zn. A larger dosage of Zn would play a more important role in the process of CD expression.

DISCUSSION

The results of the study indicated that ZN and VA had a synergistic effect on T-cell functions in the culture system. Zinc- or VA-supplement could accelerate the proliferation of T-cells, and better effect could be expected by using an appropriate dosage (Table 1), which is consistent with other studies on mice[12]. Combination of VA and Zn could enforce this effect. The findings perhaps could be explained by the experiments of cell cycle and apoptosis. Supplementation with Zn and VA could stimulate the cell cycle to increase or decrease cell apoptosis. Therefore, the number of active cells in MTT test was much larger than that in the normal control group. These two micronutrient elements seem to stimulate their respective effect when they are used in combination. The best effects can be achieved by instantaneously giving Zn and VA in appropriate combination. A similar result could be found *in vivo* studies[13].

A number of factors can change T-cell phenotype, such as a variation in the ratio of CD^{4+} to CD^{8+} T-cells or in the ratio of "naive" to "memory" helper T-cells. Changes in one or more of the parameters are related to susceptibility to infectious diseases, or disease severity. In human beings, factors that are predictive of either morbidity or early mortality of infectious disease in the aging process include a decline in CD^{4+} T-cells, a reduction *in vivo* delayed-type hypersensitivity to various antigens, or a reduction in a cluster of immune functions[14]. This study showed that proper zinc supplementation could increase the CD^{4+}/CD^{3+} percentage, and decrease the CD^{8+}/CD^{3+} percentage. Contrary to the observations in zinc-supplemented cells, VA supplemented cells significantly decreased CD^{4+}/CD^{3+} expression, but increased CD^{8+}/CD^{3+} expression, suggesting that the CD^{4+}/CD^{8+} ratio can be controlled by adjusting nutrition supplementation. The mechanism of CD expansion of T cells in culture is unknown. The following hypotheses have been proposed: by stimulating specific cytokine secretion[15], by breaking the balance of T helper cells (Th1/Th2) in internal metabolism[16] or by affecting some special receptors or other unknown actions.

In conclusion, human T-cell functions are changed in PBMC, depending on the level of zinc or/and vitamin A. The combined functions of vitamin A and zinc *in vitro* culture indicate that appropriate supplementation of these micronutrients in combination would result in better outcomes than moderate supplementation of them in a separate way. The mechanism and clinical significance of these two micronutrients remain to be elucidated.

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