

Effects of vitamin D₃ and vitamin E on prednisolone-induced alterations of phagocyte function

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Abstract. – OBJECTIVE: To evaluate the effectiveness of vitamin D₃ and its combined action with vitamin E in the correction of the impairments of phagocyte function caused by chronic glucocorticoid administration.

MATERIALS AND METHODS: Phagocytic activity was assessed by the ability of peripheral blood neutrophils and monocytes to capture FITC-labeled *Escherichia coli* using flow cytometry. Metabolic activity of neutrophils was measured cytochemically as nitro blue tetrazolium (NBT) reduction test. Intracellular reactive oxygen species (ROS) were detected by 2',7'-dichlorofluorescein fluorescence.

RESULTS: Prednisolone administration (5 mg/kg b.w., 30 days) was accompanied by vitamin D₃ deficiency and decompensation of phagocyte function associated with antimicrobial activity (decrease in NBT reduction rate, ROS formation and phagocytic activity). Vitamin D₃ co-administration with prednisolone and, to a greater extent, its combination with α -tocopherol (100 IU and 0.5 mg/rat, 30 days respectively) partially restored phagocyte function.

CONCLUSIONS: These data suggest that vitamin D₃ and α -tocopherol can prevent immunosuppressive effects of prednisolone through elevating the efficacy of oxygen-dependent mechanisms of phagocytosis and increasing the functional activity of phagocytic cells.

Key Words:

Prednisolone, Vitamin D₃, α -tocopherol, Phagocytosis.

Introduction

Glucocorticoids (GCs) are effective anti-allergic, immunosuppressive and anti-inflammatory drugs, which are widely used in clinical practice. However, it is known that prolonged treatment

with steroid anti-inflammatory drugs increases, via genomic and non-genomic mechanisms, the risk of adverse effects. By acting as immunosuppressants, they can reduce the functional activity of neutrophils and monocytes, cause lymphopenia and inhibit cellular immunological reactions^{1,2}. This markedly increases organism susceptibility to various bacterial, fungal, viral and parasitic infections³.

The presence of secondary immunodeficiency induced by chronic action of GCs encourages further research to develop effective approaches for optimization of the therapeutic use of steroid hormones. Given the significant disturbance of cellular immunity and phagocytic system in patients receiving long-term GC therapy, the use of naturally occurring immunomodulators may be promising. Recently, considerable efforts have been made for experimental confirmation of the efficacy of vitamin D₃ (cholecalciferol) application to alleviate the side effects of GCs. Vitamin D₃ is a real secosteroid hormone with known effect on calcium homeostasis, but it has been now recognized to be also involved in cell proliferation and differentiation, immunomodulation and anti-inflammatory defense. However, the impact of vitamin D₃ on phagocytic immune response is still a controversial subject because of the ability of this compound to exert immunostimulatory and immunosuppressive effects^{4,5}.

Growing evidence also suggests the pivotal role of oxidative stress in the development of GC-induced side effects⁶. Because vitamin E possesses both antioxidative and anti-inflammatory properties⁷, additional protective effects of α -tocopherol in combination with vitamin D₃ can be anticipated.

This study was designed to assess the effectiveness of vitamin D₃, as well as its combined

action with vitamin E, in the correction of impairments of phagocytic activity and reactive oxygen species (ROS) production in phagocytic cells of rat blood associated with chronic administration of synthetic glucocorticoid prednisolone.

Materials and Methods

Experimental Design

A total of 40 female Wistar rats (100 ± 5 g), were divided into the following groups: (1) the control group; (2) the prednisolone group that received GC at dose 5 mg/kg b.w.; (3) the group treated with vitamin D₃ at dose 100 IU per rat concurrently with prednisolone administration; (4) the group treated with vitamin D₃ and vitamin E (5 mg/kg b.w.) concurrently with prednisolone administration. Prednisolone, vitamin D₃ and α -tocopherol were administered once a day orally for 30 days. All studies have been carried out according to national and international guidelines and laws concerning animal welfare and are ethically acceptable.

Blood Serum 25-Hydroxyvitamin D₃ Level Assay

To assess the vitamin D₃ status in rats the level of 25-hydroxyvitamin D₃ (25OHD₃) was determined in blood serum. The concentration of this metabolite was measured by Enzyme-Linked Immunosorbent Assay (ELISA) using a commercial kit «25-OH-Vitamin D₃» (Immunodiagnostic systems, Frankfurt am Main, Germany).

Phagocytic Activity Determination

Phagocytosis (*Escherichia coli* capture by neutrophils and monocytes) was determined by flow cytometry using a commercial kit PHAGOTEST (Orpegen Pharma, Heidelberg, Germany) and EPICS XLTM flow cytometer (Beckman Coulter, Los Angeles, CA, USA).

The Nitro Blue Tetrazolium Reduction Assay

To determine the functional activity of peripheral blood neutrophils a spontaneous (unstimulated) nitro blue tetrazolium (NBT) reduction test was used according to the method⁸. Heparinized venous blood (0.1 ml) was incubated in plastic tubes with 0.1 ml of 1% NBT suspended in potassium-phosphate buffer (pH 7.3) at 37°C for 20 min and then at room temperature for 10 min.

For hemolysis the samples were mixed with 3 ml of distilled water for 40 seconds. Isotonicity was restored by immediate addition of 1.0 ml of 3.4% sodium chloride and gentle inversion. The samples were then washed twice with ice-cold PBS buffer (pH 7.4) and centrifuged at 1500 rpm for 5 min. The supernatant of each sample was removed and a smear was made of the cells remaining at the bottom of the tube on slide. The slides were air-dried and counterstained with 0.1% w/v aqueous neutral red for 5 min. As a routine 100 neutrophils with intact cytoplasmic membranes were counted under oil immersion using light microscope Axioskop 40 (Carl Zeiss, Oberkochen, Germany). Polymorphonuclear cells were interpreted as NBT-positive if dark blue formazan was present within the cells. The mean cytochemical coefficient was calculated basing on cells with different contents of formazan granules by the following formula:

Mean cytochemical coefficient = $(1A+2B+3C+4D)/100$, where A, B, C, D - number of cells in which the granules of formazan occupy respectively 1/4, 2/4, 3/4, 4/4 of cell volume.

DCFH Oxidation Assay

For analysis of intracellular ROS by flow cytometry, the oxidant-sensitive probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was used⁹. Polymorphonuclear neutrophils were isolated from blood as described previously¹⁰. Neutrophils (10^6 cells/ml) were suspended in PBS, pH 7.4, containing 2% BSA and loaded with DCFH-DA (25 μ M final concentration) in a shaking water bath (15 min, 37°C in the dark). The cells were washed twice with PBS buffer (pH 7.4), and the final pellets of neutrophils were resuspended in 0.5 ml of the same buffer solution. The samples were placed on ice and DCF-fluorescence was analyzed immediately at 525 nm using EPICS XLTM flow cytometer (Beckman Coulter, Brea, CA, USA).

Statistical Analysis

All data are expressed as mean \pm standard deviation (SD). Statistical analysis of data was carried out using the Student's *t*-test. $p < 0.05$ was considered to denote statistical significance.

Results

It was established that blood serum content of 25OHD₃ in rats administered with prednisolone

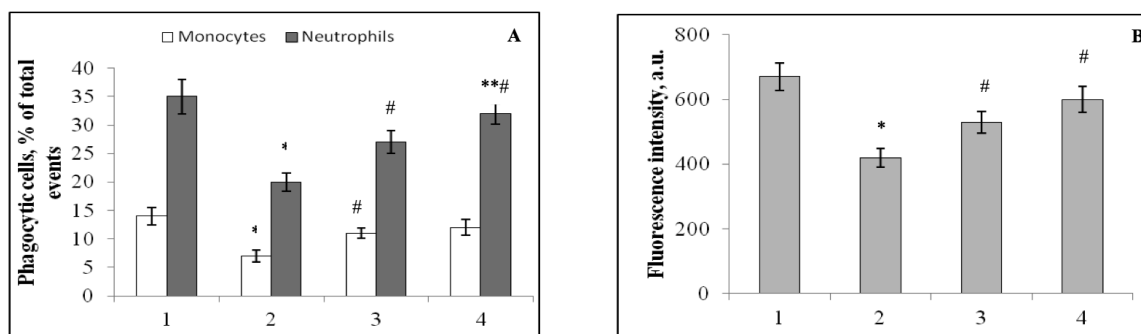


Figure 1. Percentage of phagocytic neutrophils and monocytes (**A**) and activity of phagocytic blood cells (mean fluorescence intensity that correlates with the number of FITC-labeled *E. coli* captured by cells; a.u. – arbitrary units) (**B**): 1 – control rats; 2) animals administered with prednisolone; 3) animals treated with vitamin D₃ concurrently with prednisolone administration; 4) animals treated with vitamin D₃ plus vitamin E concurrently with prednisolone administration. The values represent the mean value \pm SEM of duplicate measurements of 10 rats (* p <0.05 vs. control; # p <0.05 vs. prednisolone administration; ** p <0.05 vs. vitamin D₃ administration).

was significantly reduced to 31.2 ± 0.9 as compared with 93.2 ± 3.67 nmol/L in control, p <0.05. Administration of vitamin D₃ led to partial normalization of 25OHD₃ concentration in blood serum, while they achieved the normal values following combined treatment with vitamin D₃ and E (83.3 ± 2.0 and 95.4 ± 2.0 nmol/L respectively, p <0.05).

Figure 1 depicts the capacity of phagocytic cells (monocytes and neutrophils) to capture bacterial cells, documented by flow cytometry. As is evident from Figure 1 A prednisolone administration induced the lowering of the number of monocytes and neutrophils that capture FITC-labeled *Escherichia coli* from 15 and 33% in controls to 6 and 22% respectively after hormonal load. The estimation of mean fluorescence intensity, which correlates with the number of bacteria captured by phagocytes, has shown significant inhibition of prednisolone-induced phagocytic activity of monocytes and neutrophils (Figure 1 B). The percentage of phagocytic monocytes and neutrophils after administration of vitamin D₃ was increased more than 1.7 and 1.3 fold respectively compared with the effect of prednisolone. In addition, the increase in the mean fluorescence intensity associated with vitamin D₃ treatment also indicates a significant augmentation in the number of bacteria captured by phagocytic cells. The percentage of phagocytic neutrophils and the activity of phagocytic cells increased even more in case of combined action of vitamin D₃ and E.

The functional activity of the cells was next evaluated by reduction of nitroblue tetrazolium to formazan, which is deposited in the form of

dark blue granules in the cytoplasm of neutrophils. It was found that chronic administration of prednisolone was accompanied by decreased values of mean cytochemical coefficient of polymorphonuclear neutrophils in spontaneous NBT test to 1.6 vs. 2.3 in control (Table I). In the group of animals that received GC together with vitamin D₃, partial normalization of NBT test parameters was observed, indicative of the tendency to increase the level of redox potential in peripheral blood neutrophils. It was also found that animals treated with both vitamin D₃ and vitamin E demonstrated higher rates of NBT reduction compared with the group of animals that received vitamin D₃ alone.

The results shown in Table I also indicate that animals administered with prednisolone were characterized by a significant, almost two-fold, decrease in the intensity of ROS formation, which was fully consistent with the changes of NBT test. Vitamin D₃ treatment concurrently with hormone therapy considerably intensified the ROS formation in neutrophils compared with the effect of prednisolone. In rats, that beyond vitamin D₃ additionally received vitamin E, further increase in ROS production was seen.

Discussion

The changes related to prednisolone action indicate hormone-induced suppression of antimicrobial activity of phagocytes as the primary effector responses of nonspecific immune defense. Abnormal phagocytic activity of blood cells demonstrat-

Table I. Metabolic activity and production of oxidants in phagocytic blood cells, M ± m (n=10).

Groups	NBT reduction test (mean cytochemical coefficient)	DCFH oxidation (fluorescence intensity, a.u.)
Control	2.35 ± 0.17	940.0 ± 63.4
Prednisolone administration	1.64 ± 0.07*	720.5 ± 38.0*
Prednisolone + vit. D ₃	1.94 ± 0.14 [#]	854.3 ± 45.1 [#]
Prednisolone + vit. D ₃ + vit. E	2.21 ± 0.12 ^{**#}	903.0 ± 50.3 [#]

p*<0.05 vs. control; [#]*p*<0.05 vs. prednisolone administration; *p*<0.05 vs. vitamin D₃ administration.

ed in the present study can lead to the chronification of inflammatory and infectious processes and to the development of “aggression” against their own body tissues (autoimmunity)^{3,11}. In light of the data obtained in NBT test a significant decrease in the metabolic capacity of resting phagocytic blood cells associated with prednisolone action predictably occurred. Since formazan accumulation reflects the intensity of energy metabolism in cells that provides production of oxidants with bactericidal properties (hydrogen peroxide, superoxide anion radicals, singlet oxygen, hydroxyl radicals), NBT test not only characterizes the activity of their enzymatic systems and metabolic rate in general, but also detects, at least partially, altered phagocytic potential of neutrophils¹².

The true activation of neutrophils is known to be associated with “metabolic and respiratory burst” that greatly increases cellular energy consumption and oxygen demand. Prednisolone, as immunosuppressive drug, significantly decreased the level of reactive oxygen production in rat phagocytic cells^{13,14}. Suppression of spontaneous ROS generation in neutrophils and monocytes most likely reflects the weakening of their immune reactivity that could potentially lead to a decrease in functional activity of phagocytes in response to additional stimulation by bacterial products.

Established lowering of blood serum content of 25OHD₃ in rats administered with prednisolone suggests that chronic GC therapy can cause the development of D₃-hypovitaminosis that may contribute to alterations of nonspecific immune defense. The stimulating effects of vitamin D₃ treatment on the functional activity of phagocytic blood cells revealed in the present investigation is consistent with available scientific data indicating that chronic vitamin D₃ deficiency causes suppression of the immune system and enhances susceptibility to infectious diseases of viral and bacterial origin, including influenza, HIV infection, tuberculosis and pneumonia, as well as several autoim-

mune diseases^{4,15-17}. Furthermore, vitamin D₃ is known to facilitate, through genomic mechanisms, formation of interleukins, antimicrobial peptides and increase phagocytic activity of macrophages, etc., and its deficiency is accompanied by the development of the “incomplete phagocytosis” syndrome¹⁸⁻²⁰. In rats, that beyond vitamin D₃ additionally received vitamin E, further increase in phagocytosis was seen probably due to direct protection of cell structures from damage caused by free radicals and lipid peroxidation. Thus, our data suggest the crucial role of vitamin D₃ and α-tocopherol in stimulating cellular systems and biochemical pathways involved in phagocytic function of the peripheral blood cells.

Conclusions

Vitamin D₃ treatment results in a significant activation of oxygen-dependent bactericidal mechanisms and an enhancement of phagocytic activity of monocytes and granulocytes in peripheral bloodstream. These results may well be considered as a positive immunomodulatory effect of the vitamin D₃, especially in combination with vitamin E, that can warrant their further clinical use for attenuation or prevention of immune disorders associated with the chronic administration of prednisolone.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

- 1) QUAX RA, MANENSCHUJN L, KOPER JW, HAZES JM, LAMBERTS SW, VAN ROSSUM EF, FEELDERS RA. Glucocorticoid sensitivity in health and disease. *Nat Rev Endocrinol* 2013; 9: 670-686.

- 2) LIM HY, MÜLLER N, HEROLD MJ, VAN DEN BRANDT J, REICHARDT HM. Glucocorticoids exert opposing effects on macrophage function dependent on their concentration. *Immunology* 2007; 122: 47-53.
- 3) CUTOLO M, SERIOLO B, PIZZORNI C, SECCHI ME, SOLDANO S, PAOLINO S, MONTAGNA P, SULLI A. Use of glucocorticoids and risk of infections. *Autoimmun Rev* 2008; 8: 153-155.
- 4) DELVIN E, SOUBERBIELE JC, VIARD JP, SALLE B. Role of vitamin D in acquired immune and autoimmune diseases. *Crit Rev Clin Lab Sci* 2014; 51: 232-247.
- 5) TROCHOUTSOU AI, KLOUKINA V, SAMITAS K, XANTHOU G. Vitamin-D in the immune system: genomic and non-genomic actions. *Mini Rev Med Chem* 2015; 15: 953-963.
- 6) SHYMANSKYI IO, KHOMENKO AV, LISAKOVSKA OO, LABUDZYNSKY DO, APUKHOVSKA LI, VELIKY MM. The ROS-generating and antioxidant systems in the liver of rats treated with prednisolone and vitamin D₃. *Ukr Biokhim Zhurn* 2014; 86: 111-125.
- 7) PEH HY, HO WE, CHENG C, CHAN TK, SEOW AC, LIM AY, FONG CW, SENG KY, ONG CN, WONG WS. Vitamin E isoform γ -tocotrienol downregulates house dust mite-induced asthma. *J Immunol* 2015; 195: 437-444.
- 8) GERASIMOV IG, KALUTSKOV OA. Kinetics of nitroblue tetrazolium reduction by human blood neutrophils. *Tsitologiya* 2000; 42: 160-165.
- 9) BASS DA, PARCE JW, DECHATELET LR, SZEJDA P, SEEDS MC, THOMAS M. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J Immunol* 1983; 130: 1910-1917.
- 10) HASLETT C, GUTHRIE LA, KOPANIAK MM, JOHNSTON RB JR, HENSON PM. Modulation of multiple neutrophil functions by preparative methods or trace concentrations of bacterial lipopolysaccharide. *Am J Pathol* 1985; 119: 101-110.
- 11) DHABHAR FS, McEWEN BS. Enhancing versus suppressive effects of stress hormones on skin immune function. *Proc Natl Acad Sci USA* 1999; 96: 1059-1064.
- 12) PARAK RB, WADEE AA. The synergistic effects of gamma interferon and clofazimine on phagocyte function: restoration of inhibition due to a 25 kilodalton fraction from *Mycobacterium tuberculosis*. *Biotherapy* 1991; 3: 265-272.
- 13) DANDONA P, MOHANTY P, HAMOUDA W, ALJADA A, KUMBKARNI Y, GARG R. Effect of dexamethasone on reactive oxygen species generation by leukocytes and plasma interleukin-10 concentrations: a pharmacodynamic study. *Clin Pharmacol Ther* 1999; 66: 58-65.
- 14) CRUZ-TOPETE D, CIDLOWSKI JA. One hormone, two actions: anti- and pro-inflammatory effects of glucocorticoids. *Neuroimmunomodulation* 2015; 22: 20-32.
- 15) MIRAGLIOTTA G, MIRAGLIOTTA L. Vitamin D and infectious diseases. *Endocr Metab Immune Disord Drug Targets* 2014; 14: 267-271.
- 16) KLASSEN KM, FAIRLEY CK, CHEN M, KIMLIN MG, KARAHALIOS A, EBELING PR. Vitamin D deficiency may be associated with a more rapid decline in CD4 cell count to <350 cells/ μ L in untreated HIV-infected adults. *Curr HIV Res* 2015; 13: 517-523.
- 17) TAVAKKOLI A, DIGIACOMO D, GREEN PH, LEBWOHL B. Vitamin D status and concomitant autoimmunity in celiac disease. *J Clin Gastroenterol* 2013; 47: 515-519.
- 18) SOMMER A, FABRI M. Vitamin D regulates cytokine patterns secreted by dendritic cells to promote differentiation of IL-22-producing T cells. *PLoS One* 2015; 10: e0130395.
- 19) GUO C, GOMBART AF. The antibiotic effects of vitamin D. *Endocr Metab Immune Disord Drug Targets* 2014; 14: 255-266.
- 20) DJUKIC M, ONKEN ML, SCHÜTZE S, REDLICH S, GÖTZ A, HANISCH UK, BERTSCH T, RIBES S, HANENBERG A, SCHNEIDER S, BOLLHEIMER C, SIEBER C, NAU R. Vitamin D deficiency reduces the immune response, phagocytosis rate, and intracellular killing rate of microglial cells. *Infect Immun* 2014; 82: 2585-2594.