Vitamin complex (ascorbic acid, alpha-tocopherol and beta-carotene) induces micronucleus formation in PBMNC unrelated to ROS production

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Objectives: To evaluate the correlation between reactive oxygen species (ROS) production and micronucleus formation induced by a vitamin complex in peripheral blood mononuclear cells from healthy people aged between 40 and 85 years old.

Methods: Peripheral blood mononuclear cells (PBMNCs) were purified utilizing ficoll-hypaque gradient. ROS production by PBMNCs was quantified by luminol-dependent chemiluminescence in the presence or in the absence of the vitamin complex. DNA damage in PBMNC by the vitamin complex was detected by the micronucleus technique. Statistical analyses were made with the Student's 't' test and the Pearson correlation. $P < 0.05$ was considered significant.

Results: The vitamin complex induced MN formation in PBMNC but did not augment ROS production. There was no correlation between ROS production and MN formation either in the presence or in the absence of the vitamin complex.

Discussion: There was no increase in the ROS production in the presence of the vitamin complex. The vitamin complex induced an augmentation in the MN formation. There was no correlation between ROS production and the induction of MN formation. Since no association could be detected between ROS production and MN formation, additional studies are required in order to investigate the possible mechanism of vitamin-induced MN formation.

Keywords: Micronucleus, ROS, Vitamins, Peripheral blood mononuclear cells, Antioxidants

Introduction

Aging is associated with a progressive impairment of mitochondrial function, increased oxidative stress, reduced antioxidant defenses, immune activation, and an increase in DNA damage and DNA mutation.¹ Experimental evidence has demonstrated that a low dose supplementation with vitamins and minerals normalizes the biological nutrient status as early as 6 months of treatment. In addition, the antioxidant defense in elderly subjects was improved with low

doses of vitamin C, vitamin E, and beta carotene.² However, a pro-oxidant effect, characterized by the increased incidence of lung cancer, was described in smokers taking high (pharmacological) daily doses of beta-carotene for 6 months.³ Many authors have shown that these vitamins might act as oxidants or pro-oxidants under certain conditions.⁴⁻⁶ It has also been suggested that pro-oxidant compounds increase the production of free radical species, neutralizing antioxidant defenses and causing cell membrane, protein, and DNA damage.⁷ Antioxidants are reactive oxygen species (ROS) scavengers, but the exact role for antioxidants in aging is controversial. Several authors have reported that individual antioxidants act as

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pro-oxidants.^{8–10} In contrast, Niki *et al.*¹¹ have demonstrated that the antioxidants vitamin C, vitamin E, and beta-carotene act not only individually but also cooperatively and in some cases synergistically. Despite the vast literature concerning vitamins, the mechanism in through which these compounds might act as pro-oxidants, inducing DNA damage, remains unknown. ROS and micronuclei are biomarkers for oxidative stress and DNA damage, respectively.^{12,13} The micronucleus (MN) index is a reliable indicator and a well-accepted biomarker for oxidative stress-induced DNA damage, as this marker reflects whole chromosome loss or chromosomal breakage.¹³ Different cytogenetic methods exist to analyze DNA damage, but the MN assay is a simple, cheap, and informative cytogenetic method used to detect genotoxic-related markers.¹⁴ In addition to oxidative stress, MN might also be induced through exposure to toxic agents and genetic defects in the cell cycle.¹⁵ Thus, it is possible to envisage an association between DNA damage and increased ROS generation. The aim of the present study was to evaluate the potential association between the ROS generation from the peripheral blood mononuclear cells (PBMNCs) of aged healthy people and MN formation during 'in vitro' exposure to an antioxidant vitamin complex comprising beta-carotene, alpha-tocopherol, and ascorbic acid.

Methods and materials

Subjects

The Ethical Committee of the Federal University of Minas Gerais approved this study. A detailed medical history, complete physical examination, and laboratory data for each subject were recorded before entering the study. Appropriate informed consent was obtained from each participant. The individuals excluded from this study had one or more of the following features: infections, inflammation, malignancy, lymphoproliferative disorders, arteriosclerosis, cardiac insufficiency, hypertension, and/or dementia. In addition, individuals who were smokers, pregnant, had a history of alcoholism and drug abuse, and/or were taking drugs that directly influence the immune function were excluded. Dr Edgar Nunes de Moraes, Dr Rodrigo Santos, and Dr Marco Túlio Gualberto Cintra selected the subjects (Reference Center of Age – Professor Caio Benjamin Dias from HC-UFG). The subjects live in Belo Horizonte. The characteristics of the population are shown in Table 1 ($n = 40$).

Separation of PBMNCs

The PBMNCs were purified from 10 ml of heparinized venous blood samples obtained from the participants using a Ficoll-Hypaque gradient according to the method of Bicalho *et al.*,¹⁶ with slight modifications.

Table 1 Characteristics of the studied group

Parameters	Values (mean $\pm$ SD)
Age groups (years)	61.9 $\pm$ 13.4
Body mass index (kg/m ²)	27.3 $\pm$ 3
Glycohemoglobin (GHb%)	5.5 $\pm$ 0.3
Triglycerides (mg/dl)	143.8 $\pm$ 35
Serum creatinine (mg/dl)	0.903 $\pm$ 0.162

Number of participants (n) = 40.

The cellular viability of each sample was consistently greater than 95%, as determined using the trypan blue exclusion test.

Preparation of the vitamin complex

The vitamin complex was obtained in capsules from a compounding pharmacy and dissolved in 10 ml of dimethyl sulfoxide (DMSO – Vetec) and subsequently diluted 10 times in phosphate-buffered saline (PBS). The DMSO concentration was limited to 0.5% in accordance with the stipulations of the International Organization for Standardization.¹⁷ In all experiments, the final concentration of DMSO was never above 0.001%, and no cell toxicity or cell death was observed; thus, the addition of DMSO at this concentration did not interfere with the results. All reagents were purchased from Sigma (Labscience Instrumentos Científicos Ltda, São Paulo, SP, Brazil, USA).

Determination of ROS generation

ROS generation was determined through a quantitative chemiluminescence assay using a luminometer 1250-101 (Lumat, LB9501; EG&G Berthold, Germany). The PBMNCs (1×10^6 cells/100 μ l) were transferred to an unsealed luminescence tube together with 500 μ l of luminol (10^{-4} M) and 100 μ l of the vitamin complex. In the basal production, 100 μ l of PBS (pH = 7.3) was used instead of the vitamin complex. The chemiluminescence of the mixture was measured over a 30-minute reaction time. The results were expressed in RLU/minute (relative light units/minute).

MN technique

DNA damage was detected using the MN technique according to Fenech,¹⁸ with slight modifications. Briefly, the PBMNCs (1×10^6 cells/100 μ l) were incubated in a 24-well plate in the presence or absence of the vitamin complex, and the total volume was adjusted to 3 ml with RPMI 1640 (Sigma-Aldrich Co., Labscience Instrumentos Científicos Ltda, São Paulo, SP, Brazil) and supplemented with 15% inactivated fetal bovine serum (Gibco), antibiotic–antimycotic solution (penicillin, streptomycin, gentamicin, and amphotericin (Gibco)), and phytohemagglutinin (PHA) (20 μ g/ml) (Sigma Aldrich Co.) to stimulate cell proliferation. Treatment with the test compound

(vitamin complex) was initiated at 48 hours after PHA stimulation, lasting 20 hours, followed by the addition of cytochalasin B (6 µg/ml) (Sigma-Aldrich Co.). Subsequently, the cells were harvested at 96 hours after the initiation of the culture. The cells were subjected to a mild hypotonic treatment (KCl 0.075 M), fixed once with methanol:acetic acid (5:1), and twice with the same solution at 3:1. The cells were subsequently smeared on precleaned microscope slides and air-dried. The cells were stained with Giemsa (Dinâmica) to determine the micronuclei frequency. Negative controls were performed using DMSO (Vetec, Biosan, Belo Horizonte, MG, Brazil) (15 µl). Positive controls were performed using colchicine (12 µl–0.025 µg). The results are expressed as the number of micronuclei in 1000 binucleated cells.

Statistical analyses

The ROS data are expressed in terms of the mean values of RLU/minute $\pm$ standard error (SE). The micronuclei results are expressed as the number of micronuclei in 1000 binucleated cells. The comparison of the data between the groups was performed with Origin 6.0 software (Microcal Software Inc., Northampton, MA, USA), using the unpaired Student's *t*-test and Pearson's correlation in the analyses. In each case, $P < 0.05$ was considered significant.

Results

Determination of ROS production

The ROS production is shown in Fig. 1. The vitamin complex did not alter the basal ROS production ($P > 0.05$). The results showed that the basal (PBMNC + PBS) ROS production was 2712 ± 401 RLU/minute, and in the presence of the vitamin complex, the ROS production was 2358 ± 359 RLU/minute.

Figure 1 Reactive oxygen species (ROS) production by PBMNC stimulated by the vitamin complex. PBMNC = peripheral blood mononuclear cells; Vitamin complex = beta carotene, ascorbic acid, and alpha tocopherol; PBS = phosphate buffer saline; ns = not significantly different compared to the basal (PBMNC + PBS).

Micronucleus formation

Fig. 2 shows micronuclei formation in the presence and absence of the vitamin complex. The number of MN was significantly higher ($P < 0.05$) in the presence of the vitamin complex compared with the negative control. The following results, expressed as the number of MN in 1000 binucleated cells $\pm$ SE, were obtained: negative control (PBMNC + DMSO) = 12.95 ± 0.76 ; PBMNC + vitamin complex = 18.32 ± 0.96 ; positive control (PBMNC + colchicine) = 26.2 ± 1.06 .

Correlation between ROS production and MN formation

Fig. 3A and B shows that there was no correlation between ROS production and MN formation either in the presence or in the absence of the vitamin complex.

Discussion

Vitamin complex-induced DNA damage was evaluated through MN formation in the PBMNCs from healthy people between 40 and 85 years old (Fig. 2), and no correlation between ROS production and MN formation was observed (Fig. 3). Previous studies¹⁹ have demonstrated that people aged 40 years old or older exhibit an activated oxidizing profile without a concomitant and correspondent increase in the reducing metabolic response, characterizing oxidative stress.

The vitamin complex used in the present study comprised ascorbic acid, alpha-tocopherol, and beta-carotene in previously suggested concentrations and proportions (ascorbic acid = x , alpha-tocopherol = $x/2$, beta-carotene = $x/100$).²⁰ The vitamin complex concentration used in the present study is comparable to that of Bergman *et al.*²¹ and Prabhala *et al.*²² Bergman *et al.*²¹ used 0.011–1.1 µM of ascorbic acid and 0.002–0.2 µM of alpha-tocopherol in 2×10^6 PBMNCs, while Prabhala *et al.*²² used 0.001–10 µM

Figure 2 Micronuclei (MN) formation by PBMNC stimulated by the vitamin complex. PBMNC = peripheral blood mononuclear cells; Vitamin complex = beta carotene, ascorbic acid, and alpha tocopherol; PBS = phosphate buffer saline; DMSO = dimethyl sulfoxide; colchicine = used as positive control; * = significantly different compared to PBMNC + DMSO (negative control).

Figure 3 Correlation between ROS production and MN formation. (A) Correlation between nasal ROS production and MN negative control. (B) Correlation between ROS production and MN formation, both stimulated by the vitamin complex. ROS = reactive oxygen species (in relative light units/min), MN = micronucleus formation (in 1000 binucleated cells), VIT = presence of the vitamin complex.

of beta-carotene in 1.6×10^6 PBMNCs. In this study, we used a similar vitamin complex concentration comprising 0.08 μM ascorbic acid, 0.04 μM alpha-tocopherol, and 0.0008 μM beta-carotene. Niki *et al.*¹¹ demonstrated that antioxidants vitamin C, vitamin E, and beta-carotene act not only individually but also cooperatively and in some cases synergistically. The advantage of using a mixture of several dietary antioxidants is reflected by the fact that some of these compounds utilize synergism and partnership to achieve protection from physical and chemical genotoxins.^{23,24} Based on these assumptions, we have examined the role of a vitamin complex.

Antioxidant vitamins A, C, and E protect against many diseases and conditions associated with aging.²⁵ Thus, we examined the association among ascorbic acid, alpha-tocopherol, and beta-carotene as potential oxidants or pro-oxidants on PBMNCs obtained from an aged population.

The results demonstrated that this vitamin complex did not significantly alter ROS production in PBMNCs ($P > 0.05$) (Fig. 1). These results are consistent with those of Fusco *et al.*,²⁶ who demonstrated that only specific populations with low antioxidant profiles and/or high oxidative stress levels might benefit from antioxidant supplementation. Notably, under conditions in which high levels of ROS (oxidative stress) are detected, cellular and genome damage have been reported.²⁷ Thus, it is reasonable to suggest that an antioxidant vitamin complex would prevent MN formation in PBMNCs.

MN frequency was evaluated in the presence of the vitamin complex, and the results in Fig. 2 show that the vitamin complex activated MN formation ($P < 0.05$), suggesting a pro-oxidant effect. The literature concerning this subject is controversial. Previous studies using single antioxidants (vitamin C alone²⁸ or vitamin E alone²⁹) showed no significant increase in the micronuclei frequency. In contrast, Anderson *et al.* (1994)³⁰

reported that vitamin C causes a dose-dependent increase in DNA damage. A variety of 'in vivo' studies have shown that multiple antioxidants might act together to reduce micronuclei frequency.^{31–33} However, these studies are not directly comparable to the present study because we assayed an antioxidant mixture 'in vitro'. The 'in vivo' assays used a different antioxidant mixture comprising succinate and zinc,³¹ folic acid and rutin,³⁴ and selenium,³³ ascorbic acid, beta-carotene, and alpha-tocopherol. It has been suggested that under certain conditions, vitamins A, C, and E might have pro-oxidant effects.^{20,35,36} Vitamin C induces an increase in the formation of oxidative DNA damage in cultured lymphocytes.^{8,10,30} This 'in vitro' effect, mediated through vitamin C, corresponds to the same effect as observed with the concentrations used 'in vivo' via supplementation.^{30,37–39} A similar effect was observed with high doses of E vitamin 'in vitro'.⁴⁰ This effect reflects vitamin-induced oxidative damage and many authors have shown that ROS induces DNA damage.^{41,42} Weitberg and Weitzman (1985)³⁹ observed variable effects in the induction of sister chromatid exchanges in Chinese hamster ovary cells incubated with an enzymatic oxygen radical generating system (xanthine oxidase plus hypoxanthine) and ascorbate, as a protective effect, is observed in some cases, whereas in other cases, the induction of sister chromatid exchanges was observed.

The results (Fig. 3) obtained in the present study showed no significant correlation between ROS production and the induction of MN formation. It is reasonable to suggest that MN formation is a complex phenomenon with intricate mechanisms. In addition to increased ROS production, there are other potential agents and mechanisms involved in MN formation. The results obtained in the present study clearly demonstrated the absence of a direct or indirect association between ROS production and MN formation. Thus, the exact role of vitamins on

MN formation needs further study to elucidate the mechanisms underlying this function.

Conclusion

Vitamin complexes induce micronuclei formation in healthy people between the ages of 40 and 85 years old in a ROS-independent manner.

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