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Additive Action of Vitamins C and E against Hydrocortisone-Induced Genotoxicity in Human Lymphocyte Chromosomes

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Abstract: In earlier reports, hydrocortisone administration to human lymphocytes in culture was shown to cause chromosomal aberrations and increased sister chromatid exchanges. With a view to study the ameliorative action of some antioxidants against this effect, vitamins C and E were used separately and in combination along with hydrocortisone treatment, at different dosage and for different durations, on human lymphocyte cultures. The levels of chromosomal aberrations and sister chromatid exchanges were lowered, suggesting a protective role of vitamins against genotoxic damage. Administration of vitamins C and E combined appeared to be more effective in preventing chromosomal damage than separate administration, demonstrating the additive action of these vitamins against steroid-induced genotoxicity.

Key words: Hydrocortisone, vitamin C, vitamin E, chromosomal aberrations, sister chromatid exchange

Introduction

Steroids are active biological agents that function as natural hormones, chemical ligands, and precursors for endogenous synthesis of other chemicals. Steroidal hormones and their synthetic derivatives perform their functions by forming steroid receptor complexes that bind with DNA and influence gene expression [1, 2]. In spite of their therapeutic use, there are many reports indicating their mutagenic, carcinogenic, clastogenic, and teratogenic effect [3]. Hydrocortisone is a potent steroidal drug used therapeutically for a number of reasons. It is a potent glucocorticoid used primarily for ameliorating severe inflammation, allergic reaction in shock syndromes, neoplasia, and adrenal insufficiency [4]. Hydrocortisone induces chromosomal aberrations and sister chromatid exchanges in human lymphocyte chromosomes, with and without metabolic activation [5, 6]. Vitamins act as antioxidants and free radical scavengers, thereby acting as anticarcinogenic, anticlastogenic, and antimutagenic agents. Of these, vitamins C and E are among the best known antioxidants used in *in vivo* animal models [7]. In this paper, we present the effects of vitamins C and E on human lymphocyte chromosomes treated with hydrocortisone.

Materials and Methods

The details of the experimental design are as follows

Treatments	Dosage
Controls	
1. Normal blood	Nil
2. Negative control (with DMSO)	5 μg/mL
3. Positive control (Cyclophosphamide)	1×10^{-7} M
Vitamins used	
4. Ascorbic Acid	20 μg/mL, 30 μg/mL
5. α-Tocopherol	15 μg/mL, 20 μg/mL
Hydrocortisone + Vitamins	
6. Hydrocortisone+Ascorbic Acid	25 μg/mL + 20 μg/mL
	50 μg/mL + 30 μg/mL
7. Hydrocortisone+α-tocopherol	25 μg/mL + 15 μg/mL
	50 μg/mL + 20 μg/mL
Hydrocortisone + Combined Vitamins	
8. Hydrocortisone+Ascorbic Acid + α-tocopherol	
	25 μg/mL + 20 μg/mL + 15 μg/mL
	50 μg/mL + 30 μg/mL + 20 μg/mL

Chromosomal aberration analysis

Steroid treatment effects on metaphase

Lymphocyte cultures were carried out in TC-199 medium (Flow Laboratories) supplemented with 15% fetal calf serum (Gibco), antibiotics (penicillin and streptomycin; 100 IU/mL each, Hoechst) and L-Glutamine (1 mM, Gibco). Around 0.5 mL of heparinized whole blood taken from two healthy adult male donors (who were known to be occupationally unexposed) was added to 4 mL each of the medium. The lymphocytes were stimulated to divide by adding 0.1 mL of phytohemagglutinin – M (PHA M, Microlab) and incubated at 37°C with 5% CO_2 for 72 hours, in the dark. Hydrocortisone at a final concentration of 25 and 50 μg/mL, respectively, was added and kept for 24, 48, and 72 hours of duration each by adding the hydrocortisone after 24 and 48 hours after initiation of cultures. Dimethylsulfoxide (DMSO; 5 μg/mL, SRL, Bombay, India) was added to the simultaneously kept cultures for 72 hours of incubation as negative control. The positive control cyclophosphamide (CP; 1×10^{-7} M) was added and kept for 24, 48 and 72 hours of incubation.

Vitamin treatment

Synthetic sodium salt of L-ascorbic acid manufactured by Roche India Ltd., with the trade name Redoxon, was used at a concentration of 20 μg/mL and 30 μg/mL per culture; the vitamin was used alone, along with steroid, and also concurrently with Vitamin E.

The source of vitamin E was Ephynal, manufactured by Roche Chemicals and Pharmaceuticals, procured from the local market. The concentrations were 15 μg/mL and 20 μg/mL. The reason for choosing this combination of vitamins is that they are generally recommended by physicians in the same qualitative and quantitative combination.

Metabolic activation

The same experiment was repeated under metabolic activation. In this series, six- to 30-hour-old cultures were treated with 25 and 50 μg/mL concentrations of hydrocortisone in the presence of rat liver S_9 microsomal fraction. Liver S_9 fraction was prepared from healthy albino rats induced with phenobarbital, and stored in liquid nitrogen until use. S_9 was freshly prepared for use. The cells were collected after centrifugation and the pellets washed twice in the prewarmed medium to remove the chemical and the S_9 mix. The parallel cultures receiving the same dose of the drug for similar duration but without S_9 mix were also set simultaneously for comparison.

Colchicine treatment (0.20 μg/mL, Microlab) was performed prior to harvesting. The cells were recollected by centrifugation and fixed in methanol and acetic acid (3:1) for preparation of slides. Staining and scanning was done under code. A total of 150 well-spread metaphase plates were analyzed at treatment duration for all types of chromatid and chromosome type of aberrations. For metabolic activation, only 100 well-spread metaphase plates were analyzed.

Sister Chromatid Exchange Analysis

Analysis of sister chromatid exchanges (SCEs) was carried out following fluorescent plus Giemsa techniques [8]. Cells were exposed to 5-Bromo-2 deoxyuridine (BrdU, 2 μg/mL Sigma) 24 hours after culture initiation. The test compounds were added together with BrdU. To minimize photolysis of BrdU, another set of cultures was maintained in the dark for 48 hours.

For SCE analysis in the presence of metabolic activation, hydrocortisone plus the S_9 mix was added into the culture 48 hours after initiation and reincubated at 37°C. After 90 minutes of treatment, the cells were spun down and the supernatant discarded. The cells were washed twice to remove traces of excess steroid and the liver metabolites. Finally the cells were resuspended in fresh medium supplemented with fetal calf serum, PHA-M, and BrdU, and cultured for another 24 hours in the dark at 37°C. Parallel cultures were also set simultaneously without adding S_9 mix. Hoechst stain (0.5 μg/mL, Sigma) was

used along with 10% Giemsa stain for differential staining of sister chromatids. The slides were coded prior to scoring and 50 well-spread metaphase cells were scanned per concentration and the number of exchanges scored. Hydrocortisone, vitamins plus hydrocortisone, and vitamin treatments separately were given as usual.

Results

The chromosomal aberrations calculated per cell show higher values in the steroid-treated cultures (25 and 50 μg/mL) at 24, 48, and 72 hours: (0.18, 0.19, 0.49) and (0.33, 0.05, 0.04) respectively. The values for normal controls and negative controls (DMSO) were lower: 0.02 and 0.02 aberrations (for 72-hour cultures only). The cyclophosphamide (CP) treated group (positive control) showed values of 0.30, 0.41, and 0.68, respectively, for the 24-, 48-, and 72-hour cultures. The differences between these values are statistically significant, showing that the steroid increased chromosomal aberrations with increasing dose concentration and duration of treatment.

Vitamin C and E treatments, when administered separately, were associated with values of 0.02 and 0.01 at 20 μg/mL and 30 μg/mL dosages, respectively for vitamin E, it comes to 0.02 and 0.01 at 15 μg/mL and 20 μg/mL respectively for 72 hours culture, there being almost similar effect. However, in the case of vitamins and steroids administered together, the chromosomal aberration scores were lower than those for steroids used alone. For Vitamin C and hydrocortisone, these values were 0.12, 0.13, and 0.34 for different durations at the first concentration and 0.23, 0.03, and 0.03 at the second concentration. The values for vitamin E and hydrocortisone were slightly higher: 0.14, 0.14, 0.36 at the first concentration and 0.25, 0.03 and 0.03 at the second concentration. The additive effect of vitamins C and E plus hydrocortisone resulted in values of 0.11, 0.11, 0.30 at the first concentration and 0.22, 0.30 and 0.02 at the second concentration. When metabolic activation systems were used, we found the higher level of aberrations per cell in the presence of the S_9 mix than cells cultured without the S_9 mix, thus showing that vitamins decrease the genotoxic effects of hydrocortisone even in the presence of metabolic activation (Fig. 1 and 2).

Sister Chromatid Exchange

The number of SCEs in human lymphocytes following hydrocortisone treatment were 3.70 and 2.50 at two different concentrations, higher than the normal and DMSO control values of 1.70 and 1.80. For CP the exchanges were 4.50 per cell. Vitamins reduced the SCE levels to 1.60 and 1.54 (vitamin C) and 1.66 and 1.60 (vitamin E). The cells

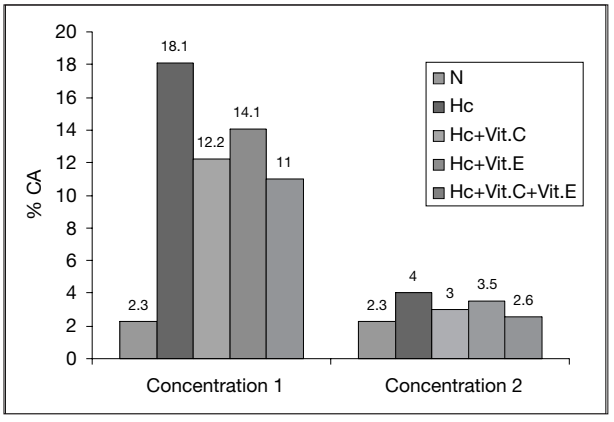

Figure 1: Additive effects of vitamins on chromosomal aberration without S_9 mix.

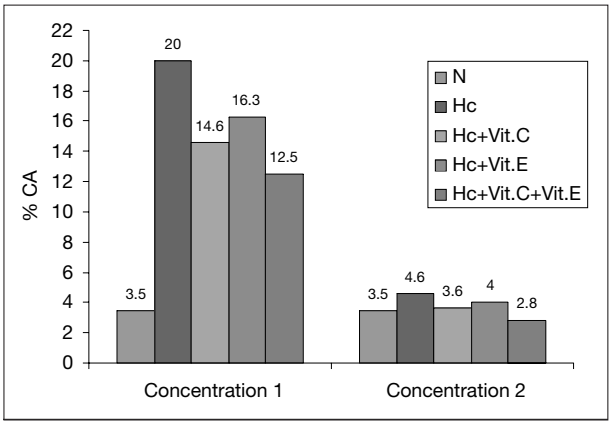

Figure 2: Additive effects of vitamins on chromosomal aberration with S_9 mix.

N	=	Normal
Hc	=	Hydrocortisone
Hc+Vit.C	=	Hydrocortisone+Vitamin C
Hc+Vit.E	=	Hydrocortisone+Vitamin E
Hc+Vit.C+Vit.E	=	Hydrocortisone+Vitamin C+Vitamin E

treated with combined vitamins C and E, plus hydrocortisone at two different concentrations, showed SCE values of 2.30 and 1.70 respectively. With the S_9 mix treatment, the trend is similar: the lowest value of SCE was 1.50 for vitamin C alone and the highest value for CP was 6.50. There is thus a distinct effect of vitamins of C and E separately and additively in bringing down the SCE level, the effect being statistically significant (Fig. 3 and 4).

Discussion

The field of antimutagenesis is as old as Norvick and Szilard [9] who developed this idea in their seminal contri-

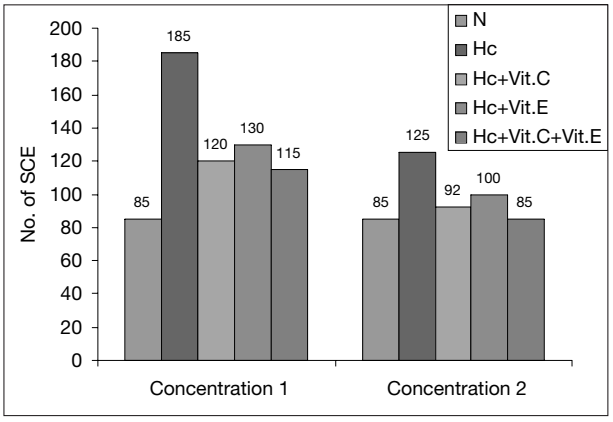

Figure 3: Additive effects of vitamins on sister chromatid exchange without metabolic activation.

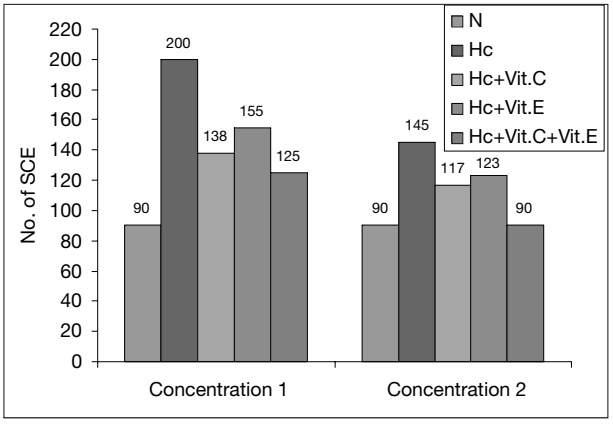

Figure 4: Additive effects of vitamins on sister chromatid exchange with metabolic activation.

N	=	Normal
Hc	=	Hydrocortisone
Hc + Vit.C	=	Hydrocortisone + Vitamin C
Hc + Vit.E	=	Hydrocortisone + Vitamin E
Hc + Vit.C + Vit. E	=	Hydrocortisone + Vitamin C + Vitamin E

bution to antigenotoxicity assays. Subsequently, mechanisms were suggested by a number of researchers [10, 11]. Among antimutagens, vitamins occupy a principal place for their anticarcinogenic, anticlastogenic, and antimutagenic action, e.g. vitamin A [12, 13], vitamin B complex [14], riboflavin [15], thiamine [16] and most conspicuously, vitamin C [13, 17] and vitamin E [18]. The effects of vitamins C and E in human lymphocyte chromosomes has been reported by Gebhart *et al* [19]. Studying the chromosome aberrations in the blood cells of coal tar workers, Sram *et al* [20] and Krishna *et al* [21] reported inhibition of sister chromatid exchanges in mice by cyclophosphamide and mitomycin treatment. Most of these studies show protective action of vitamin C and E against genotoxicity [22].

Few studies, however, report toxic effects of vitamins of C and E at high dosages [23]. Alberts *et al* [24] reported potentiation of doxorubicin (DXR)-induced toxicity due to vitamin E. The conflicting reports in fact are related to the mechanism of action and threshold values of both aberrations and concentrations of protective substances [25]. Most of these vitamins act as free radical scavengers and antioxidants [26, 27]. Wayner *et al* [28] have shown the antioxidation of vitamin C itself, apart from its radical trapping antioxidant property. Vitamin C increases production of hydroxyl radicals for formation of hydrogen peroxide via the Fenton reaction [23]. Our study uses only milder doses of vitamin C, eliciting its anticlastogenic effect in lymphocytes. The synergistic action of vitamins C and E has also been reported by Niki *et al* [29] and by Niki [30]. Lack of synergistic effect, however, has been reported by Renner [31] in combating genotoxicity.

It has been suggested that ascorbic acid reduces tocopheroxyl radicals *in vitro* and thereby regenerates vitamin E. The tocopheroxyl radicals are produced by scavenging of free radicals by vitamin E during normal metabolism. This mechanism of tocopherol regeneration also links soluble ascorbic acid to the protection of membranes against free radical damage. A synergistic relationship between ascorbic acid and vitamin E *in vitro* has been reported by Niki *et al* [32], and Packer *et al* [33]. Mulholland and Strain [34] have found little effect of large doses of alpha-tocopherol and ascorbic acid supplement action in young healthy volunteers using total radical trapping antioxidant potential (TRAP) levels in plasma. The lack of response, they argued, might in fact be due to high levels of dietary antioxidants already in the diet. Our study shows an additive effect, rather than synergistic effect, which has been also noted by Loebitz *et al* [35]. Burton *et al* [36] have also found a negligible effect studied *in vivo*, in the case of nonoxidatively stressed guinea pigs. The synergistic action of tocopherol and ascorbic acid however resulted in an increase in total radical trapping antioxidant potential (TRAP) levels. Vitamin C is known to stimulate 7-hydroxylation of lipids and cholesterol rings, thus enhancing their biodegradation [37]. Most of these studies were performed *in vivo* in animal models. Our study emphasizes the additive action of these vitamins in human lymphocytes, with chromosomal aberrations and SCE as genetic end points, and hence it may be suggested that these vitamins be used as antidotes to steroid-induced genotoxicity during hydrocortisone therapy.

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