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Effect of Vitamin C and Vitamin E Analog on Aged Fibroblasts

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Human dermal fibroblasts were cultured and aged *in vitro*. Survival of young and aged fibroblasts was determined in the presence and absence of different concentrations of two vitamins. Vit C at doses of 5, 12.5, 25 and 50 µmol/L and water-soluble Vit E (Trolox) at 1, 5, 10 and 50 mg/L, were added 30 minutes before oxidative stress, consisting of exposure to 5 mM hydrogen peroxide for 30 minutes. A non-radioactive cell proliferation cytotoxicity assay (MTT) was used to determine the protective effect of the vitamins studied. Vit C produced a clear cytoprotective effect on aged cells over the entire range of doses applied. The protection provided by Vit E, was less pronounced.

Key words: Ascorbic acid, Hydrogen peroxide, Fibroblasts, α-Tocopherol, Antioxidants.

It has been well-established that under normal conditions aerobic cell metabolism generates reactive oxygen species. For experimental purposes, cell models may be set up *in vitro*, then injured or killed, and depending on the system used different free radicals are produced. Contact with oxidants and, specifically, exposure to hydrogen peroxide (H_2O_2), induces changes which include among others, a decrease in intracellular ATP, DNA damage, alterations of the cytoskeleton, glycolysis, oxidation of proteins and peroxidation of unsaturated fatty acids (20). The intrinsic mechanisms by which oxygen radicals kill cells is not completely understood and the precise identity of the metabolic pathways involved in cell death remain controversial (10).

Oxidative stress seems to play a role in the process of aging and in various diseases. Specific therapies are being developed to provide effective nutritional and pharmaco-

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logical protection against metabolic disturbances in cells caused by free radicals and oxidative stress (21). Experimental and clinical trials designed to gain insight into the effectiveness of antioxidant substances in different cell types are providing information along this line.

Ascorbic acid (Vit C) and α-tocopherol (Vit E) are two natural antioxidants that have been observed to restrict the chain of reactions induced by free radicals acting synergistically in different cell type cultures (6, 18). Among its many biological functions, Vit C is a reducing and chelating agent and is one of the most effective aqueous-phase soluble antioxidants present in cytosol and plasma (7). Vit C scavenges free radicals and regenerates α-tocopherol but may be toxic or even lethal to several types of malignant tumor cells depending on the dosage used (11). Vit E, a lipid-soluble compound, is highly reactive with organic peroxyl radicals and protects mainly membrane lipids.

Cultured human dermal fibroblasts may be propagated and aged, and provide easily obtainable human diploid nucleated cells as a working model. In the present study cultured human fibroblasts were exposed to the oxidative stress of hydrogen peroxide in the presence of different concentrations of Vit C and Vit E analog. The survival rate of both young and aged fibroblasts was determined to evaluate the antioxidant efficacy of the two vitamins studied.

Materials and Methods

Cell culture.– Human dermal fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) and 20 % Ham's F-12 medium to which 10 % foetal bovine serum, penicillin (100 U/L) and streptomycin (100 U/L) were added, at 37 °C in a 95 % O_2 and 5 % CO_2 atmosphere, and passaged to confluence by trypsinization. Fibroblasts were aged *in vitro* by subculturing the cells to a population doubling level (PDL) > 56 (2). All experiments were done using the same cell line, with cells from passages 5 to 20.

As a preliminary step, to determine cell survival/dose concentration, it was necessary to study the half lethal dose (LD50) of H_2O_2 in the situation under examination. Cell suspension was diluted to different densities, placed in plate wells and incubated for 15, 30, 45 and 60 min with H_2O_2 at 1, 2, 5 and 10 mM concentrations. Each point was the average of results from 9 assays.

Vitamins and hydrogen peroxide solutions were prepared immediately before use. On the day of the experiment the cells were harvested by tripsinization and 1.0×10^4 cells were placed in each well of the 96-well plate for determination. After 24 h of incubation, Vit C at 5, 12.5, 25 and 50 µmol/L (normal plasma level doses) or 100, 250 and 500 µmol/L (high doses), or water soluble Vit E (Trolox) at doses of 1, 5, 10 and 50 mg/L, were added and the plates were incubated for 30 minutes more. To induce oxidative stress, 5 mM H_2O_2 was added to the wells; plates were incubated for another 30 minutes and immediately after, cytotoxity tests were performed.

Cytotoxicity assay.– A non-radioactive cell proliferation cytotoxicity assay (CellTiter^{96}, Promega) was used to determine the protective effect of the two vitamins studied. The assay is based on the cellular conversion of yellow 3-(4,5-dimethylthiazol-2)-2,5-diphenyl tetrazolium bromide (MTT) to a blue formazan product by viable mitochondrial dehydrogenases, easily detected using an ELISA plate reader at a wavelength of 570 nm (14). The absorbance of blue color developed is directly proportional to the

number of living cells. Each point was the mean of six determinations.

Statistical analyses were carried out using the Mann-Whitney U test. P values of less than 0.05 were taken as significant.

Results

Under the culturing conditions used, control cell viability was 98 % (Trypan blue exclusion dye method). The MTT microassay results are expressed in relation to controls present in each plate.

The effect of cell density (0-2 x 10^4) on absorbance at 570 nm was studied to standardize the method. A linear relationship ($r = 0.98$) was observed between number of cells and absorbance of formazan generated by viable mitochondria. The optical density obtained (0.81 ± 0.08) at approximately 1×10^4 cells per well was considered adequate for our purposes.

Fig 1 depicts results obtained with the study of H_2O_2 half-lethal dose and exposure time. Five mM H_2O_2 and thirty minutes exposure were chosen as oxidative stress for the vitamin testing, being the effective dose which resulted in 50 % cytotoxicity.

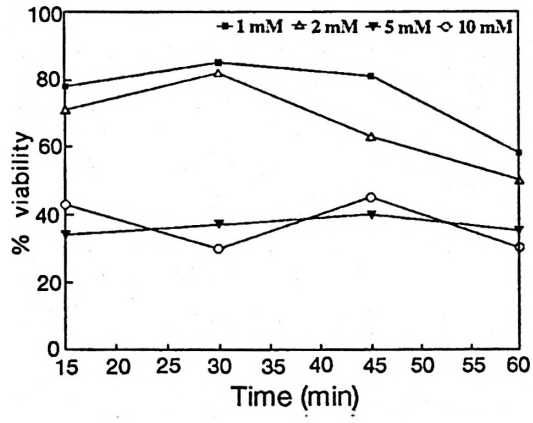

Fig 1. *Effect of different H_2O_2 concentrations on cell viability at 15, 30, 45 and 60 minutes.*
Five mM H_2O_2 and 30 min were chosen as oxidative stress.

Vit E added 30 minutes before oxidative stress to young fibroblasts showed no protective effect (data not shown). When tested under the same conditions on aged fibroblasts, however, the doses lower than 50 mg/L produced increases in cell viability as compared to the unprotected stressed cells (fig. 2, black bar), while the dose of 50 mg/L produced a statistically

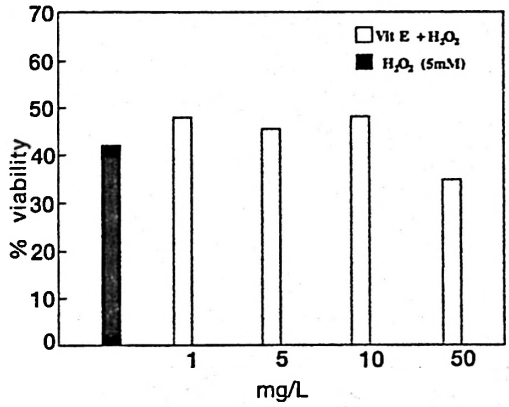

Fig 2. *Effect of Vitamin E on aged fibroblasts (PD L= 62).*

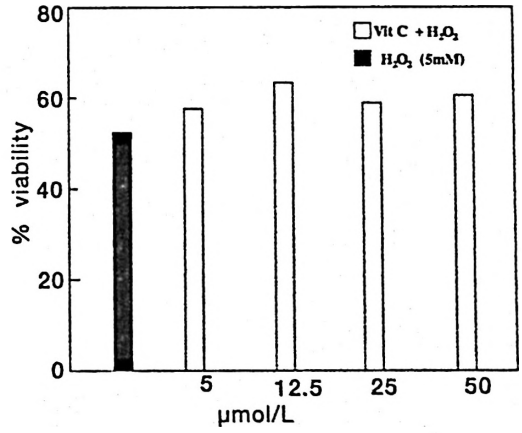

Fig 3. *Effect of Vitamin C on aged fibroblasts (PDL = 87).*
Cytoprotection at all doses studies.

significant decrease in cell viability ($p < 0.05$). Similar results were obtained with Vit C supplementation: a clear cytoprotective effect was observed with all the normal doses studied (fig. 3). Cultured cells receiving high doses of Vit C produced optical density results beyond the range of the assay and were excluded from consideration. The beneficial doses of Vit C and Vit E were within the reported normal plasma levels (19).

Discussion

Vitamins can enhance the protection of cultured cells against aggression, such as oxidative stress or exposure to chemicals, but resistance may vary according to the "age" of the cell. It has been demonstrated in culture systems that hydrogen peroxide, a small, uncharged and relatively stable molecule, can cross cell membranes easily and cause cellular injury. This toxic action has been well documented (12). The cytotoxic effect is strongly dependent on the density of the cells and the composition of the medium in which the cells are treated.

Hydrogen peroxide is the main cytotoxic product formed in chronic oxidative injury by xantine/xantine oxidase systems and may be used in *in vitro* acute stress experiments. The cytotoxity of H_2O_2 over low density cultured cells was observed in this study, underlining the importance of determining adequate cell concentrations for *in vitro* systems. Our results concerning the appropriate level of H_2O_2 in relation to cell density agree with data previously published (9, 17).

The mechanism(s) involved in oxidative cell damage are still unclear, although a number of changes in cytoplasm and nucleus have been documented (8). When endogenous protective mechanisms are exhausted, H_2O_2 may enter the cell and damage internal components. Alternatively, the relatively vulnerable external cell membrane may receive a fatal lesion while the internal cellular antioxidant systems remain intact due to catalase and peroxidase actions.

Lipid-soluble vitamins, and specific water-soluble micronutrients are the principal nonenzymatic antioxidants in plasma (5, 22). Vit C interacts with H_2O_2 and other forms of reactive oxygen and its protective role has been well-established (8). The low affinity ascorbic acid transport system in human fibroblasts may increase intracellular Vit C levels in stress conditions. Cytoprotection has been demonstrated with doses of vitamin C below 50 μM, while toxicity appears with higher concentrations (4). Our findings demonstrate cytoprotection to 5 mM H_2O_2 stress at all normal doses studied suggesting the benefits of a preventive vitamin intake. However, the optimum dose has still to be defined: low doses of ascorbic acid offer poor protection or are uneffective, while high doses may be toxic.

It is well-known that Vit E prevents the radical chain of reactions leading to lipid peroxidation. A hydrosoluble Vit E analogue is frequently used in experimental studies (15). In our experiments the low protection afforded by Vit E was probably due to the fact that hydrogen peroxide and hydroxyl radicals cannot be scavenged by Vit E because it is mainly a singlet oxygen scavenger (16).

In evaluating the results another relevant fact to consider is that different cell types have different susceptibilities to reactive oxygen metabolites (1). This is not due to spatial or environmental factors but is inherent to the cells. Several theories have been proposed to explain this, such as differences in membrane structure and endogenous antioxidant defense mechanisms.

The fact that Vit C seems to have a protective effect on aged fibroblasts implies the need to determine the best doses and the best combinations of antioxidant vitamins and other antioxidant nutrients to gain information that might be useful in clinical situations (13). In a recent trial involving a population of approximately 30000 subjects having a high-risk for cancer it was demonstrated that combinations of vitamins and minerals reduce the incidence and mortality rate in neoplastic diseases (3). Moreover, antioxidant nutrient therapy is potentially a valuable, non-invasive tool for treating elderly patients. Cultured cell studies contribute valuable knowledge which can lead to the practical application of this nutrient arsenal.

M. FARRIOL, M. MOURELLE y S. SCHWARTZ. *Efecto de la vitamina C y de un análogo de la vitamina E en fibroblastos envejecidos.* Rev. esp. Fisiol. (J. Physiol. Biochem.), 50 (4), 253-258, 1994.

Se mide la supervivencia celular en fibroblastos humanos de origen dérmico jóvenes y envejecidos *in vitro*, en presencia y ausencia de diferentes concentraciones de vitamina C (5, 12,5, 25 y 50 µmol/L) y un análogo soluble de la vitamina E (Trolox) a 1, 5, 10 y 50 mg/L). Ambos compuestos vitamínicos se añaden al medio de cultivo 30 min antes del estrés oxidativo consistente en la exposición, durante otros 30 min, a la acción del H_2O_2 5 mM. La viabilidad celular se determina mediante un test de proliferación celular no radiactivo (MIT). La vitamina C tiene efectos citoprotectores en los fibroblastos envejecidos a todas las dosis ensayadas. Efectos similares se observan con el análogo de la vitamina E, aunque a la dosis mayor (50 mg/L) hay un descenso significativo que podría indicar un menor efecto de este derivado.

Palabras clave: Acido ascórbico, Peróxido de hidrógeno, Fibroblastos, Tocoferol, Antioxidantes.

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