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Protection by α-tocopherol against skin ulcers induced by ionizing radiations

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Abstract

An in vivo study was carried out to determine the protective character of α-tocopherol against lesions caused by free radicals of ionizing radiation. Emulsified vehicles (o/w) were used at dosages of 1.5 and 2.5% w/w of α-tocopherol. To promote generation of free radicals, we used 6 MeV electrons from a lineal accelerator; a dosage of 2800 cGy was selected. The lesions were assessed according to the indications of the WHO, modified for such an effect. Biopsies of the treated areas were taken at the moment of maximum acute toxicity in order to subject them to an anatomopathological study, evaluating five parameters: thickness of epidermis, squamous metaplasia of the adnexa, overall impression, follicular atrophy and density of fibroblasts. The conclusions of this study indicate that under the experimental conditions used, α-tocopherol, applied topically, protects the skin from ionizing radiation, giving rise to a thicker epidermis than found in healthy animals.

Key words: α-Tocopherol; Free radical; Ionizing radiation; Epidermal thickness

1. Introduction

For some years, theoretical knowledge as well as knowledge derived from scientific experiments has been accumulating, along quite coherent lines, relating the existence of free radicals (peculiar to the aerophile character of cellular metabolism) to the factors that set off physiological aging and as an integral part of various pathologies associated with abnormally accelerated aging, whether cellular or tissue.

Among the protective elements on which the human organism relies, it is perhaps vitamin E (α-tocopherol) that is the most effective in combatting the aforementioned phenomena (Pryor, 1978). This is due to the fact that because of its antioxidizing character (Skinner and Parkhust, 1970; Bieri et al., 1983; Burton et al., 1983), it impedes the cellular morphological changes un-

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derlying the peroxidizing effect of free radicals on polyunsaturated fatty acids; constituents of membranes, above all mitochondrials (Del Maestro, 1980; Kappus and Sies, 1981; Freeman and Crapo, 1982; Vigo et al., 1992a). This degrading effect, if not prevented, would cause an energy deficiency (Trush et al., 1982), accumulation of lipopigments (degeneration of membranes) (Kappus and Sies, 1981; Harman, 1984) etc., with the immediate consequence of a decrease in the function levels of tissue and organ (Pryor, 1976; Doba et al., 1984; Vigo et al., 1992b).

Therefore, α-tocopherol, integrated in the lipid-proteic structure of membranes, has a protective, antioxidizing and scavenging action (Niki, 1987). In accordance with this, Bisby et al. (1984) suggested that one of the functions of vitamin E could be the reparation of radicals in its integrating proteins. When oxidized by peroxides, this substance is converted into its inactive form (tocopherylquinone) which can recover its activity from the reducing action of glutathione (Furuse, 1987).

Furthermore, α-tocopherol also acts as a protector against oxidations of collagen and mucopolysaccharides, inhibiting sclerotic development in the conjunctive tissue. This is due to the fact that, in a manner parallel to the general cellular action of the aforementioned radicals, the specific deterioration that free radicals have on this dermic conjunctive tissue must be added, especially at the macromolecular level (Chiu et al., 1982).

The central objective of this study was to investigate the protective capacity of α-tocopherol, administered topically, against noxious agents that produce free radicals, specifically ionizing radiation. From this study, we intend to determine the viability of these topical preparations which are still not available in specific therapy.

2. Materials and methods

2.1. Excipients

The following were used: 8 g Sedefos 75^{R} (Gattefossé, Saint-Priest, France), 14 g liquid paraffin (Acofarma, Barcelona, Spain), 20 g propylene glycol (Acofarma, Barcelona, Spain) and 60 g purified water.

Once the emulsion had been obtained, α-tocopherol (α-T) (dl-α-tocopherol, F. Hoffman-La Roche & Co., Basel, Switzerland) was mixed at room temperature, in two different concentrations: 1.5 and 2.5% w/w. These formulas also included 0.1% w/w of butylated hydroxytoluene (Acofarma, Barcelona, Spain) as an antioxidant of the formulas. Once the creams had been formulated, they were left to rest at room temperature for 24 h prior to carrying out the experiment.

2.2. Accelerated aging: ionizing radiation

2.2.1. Preliminary study

Because the rats used in our study are animals that are quite primitive on the zoological scale, their radiobiological sensitivity is unknown. As a consequence, in order to attain the objectives of our study, it was necessary to determine, with the greatest possible accuracy, the noxious levels that ionizing radiation produces in these animals.

All animals were irradiated with a linear electron accelerator (Mevatron 74. Siemens). Electrons with 6 MeV of energy were selected, being of lower penetration and providing a greater dosage in the skin compared to the intense dosage. A 3 cm circular localizer, designed for this purpose, was used. The beam of irradiation was cast over the entire proximal region of the thigh, considered as beginning at about 1.5 cm above the heel of the animal.

Before selecting the optimal dosage, an experimental model was carried out: The animals were divided into eight groups which were administered 600, 900, 1000, 1200, 1500, 1800, 2800 and 4000 cGy, respectively.

With respect to the previous model, the dosages chosen in this case are, in general, different and higher than those of the first irradiation due to the lack of macroscopic alterations evident from that irradiation.

2.2.2. Definitive study

Once the optimal dosage of radiation needed to produce the most adequate lesions for our

study was chosen (2800 cGy), 30 rats (6-month-old female Wistar) were employed for our purposes. The following groups were established: group 1.5: 10 animals received α-T at 1.5% w/w; group 2.5: 10 animals treated with α-T at 2.5% w/w; group C: the remaining 10 rats, which were not treated with this drug, but only with the excipient and which served as a control in this experiment.

The posterior extremities of the animals in groups 1.5 and 2.5 were shaved (Gurelan^R no. 0000-A) 24 h before beginning the treatment. After this time they were anesthetised with 0.04 ml of ketamine hydrochloride (100 mg/ml, Ketolar^R, Parke Davis). The rats were then administered 0.2 g of cream with 1.5% w/w of α-T and 2.5% w/w as indicated for the established groups. This procedure was carried out once a day for 10 days. Conversely, the animals in group C were shaved and subjected to irradiation, but only the excipient was administered.

Once the treatment had been completed, all 30 rats received radiotherapy on the posterior extremities according to the technique previously described. Immediately after groups 1.5, 2.5 and C were irradiated, they were administered the respective formulations. This was repeated a final time 24 h later.

2.2.3. Biopsies

Upon establishing that the acute lesions developed during the 2 weeks following irradiation and reached maximum toxicity on the 15th day, biopsies were taken from the skins of all animals and the macroscopic changes were simultaneously described.

Cutaneous trepans, 3 mm in diameter (Medicon^R Instruments) were used to take the biopsies. Once extracted, the biopsies were conserved in a buffered solution with 10% formol.

2.2.4. Macroscopic alterations

For the assessment of the macroscopic changes, a table was established (Table 1) according to the WHO scale (OMS, 1980), modified by us. In the table, only the maximum toxicity shown by each animal was assigned a value.

Because observational bias is possible, and given that we are interested in establishing the lesional degree of the established dosages, a histological study becomes essential. The study and macroscopic observations were carried out in double-blind fashion.

Table 1
Maximum macroscopic toxicity shown by each animal irradiated with 2800 cGy in the established groups

Alterations	Value	1.5	2.5	C
Erythema or hyperpigmentation	10	2	4	2
Minimum exudation-ulceration	20	3	3	1
Moderate exudation-ulceration	30	1	1	4
Exudation-ulceration	40	2	1	–
Wide ulceration-exudation with tendency to bleeding	50	2	1	3

2.2.5. Histological study

The biopsies were enclosed in paraffin wax, cut with a microtome, and dyed with hematosylin-eosin. Once the preparations had been dyed, the study was carried out using an optical microscope (Leitz Model Dialux 20 EB).

Five anatomopathological parameters were evaluated in the histological analysis: epidermal thickness (ET) of the various groups of animal relative to that of healthy rats; squamous metaplasia of the adnexa (SMA); overall impression (OI) given by the preparations; degree of follicular atrophy (FA); and finally, density of fibroblasts (DF) shown by the specimens being studied. All of these factors lead to the determination of the degree of radioinduced lesion. These aspects, while not specific, are the most representative of radiological damage.

In all cases, an assessment was made of the macroscopic alterations, assigning a numerical value from 1 to 10 according to the pathological level observed.

3. Results

3.1. Macroscopic alterations

As previously explained, the irradiation with small dosages was discarded for not producing adequate lesions. Similarly, we rejected the dosage of 4000 cGy because it produced massive damage which even the most potent radioprotec-

tors could not modify. Finally, we estimated a dosage of about 2800 cGy as being ideal for the irradiation of the animals in the central phase of the experiment because this produced clinically evident, but not massively destructive lesions. This dosage allowed us to observe the speed and degree of recuperation of the acute lesions and ensuing consequences.

In the animals irradiated at 2800 cGy, the following sequence was evident: hyperemia in the first 24–48 h, marked erythema and an increase in vascularization at the end of 1 week, continued erythema and a marked delay in the healing of the biopsies at 2 weeks. At 16 days, exudative epithelitis and partial ulceration of the skin was observed. Healing and reepithelization of the lesions began around the third week. At 6 weeks the only lasting consequences of the acute effects were complete alopecia and moderate atrophy of the skin.

Once the most suitable dosage of radiation for our investigation had been determined, the macro- and microscopic assessments of the lesions were carried out at the moment of maximum acute toxicity produced by the radiation, according to the scheme established through previous studies. This is due to the fact that, in general, the degree of acute lesion has a direct relation to the intensity of later effects (permanent alopecia or loss of hair, loss of sweat and sebaceous glands, hardening of the skin accompanied by loss of elasticity, etc.) that lead to accelerated aging of the skin.

After carrying out our proposed treatment and subsequent irradiation with 2800 cGy, macroscopic results for the three groups of animals were obtained and are shown in Table 1. These alterations can be better observed in Fig. 1, where they are grouped according to the value assigned to their maximum toxicity. In Fig. 1, it can be seen that group 1.5 showed lower toxicity than group 2.5 and group C.

To determine the existence of significant differences between the three groups of animals, we subjected the results to the Kruskal-Wallis statistical test which showed an absence of statistically significant difference ($H = 2.09$, $p > 0.05$) among the three groups of animals studied.

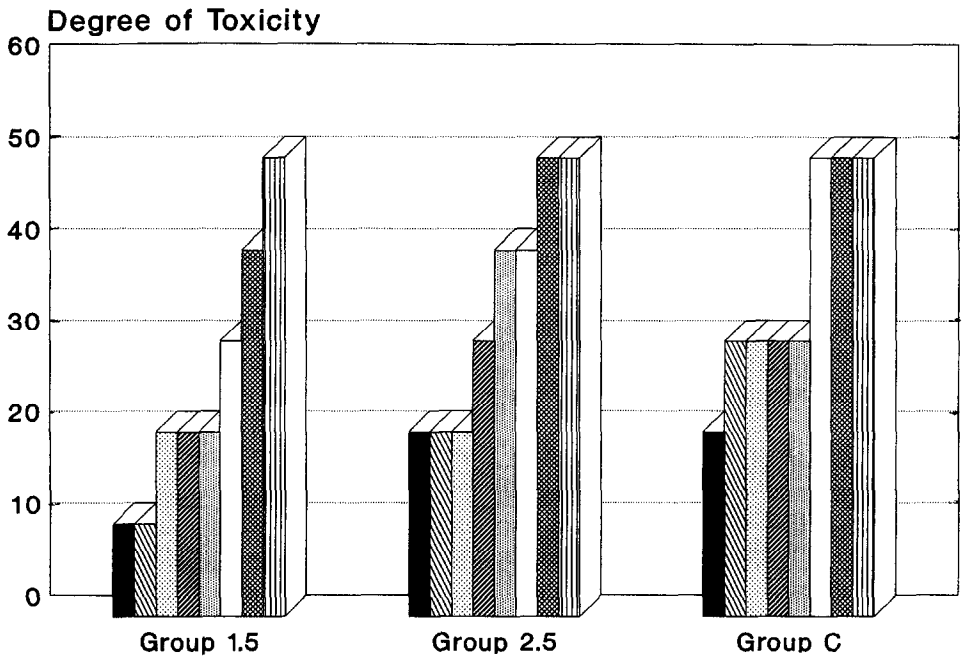

Fig. 1. Maximum macroscopic toxicity in the established groups.

Because α-T acts on cells by protecting their membranes from oxidation, it was necessary to complete the macroscopic study with a histological study.

3.2. Anatomopathological study

Fig. 2–5 summarize the histological observations found in the various groups of animals.

Regarding the first parameter under study, epidermal thickness (ET), Fig. 2 summarizes the overall aspect that the skin of a healthy animal possesses, showing a thin epidermis upon which the keratin layer is situated. Considering this skin as normal, it was compared to those of the various groups of animals subjected to irradiation. An atrophic epidermis can be observed in group C (Figure3), while in groups 1.5 and 2.5 (Fig. 4 and 5) an epidermis which is perfectly differentiated in its distinct layers is seen, with an evident increase in thickness as compared to group C and even healthy animals.

In Table 2 a great difference is evident between the treated animals and the control. The statistical study, carried out by means of the Kruskal-Wallis test, shows the existence of a statistically significant difference ($H = 12.65$, $p < 0.05$) among all variables compared. To determine which group is responsible for this result, the same data were subjected to the Mann-Whitney test, in which a significant difference was again shown between groups 1.5 and C ($|z| = 2.78$, $p < 0.05$) and 2.5 and C ($|z| = 3.1$, $p < 0.05$). Conversely, a significant difference was again shown between groups 1.5 and 2.5 ($|z| = 0.79$, $p > 0.05$).

Squamous metaplasia of the adnexa (SMA), shown in Table 3, was detected in the majority of the animals treated (groups 1.5 and 2.5), but not in the control rats. The exact Fisher test was

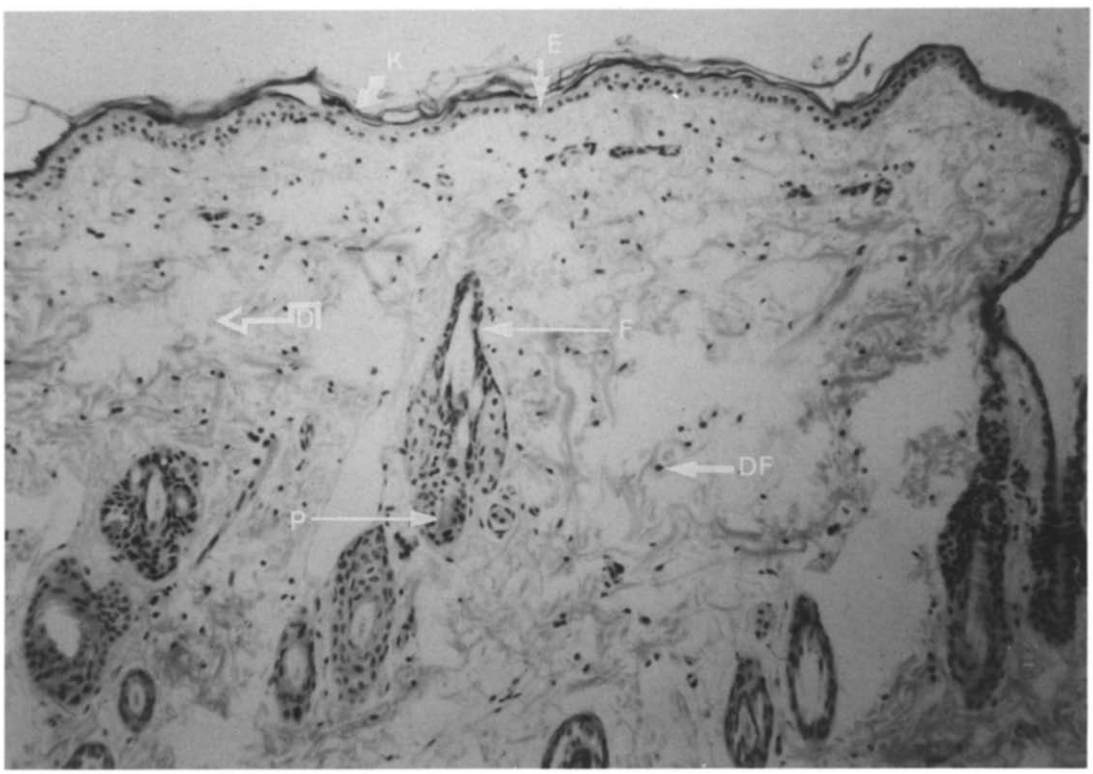

Fig. 2. Vertical section of the skin of a healthy animal. Observe the thin epidermis (E) upon which is situated its keratin (K) layer, a wide dermis (D) into which papillas (P) and follicles (F) penetrate and the density of fibroblasts (DF) (magnification $\times 25$).

employed, obtaining a statistically difference ($p < 0.05$) between groups 1.5 and C and between groups 2.5 and C. This difference did not exist between groups 1.5 and 2.5 ($p > 0.05$).

Regarding what has be referred to as overall impression (OI), in Fig. 6 a higher toxicity, in general, can be seen in groups 2.5 or C and lower toxicity for group 1.5. The Kruskal-Wallis test determines the absence of significant differences ($H = 1.28$, $p > 0.05$) between the control animals and those treated with concentrations of 1.5 and 2.5% w/w.

Fig. 7 shows the results of follicular atrophy (FA). They indicate again that the rats of group 1.5 show greater atrophy than the animals of group 2.5 and also greater than the control animals. In this case, in the corresponding statistical study (Kruskal-Wallis), a significant difference is

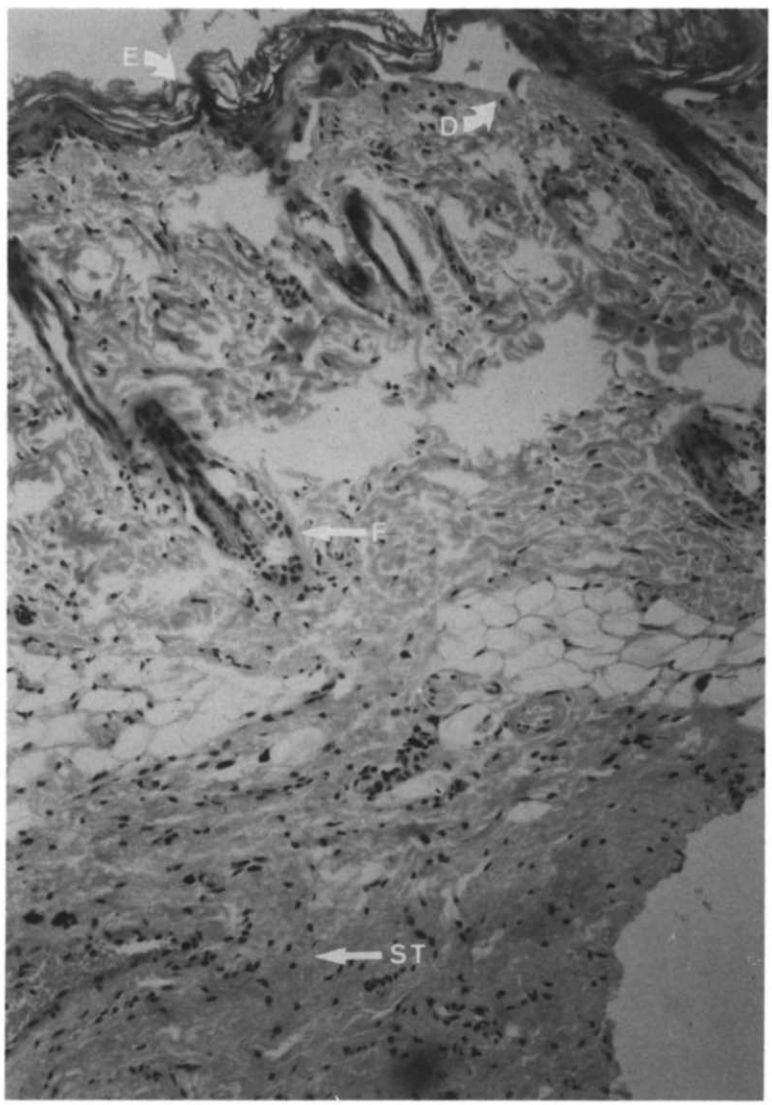

Fig. 3. Vertical section of the skin of an animal subjected to irradiation with prior treatment of the excipient without α-tocopherol. Note the atrophic epidermis (E) with separation of the dermis (D), few follicles (F) and intense fibrosis of the subcutaneous cell tissue (ST) (magnification $\times 25$).

Table 2
Anatomopathological study of epidermal thickness in the established groups

	1.5	2.5	C
	5	5	0
	4	4	3
	7	7	0
	6	9	0
	5	5	4
	3	4	0
	2	5	0
	3	3	0
$\bar{x}$	4.37	5.25	0.87
SD	1.68	1.91	1.64
SE	0.60	0.67	0.58

not shown ($H = 0.76$, $p > 0.05$) among any of the groups studied.

Finally, concerning the density of fibroblasts (DF) (Fig. 8), no divergence was observed in the quantity of this type of cell shown by the three groups tested. This is supported by the statistical study (Kruskal-Wallis) in which no significant difference was found between any groups ($H = 0.82$, $p > 0.05$).

4. Discussion

In the scientific bibliography, neither preventive nor curative treatments using α-T against lesions produced by ionizing radiations are discussed.

The principal species of the disintegration produced by ionizing radiation, such as charged and neutral free radicals like electrons, are capable of causing mutations and even cell death as a consequence of their interaction with DNA (Kappus and Sies, 1981; Freeman and Crapo, 1982; Trush

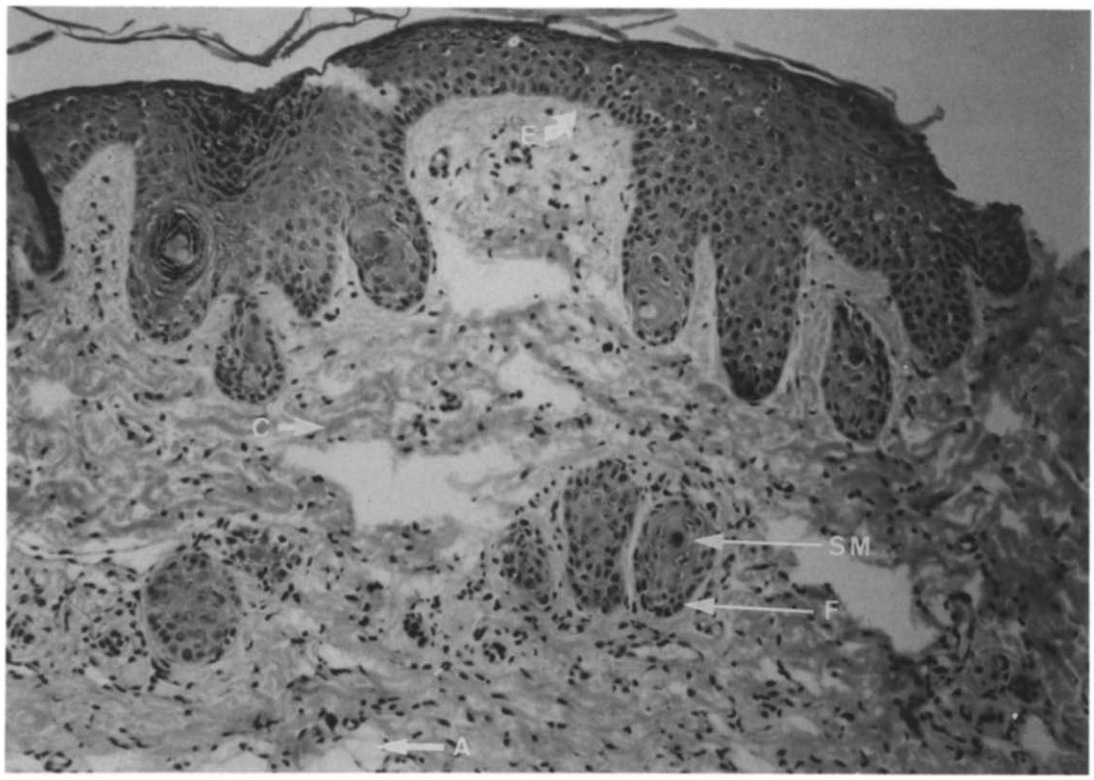

Fig. 4. Vertical section of the skin of an animal subjected to irradiation with prior treatment of α-tocopherol at 1.5% w/w. Observe the distinct layers of epidermic cells (E), follicles (F) with squamous metaplasia (SM), enlargement of the collagen fibres (C) and adipocytes (A) without fibrosis (magnification $\times 25$).

Table 3
Anatomopathological study of squamous metaplasia of the adnexa in the established groups

1.5	2.5	C
+	+	–
+	+	–
+	+	–
–	–	–
+	+	–
+	+	–
+	–	–
+	+	–

et al., 1982; Harman, 1984; Lucero et al., 1992). In 1896, Ostwald stated that "the very nature of organic radicals is such as to preclude the possibility of isolating them" (Pryor, 1976).

For these reasons our treatment with α-T was performed prior to and immediately after irradiation for the purpose of ensuring a sufficient quantity of the active substance when these reactive species are produced. Furthermore, because α-T is a liposoluble substance, it accumulates in the stratum corneum of the epidermis (Shah et al., 1991), a fact which has already been demonstrated by our own experiments (Lucero, 1989).

Faced with the disparity in the criteria among the many authors with respect to the concentration of α-T, vehicles used, means of administration and objectives pursued – whether clinical (Svingen et al., 1981a,b; Dorr and Alberts, 1983; Lucero et al., 1993) or cosmetic (Roquiers-Charles, 1988) – we established dosages of 1.5 and 2.5% w/w for our investigation so as to avoid possible secondary effects from massive dosages (Marx et al., 1984; Saperstein et al., 1984) while at the same time ensuring therapeutic efficiency.

The macroscopic alterations do not show a clear difference between the treated animals and

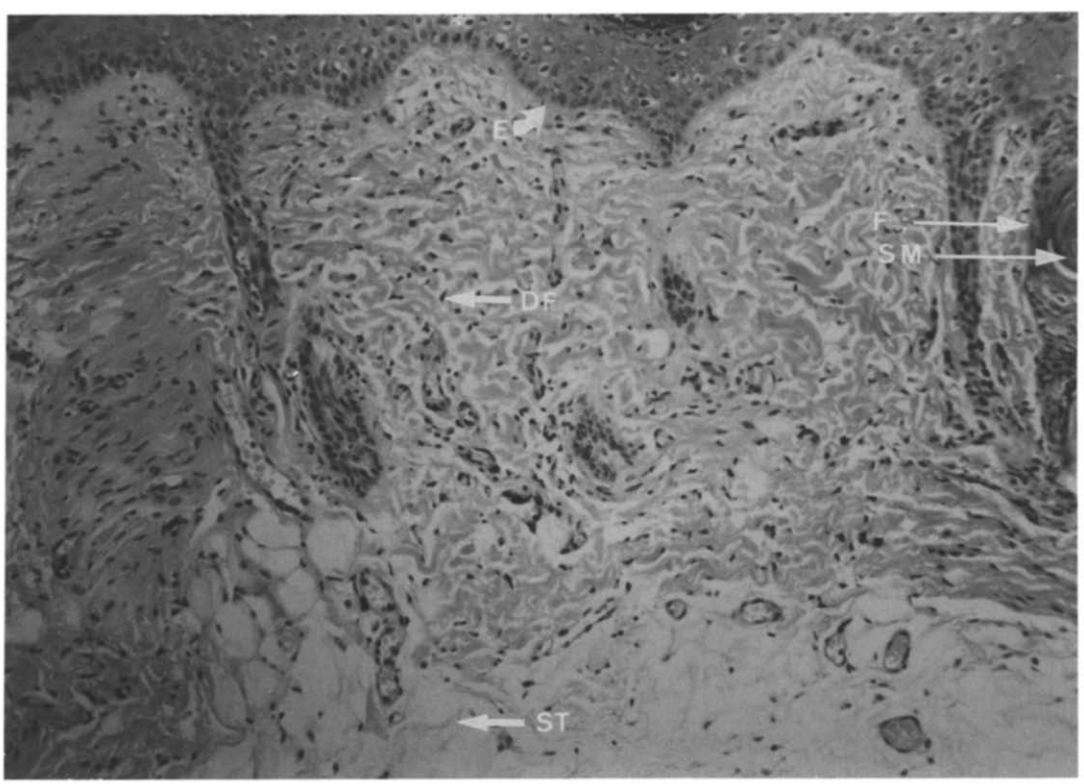

Fig. 5. Vertical section of the skin of an animal subjected to irradiation with prior treatment of α-tocopherol at 2.5% w/w. Note the distinct layers of epidermic cells (E), follicles (F) with squamous metaplasia (SM), the density of fibroblasts (DF) and an absence of fibrosis of the subcutaneous cell tissue (ST) (magnification $\times 25$).

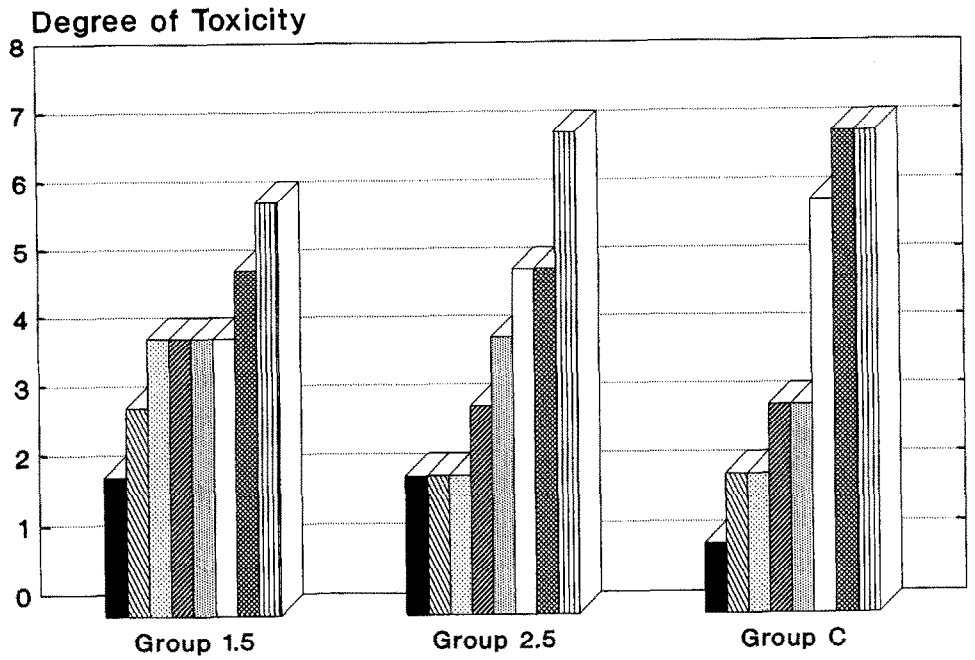

Fig. 6. Anatomopathological study of overall impression in the established groups.

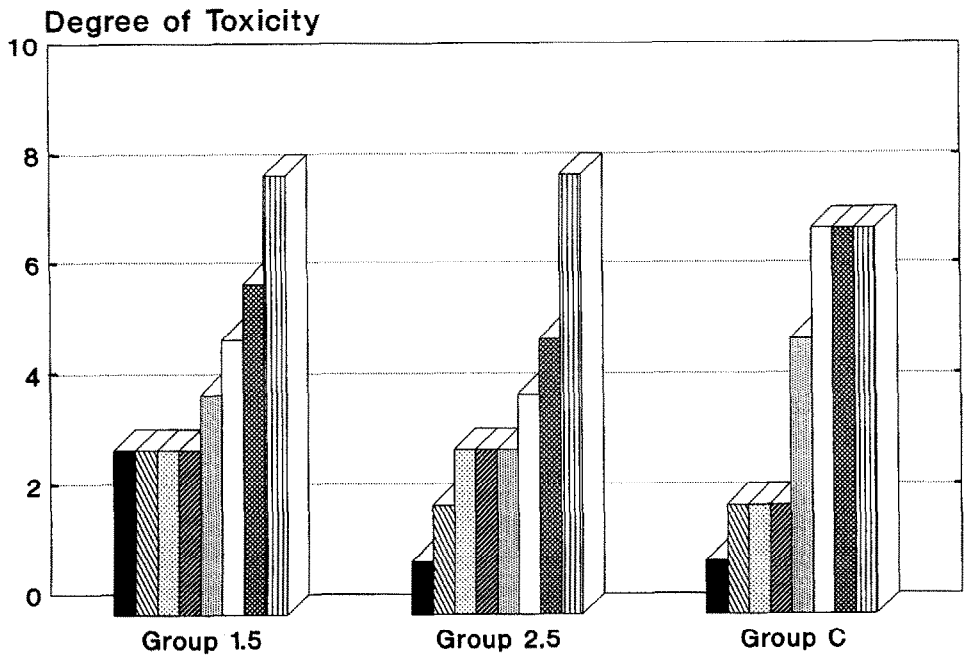

Fig. 7. Anatomopathological study of follicular atrophy in the established groups.

the control group. This could be due to the fact that the assessment of the lesions was made by observing the entire irradiated area, in which two distinct zones can be distinguished:

Zone 1: The area on which the beam of electrons falls perpendicularly on the surface. Here, the dosimetry of the radiation beam as well as its distribution are suitable for the theoretical studies. It is in this area that a dosage of 2800 cGy is unmistakably administered.

Zone 2 (also called fall off zone): The area found close to the edges of the field of radiation. Because of the shape of the irradiated limb (elliptical form in cross-section), this area is somewhat removed from the irradiation localizer. The beam falls on this area in an oblique manner, causing complete overdosage which is uncontrollable from a dosimetric point of view, since it cancels out the small build-up effect of the beam of electrons.

When macroscopically describing the lesions in this irradiated zone, the descriptions include the lesions in the area designated zone 2 which are always larger than those in zone 1. Conversely, in the histological analysis the biopsies were always taken from inside zone 1, at the center of the irradiation beam, to ensure that at this point the dosage received by all animals is the same. The degree of fall off in zone 2 is different for every animal and is always uncontrollable as it depends on the anatomical morphology of the irradiated limb.

As a consequence of these factors it was necessary to complete the macroscopic study with the histological study.

With respect to the first anatomopathological parameter under study, ET, the results obtained lead us to believe that α-T, under the experimental conditions used, not only protects the skin from ionizing radiation, but also appears to have a regenerating effect, giving rise to perfectly differentiated, thicker epidermis.

SMA was detected only in animals subjected to treatment with α-T. These animals developed tissue which is different from that normally produced in hair follicles and with no atrophy of these. Based on the findings of some authors (Cleary, 1984) on the importance of percutaneous

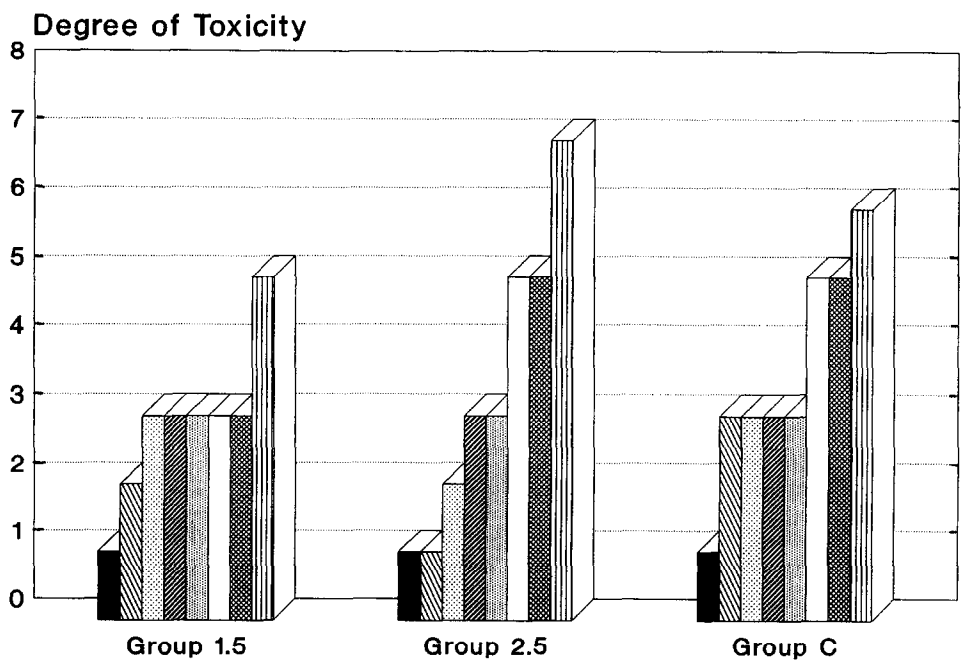

Fig. 8. Anatomopathological study of density of fibroblast in the established groups.

penetration by transfollicular way in animals, the ASM observed would indicate that α-T, applied on the skin, develops tissue in the follicles, forming distinct layers that give rise to a thickening of the skin; a process which resembles that observed in the irradiated animals treated with the distinct concentrations of α-T.

The remaining parameters studied (OI, FA, DF) did not show differences between any of the groups of animals tested.

These results could be explained by the theories developed by various authors (Del Maestro, 1980; Kappus and Sies, 1981; Freeman and Crapo, 1982; Roquier-Charles, 1988; Ross, 1988; Bast and Goris, 1989) who indicate that this active substance acts to inhibit lipid peroxidation of cell membranes. In this way, cell death would be avoided and therefore, the aging of the tissue as well.

5. Conclusions

We can affirm that under the conditions of our study, α-T, applied topically in an emulsified vehicle, has a protective effect on the skin against ionizing radiation, favoring the formation of thicker, perfectly differentiated epidermis.

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