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Radioprotective Effect of Vitamin E in Parotid Glands: a Morphometric Analysis in Rats

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The aim of this study was to evaluate the radioprotective effect of vitamin E on rat parotid glands by morphometric analysis. Sixty male rats were divided into 5 groups (n=6): control, in which animals received olive oil solution; olive oil/irradiated, in which animals received olive oil and were irradiated with a dose of 15 Gy of gamma radiation; irradiated, in which animals were irradiated with a dose of 15 Gy gamma radiation; vitamin E, which received α-tocopherol acetate solution; vitamin E/irradiated, which received α-tocopherol acetate solution before irradiation with a dose of 15 Gy gamma rays. Half of the animals were euthanized at 8 h, and the remaining at 30 days after irradiation. Both parotid glands were surgically removed and morphometric analysis of acinar cells was performed. Data were subjected to two-way ANOVA and Tukey's test (α=0.05). Morphometric analysis showed a significant reduction in the number of parotid acinar cells at 30 days in olive oil/irradiated and irradiated groups. In groups evaluated over time a significant reduction was shown at 30 days in olive oil/irradiated and irradiated groups, indicating that ionizing radiation caused tissue damage. The vitamin E/irradiated group presented more acinar cells than the irradiated group, but no statistically significant difference was observed (p>0.05). In conclusion, vitamin E seems to have failed as a radioprotective agent on acinar cells in rat parotid glands.

Key Words: ionizing radiation, acinus, parotid glands, vitamin E, radioprotective.

Introduction

The effects of radiation on salivary glands are of particular interest in clinical radiotherapy for head and neck malignancies. The loss of salivary gland function can reduce the quality of life and may lead to impairment of social activities for long-term survivors (1). Several patients who receive radiation in cancer therapy may complain about numerous side effects including xerostomia, dysphagia, taste changes, high dental caries risk, difficulty in swallowing and speaking (2). Pain and discomfort associated with hyposalivation and mucositis are also related and may affect nutritional intake and oral function. Disruption of mucosal integrity as a direct effect of radiation therapy has been shown to increase sensitivity to physical, chemical and microbial insults in the mouth, which contribute to reduce the patient's quality of life (3). In addition to a drastic decrease in the amount of saliva, qualitative changes, such as modifications in viscosity, pH, immunoglobulin and electrolyte levels, have been noticed. Furthermore, the symptoms are usually permanent, which emphasizes the importance of prevention (4).

The parotid glands are more radiosensitive than the submandibular or sublingual glands (5). Radiation therapy also inflicts tremendous damage to healthy cells surrounding tumor cells. The effects of ionizing radiation are mediated by the formation of free radicals, which are reactive, removing hydrogen atoms from fatty acids, causing lipid peroxidation and consequently cell death (6). However, there are specific nutrients that have been shown to act as radioprotectors (7). Antioxidant therapy is beneficial to avoid damage caused by radiotherapy.

Vitamin E is a natural component of cell membranes and is considered the main defense against membrane lipid peroxidation. There are several types of tocopherol, α-tocopherol being more reactive and with stronger antioxidant power. It reacts quickly with peroxyl free radicals interrupting the free radical chain reaction and consequently protecting cells from damage (8). A wide range of radioprotective effects associated with vitamin E have been demonstrated, such as preservation of the small bowel crypt, increase in the rate of DNA repair process (8), salivary dysfunction (9), against mutagenic and/or carcinogenic agents in animals and cell cultures, and a reduction in the number of micronuclei in human lymphocytes *in vitro* (10).

A reduction in the adverse effects of radiotherapy will improve the quality of life of surviving patients (9). Thus, the aim of this study was to evaluate the radioprotective effect of vitamin E on the parotid glands of irradiated rats, by morphometric analysis.

Material and Methods

This research protocol was reviewed and approved by the Ethics Committe on Experiments with Animals of Piracicaba Dental School, University of Campinas, Brazil (Protocol #600-1).

Sixty male Wistar rats between 8 and 10 weeks old were used. The animals were kept in polycarbonate cages under an alternating 12 h light/dark cycles and were maintained on laboratory chow and water *ad libitum* throughout the entire experimental period.

The animals were randomly divided into 5 groups (n=6): control, in which animals received three doses of 4 mL/kg of olive oil orally at 24, 48 and 72 h before sham irradiation; olive oil/irradiated, in which animals were given three doses of olive oil (4 mL/kg) orally at 24, 48 and 72 h before receiving gamma radiation; irradiated, in which animals were only irradiated; vitamin E group, in which animals received three doses of vitamin E (360 mg/kg) orally at 24, 48 and 72 h before sham irradiation; and vitamin E/irradiated group, in which animals were given three doses of vitamin E (360 mg/kg) orally at 24, 48 and 72 h before receiving gamma radiation.

Thirty minutes before the actual and sham irradiations, the animals were weighed and anesthetized by an intramuscular injection of ketamine chlorhydrate (0.1 mg/kg) and xylazine (0.05 mg/kg), and then placed on a table for irradiation of the head and neck region by an acute single dose of 15 Gy gamma rays, delivered by an Alcion GR II apparatus (Siemens, São Paulo, SP, Brazil) with an output of 84 cGy/min at 80 cm and operating with 1.25 Mev energy. The irradiation field corresponded to 30 cm x 30 cm. The groups receiving only olive oil and vitamin E group were anesthetized but not irradiated (sham-irradiated). The irradiation procedure was carried out between 9:00 and 11:00 a.m. (Table 1).

Eight hours and thirty days after irradiation, the animals were anesthetized by an intramuscular injection of ketamine chlorhydrate (0.1 mg/kg) and xylazine (0.05 mg/kg) and the right and left side parotid glands were surgically removed. After surgical excision, the animals were sacrificed under deep anesthesia. The specimens were removed and immersed in 10% formaldehyde buffer for 48 h and submitted to a standardized histological procedure. One-micrometer-thick serial histological sections were obtained and stained with hematoxylin and eosin for morphometric analysis. Three fields in each cut were evaluated, totaling twelve fields per slide. The histological fields were chosen at regular intervals in order to cover a representative sample of each cut (11). A light microscope (Zeiss Axiolab; Zeiss, Berlin, Germany) with an objective lens of 40× and a microcamera (Sony CCD IRIS RGB Color, Tokyo, Japan) was used for analysis. The morphometric analysis was performed using the KS400 2.0 (Kontron Electronics, Munich, Germany) software. The fractions of glandular volume occupied by acinar cells were determined by the histomorphometric analysis, using a reticulate grid with an area of 12,600 μm^2. Data were analyzed statistically by two-way ANOVA and Tukey's test with a significance level of 5%.

Table 1. Summary of the different experimental groups used in this study

Groups (n=6)	Irradiation dose	Treatment
Olive oil	No	4 mL/kg olive oil
Olive oil/irradiated	15 Gy rays	4 mL/kg olive oil
Irradiated	15 Gy rays	No
Vitamin E	No	360 mg/kg α-tocopherol acetate
Vitamin E/irradiated	15 Gy rays	360 mg/kg α-tocopherol acetate

Results

The qualitative analysis revealed the control group with normal acinar units interspersed by normal striated ducts, without inflammation or other degenerative processes. The specimens from the irradiated group presented acinar atrophy associated with loss of glandular parenchyma replaced by fibrous tissue. Similar findings were observed in the irradiated/olive oil group. On the other hand, most of the vitamin E/irradiated group specimens showed glandular parenchyma with preserved acinar cells and normal striated ducts (Fig. 1).

The morphometric analysis at 8 h post-irradiation showed that the olive oil/irradiated group had the largest number of acinar cells, though without significant difference from the olive oil and irradiated groups ($p>0.05$). The values for the vitamin E and vitamin E/irradiated groups were close, with no statistically significant difference between them ($p>0.05$).

Table 2. Morphometric analysis of acinar cells in times of 8 h and 30 days after irradiation

Groups	8 h	30 days
Olive oil	24.57 (2.79) B ab	28.83 (2.12) A a
Olive oil/irradiated	29.37 (3.60) A a	21.72 (1.28) B c
Irradiated	27.28 (2.99) A ab	20.97 (3.93) B c
Vitamin E	24.70 (4.80) A ab	26.96 (1.50) A ab
Vitamin E/irradiated	24.03 (3.08) Ab	23.05 (1.10) A bc

Different lowercase letters in columns and uppercase in the rows indicate statistically significant difference ($p<0.05$; Tukey's test).

At 30 days, the olive oil and vitamin E groups presented the largest number of acinar cells without significant difference between them (p>0.05), whereas olive oil/irradiated and irradiated groups showed the lowest values (p<0.05) (Table 2). Significant differences (p<0.05) were observed when the olive oil group was compared with the olive oil/irradiated and irradiated groups, emphasizing the deleterious effect of irradiation and demonstrating that olive oil did not exert a radioprotective action. Oil was only used as a vehicle to dissolve alpha-tocopherol. The vitamin E/irradiated presented more acinar cells than the irradiated group, but no significant difference was observed (p>0.05).

The evaluation of groups over time showed a significant reduction of cells in the olive oil/irradiated and irradiated groups, showing that ionizing radiation was able to cause tissue damage at a later stage. Vitamin E and vitamin E/irradiated groups had similar results over the time intervals.

Discussion

The radiosensitivity of the salivary glands has been studied in order to understand the mechanism of salivary gland hypofunction, an adverse effect that causes dry mouth, changes in oral microflora and oral mucosa, as well as in chewing, swallowing, speech and taste (12). Maintaining patients quality of life is the greatest concern of professionals involved in this treatment.

Although acinar cells are well differentiated, the salivary glands have shown a fast response to radiation. Some hypotheses have been raised in an effort to explain this acute radiation-induced damage. This radiosensitivity could be explained by the presence of granules in serous cells, rich in transition metals such as zinc, iron and

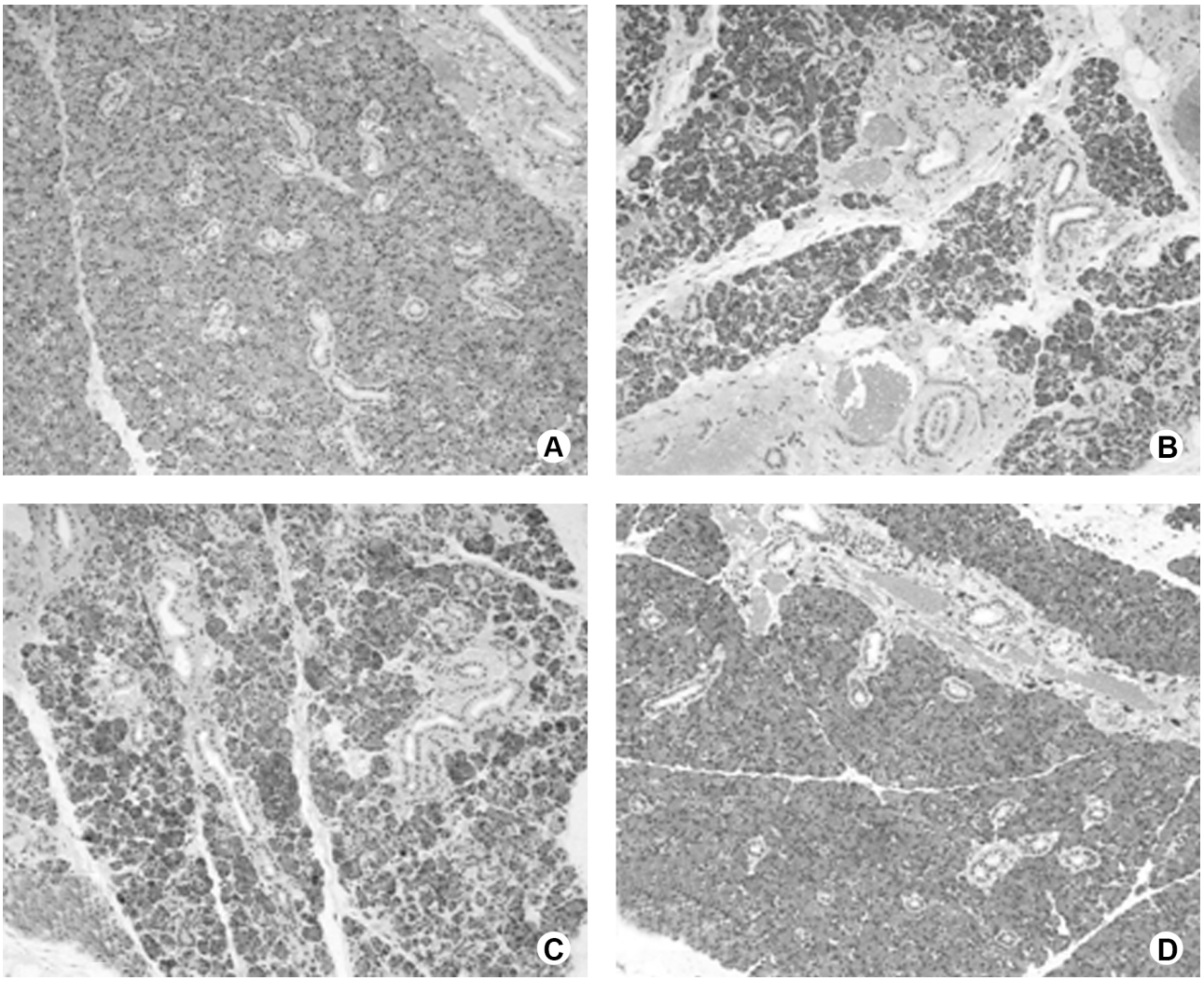

Figure 1. A: Glandular parenchyma showing preserved acinar cells and normal striated ducts in the irradiated vitamin E group (HE, 10×). B: Sample from the irradiated group showing acinar atrophy associated with loss of glandular parenchyma replaced by fibrous tissue (HE, 10×). C: Acinar atrophy and slight tissue fibrosis within the parenchyma in specimen from olive oil/irradiated group (HE, 10×). D: Normal acinar units interspersed by striated ducts in the olive oil group (HE, 10×).

manganese, which are released into cytoplasm, causing autolysis and cell death (13). On the other hand, Peter et al. (14) stimulated the degranulation of acinar cells, causing release of these metals prior to irradiation and observed that cell recovery was attributable rather to the action of isoproterenol than to the removal of metal ions from the acinar cells. Nagler et al. (2) also evaluated the protective ability of prior degranulation and concluded that there is another mechanism of damage to the submandibular glands, which is not related to the presence of metal ions in the secretory granules. Furthermore, Stephens et al. (5) demonstrated that acute functional impairment was directly caused by acinar cell apoptosis.

According to the morphometric analysis at 8 days, there was no significant reduction in the number of acinar cells in the irradiated groups. The adverse effect of irradiation was evidenced at 30 days by the significant reduction in the number of acinar cells in oil irradiated and irradiated groups. Grehn et al. (15) evaluated the long-term effects on the parotid acinar cells by morphometric analysis, after fractionated radiation with a total dose of 30 and 40 Gy. After 180 days, a significant decrease in these cells could be observed with a 40 Gy exposure only. Those authors described such findings as an ongoing degenerative process resulting in a progressive loss of acinar cells, especially serous cells.

Acinar tissue disorganization and nucleus atrophy with condensed chromatin indicating apoptosis were found 4 h and 8 h after a single exposure dose of 15 Gy gamma rays to the head and neck in rats, by ultrastructural analysis (16). O'Connell et al. (17) demonstrated that submandibular glands irradiated with a single exposure dose of 10 Gy appeared to have fewer and smaller acinar cells than the control groups at 8 and 13 months, emphasizing the adverse effect of irradiation on glands, which may impair function and subsequently produce mouth dryness. Ten days after fractionated doses totaling 20, 30, 35, 40 and 45 Gy, Franzen et al. (18) reported no significant changes in parotid acinar cells. However, the authors demonstrated changes in the noradrenaline-evoked potassium efflux that caused early damage to the cell membrane, which may lead to interphase death.

The significant reduction in acinar cells shown in irradiated olive oil and irradiated groups at 30 days, characterizes the property of ionizing radiation to cause damage in tissues. Although most specimens from the vitamin E/irradiated group presented glandular parenchyma with preserved acinar cells, no statistical difference was encountered between vitamin E/irradiated and irradiated groups at 30 days. Vitamin E and vitamin E/irradiated groups presented similar results over the time intervals, indicating some beneficial effect of this anti-oxidant on the parotid gland. The radioprotective action of 360 mg/kg α-tocopherol acetate on salivary function has been demonstrated 30 days after a single exposure dose of 15 Gy gamma rays (9). Funegard et al. (19) reported a significant decrease in acinar cells of submandibular and parotid glands 26 weeks after a 35 Gy fractionated irradiation dose. Supplementation with 3.4 mg of α-tocopherol and 6 mg β-carotene exerted a radioprotective effect only on salivary function and not on the glandular morphology. Those authors (19) believe that factors other than acinar cell damage could be involved in the diminished flow rate.

Felemovicius et al. (6) observed a significant radioprotective effect on the small bowel crypt after intraluminal administration of vitamin E. They did not find any side effects even at supraphysiological doses. In the present study, 360 mg/kg was administered orally 24, 48 and 72 h before gamma irradiation was performed and no adverse effects were noted in the studied animals.

The role of vitamin E is to scavenge free radicals thereby preventing radiation-induced damage to the cell membrane (19). The action of free radicals leads to oxidative stress and lipid peroxidation, which may result in cell death (20).

In conclusion, based on the data of the present study, the positive action of vitamin E was demonstrated in the qualitative analysis. However, the morphometric analysis suggested that vitamin E was not effective as a radioprotective agent. Further investigation is required to assess other radiation doses and evaluation times and mainly for application in humans.

Resumo

O objetivo neste estudo foi avaliar o efeito radioprotetor da vitamina E sobre glândulas parótidas de ratos por meio de análise morfométrica. Sessenta ratos machos foram divididos em cinco grupos: controle, no qual os animais receberam solução de óleo de oliva; óleo de oliva irradiado, em que os animais receberam óleo de oliva e foram irradiados com uma dose de 15 Gy de radiação gama; irradiado, em que os animais foram irradiados com uma dose de 15 Gy de radiação gama; vitamina E, no qual receberam solução de acetato α-tocoferol; vitamina E irradiado, os quais receberam solução de acetato de α-tocoferol antes da irradiação com uma dose de 15 Gy de radiação gama. Metade dos animais foi eutanasiada em 8 h, e o restante aos 30 dias após a irradiação. Ambas as glândulas parótidas foram removidas cirurgicamente e análise morfométrica das células acinares foi realizada. Os dados foram submetidos à Análise de Variância com 2 fatores e teste de Tukey (α =0,05). A análise morfométrica mostrou uma redução significativa no número de células acinares da glândula parótida aos 30 dias nos grupos óleo irradiado e irradiado. Nos grupos avaliados ao longo do tempo uma redução significativa foi mostrada aos 30 dias nos grupos óleo irradiado e irradiado, indicando que a radiação ionizante causou danos teciduais. O grupo vitamina E/irradiado apresentou mais células acinares que o grupo irradiado, mas diferença estatisticamente significante não foi observada. Em conclusão, a vitamina E parece ter fracassado como um agente radioprotetor nas células acinares das glândulas parótidas de ratos.

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