Effect of homeopathy in the function and morphology of salivary parotid glands of irradiated rats

Efeito da homeopatia na função e morfologia das glândulas parótidas de ratos irradiados

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Abstract

Objective: To evaluate the radioprotective effect of a homeopathic solution in salivary function and parotid glands morphology of irradiated rats. Materials and Methods: The sample consisted of 150 rats randomly divided into 6 groups. The groups were named based on the substance administered: Control—saline solution; Irradiated Control—saline solution and 15 Gy of X radiation; Alcohol-hydroalcoholic solution dynamized at 15 CH and 15 GY of X radiation; Homeopathy—0.25 ml (1mL/kg) of the irradiated hydroalcoholic solution and dynamized at 15 CH; Irradiated homeopathy—homeopathic solution and 15 GY of X radiation. Each group was subdivided into 5 different subgroups, based on the time point of euthanasia: 12 hours, 3, 10, 17, and 24 days. The medication was administered for 7 days before and 7 days after the radiation treatment. On the day of euthanasia, salivation was induced with pilocarpine and collected. The animals were then sacrificed and the parotid glands were removed. Results: Salivary function analysis showed that only group irradiated homeopathy euthanized on day 17 had a statistically significant difference when compared to other irradiated groups, presenting a higher salivation flow rate. The only group that showed a statistically significant difference in the number of acini over time was the irradiated alcohol group, which presented a tendency of reduction. Conclusion: The homeopathic solution presented a late radioprotective effect based on salivary function and morphological analysis of the parotid gland.

Keywords: Radiotherapy. Homeopathy. Salivary Glands.

INTRODUCTION

Among the types of therapies used in the treatment of cancerous lesions, radiotherapy is widely used, either alone or combined with chemotherapy and/or surgical procedures¹. This therapy still causes several deleterious effects on healthy structures comprised in the irradiation area². When the treatment is focused on the head and neck region, damages caused to the oral mucosa, teeth, and salivary glands have been reported³,⁴. When exposed to high doses of radiation, the salivary glands have decreased or destroyed function, either temporarily or permanently, which may be evidenced in the first week of treatment⁴. Xerostomia, mucositis, dysphagia, and dental caries are some of the complications caused, in the majority of cases, due to reduction in salivary flow³. Among the major salivary glands, the parotid gland is the most radiosensible, and therefore, the most affected by radiotherapy treatment⁶.

One way to minimize these sequelae in irradiated individuals is by using radioprotective substances that act as antioxidants capturing free radicals⁷. In a search for more-efficient...
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Radioprotective agents, homeopathic substances show up as a promising alternative due to its efficiency in the treatment of various diseases\(^8,9\), even in the alternative treatment of cancer patients\(^7\).

Based on this hypothesis, the aim of the present study aimed to evaluate the radioprotective effect of a homeopathic solution in the salivary function and parotid gland morphology of rats submitted to 15 Gy of radiation X.

**MATERIALS AND METHODS**

**Experimental procedure**

After approval of the Ethics Committee for Animal Research, 150 adult male rats (Rattus norvegicus albinus, Wistar) aging 6 to 10 weeks were used. The study has been carried out by following the EU Directive 2010/63/EU for animal experiments. The animals were randomly divided into 6 groups of 25 animals named according to the substances administered: Control (G1), Irradiated control (G2), Alcohol (G3), Irradiated alcohol (G4), Homeopathy (G5) and Irradiated homeopathy (G6).

The groups control and irradiated control (G1 and G2) received saline solution; groups alcohol and irradiated alcohol (G3 and G4) received a hydroalcoholic solution dynamized at 15 CH, and; groups homeopathy and irradiated homeopathy (G5 and G6) received 0.25 ml (1mL/kg) of a hydroalcoholic solution irradiated at 15 Gy and dynamized at 15 CH. The medications were administered for a total of 14 days. The animals of the irradiated groups (G2, G4, and G6) were also exposed to irradiation of 15 Gy of X radiation. Each group was subdivided into 5 different subgroups, based on the time point of euthanasia: 12 hours, 3, 10, 17, and 24 days. Seven days after initiation of treatment, the animals underwent the irradiation procedure. For this, they were anesthetized intramuscularly with 1.0 ml/kg of ketamine solution (Dopalen®, Agribands of Brazil Ltda., Paulínea, SP, Brazil), and 0.15 mL/kg of xylazine hydrochloride solution (Rompun®, Bayer SA, São Paulo, SP, Brazil). The animals of the irradiated groups (G2, G4, and G6) were positioned on the linear accelerator at a focal distance of 100 cm. The collimation was adjusted to an irradiation area of 18 x 30 cm, covering only the head and neck region of the animals that were exposed to a single dose of 15 Gy of radiation X (Figure 1).

**Determination of the salivary volume**

After the period determined for each subgroup (12 hours, 3, 10, 17 and 24 days), the animals were again anesthetized, now using a solution of sodium pentobarbital 30 mg/kg intraperitoneally. The salivation was induced with pilocarpine (Sigma Chemical Co., St Louis, MO - 4 mg/kg) and, after the lag phase (time required to start salivation after pilocarpine administration), saliva was collected for 30 minutes. The volume of saliva secreted was estimated by its weight assuming that the specific gravity of saliva is 1.0 g/cm\(^3\).

**Histological assessment**

After saliva collection, the animals were sacrificed through deep anesthesia and the parotid glands were removed, processed and embedded. Six-micrometer thick alternating sections were cut and stained with hematoxylin-eosin. Three slices of different depths were chosen: initial, middle and final depths. Three photomicrographs of each histological section were acquired (two from peripheral regions and one from the central region) with a Leica DM LP light microscope (Leica Microsystems, Wetzlar, Germany), using an objective lens of 20 times with conventional lighting.

**Morphometric investigations**

For the morphometric analysis, the software Image-Pro Plus version 4.5 (Media Cybernetics, Silver Spring, USA) was used. An individualized grid was made and calibrated in 20x magnification. The grid (480 x 360 microns) had an area of 172,800μm\(^2\) and 99 reference points of intersection to calculate the structures present on each image. The same grid was superimposed on histological fields and the numerical values of intersections containing acini and ducts were obtained. The ratio of the number of acini and ducts of each image was also performed (Figure 2).

**Figure 1.** Animals positioned for irradiation of head and neck region.

**Figure 2.** Morphometric analysis using the software Image-Pro Plus 4.5 with the individualized grid for acini and ducts count.
**Statistical analysis**

The following tests were used in the study: Levene’s test to assess the homogeneity of variance; Shapiro-Wilk for analysis of data distribution; Kruskal-Wallis and Student-Newman-Keuls tests for cell counts among the groups; Friedman for cell count among times; Two-way ANOVA and Tukey’s tests for sialometry analysis; considering a significance level of 5%, using BioEstat software version 5.0 (Belém, Pará, Brazil).

**RESULTS**

1 - Salivary function analysis (sialometry)

No statistically significant differences (ANOVA, P>0.05) were observed among the groups at any isolated period, except for the 17-day period in which the irradiated animals treated with homeopathic solution showed increased salivation when compared to the others groups in the same condition. Concerning the non-irradiated animals, the alcohol group showed significant sialometry reduction (P<0.05) at 12 hours and 24 days.

There was no statistically significant difference (Friedman, P>0.05) in the values of phase lag among the periods when irradiated and non-irradiated animals were evaluated, thus, the treatments were not influenced by the time (Figure 3).

**Figure 3.** Mean of salivary function analysis values among the groups and study periods

2 - Quantitative analysis of the ratio between the number of ducts and acini

The analysis of acini and ducts proportions revealed no statistically significant difference (Kruskal-Wallis, P>0.05) among the different groups considering the same period, except for the animals that were exposed to radiation and alcohol, which presented smaller proportion than the non-irradiated group at 17 days (Kruskal-Wallis, P = 0.0219) and 24 days (Kruskal-Wallis, P = 0.0041). Furthermore, the comparison among study periods, considering the same group, showed that there was a progressive and significant decrease (Friedman test, P = 0.0078) in the acini and ducts proportion of animals submitted to alcohol therapy and irradiation at 10 days. In non-irradiated animals treated with a homeopathic solution, there was an increase (Kruskal-Wallis test, P = 0.0181) in the proportion after 12 hours, which was maintained up to 24 days (Figure 4).

**Figure 4.** Mean values of the ratio between the number of ducts and acini among the groups and study periods

3 - Quantitative analysis of the number of acini and ducts

To observe how the proportion of acini and ducts was affected, the number of ducts and acini was analyzed separately. Data analysis (Kruskal-Wallis) showed that at the 12-hours, the homeopathy group had a lower number of acini than irradiated animals of control and alcohol groups (P <0.05). Moreover, it could be observed a trend of smaller acini numbers in irradiated animals, regardless of the study group / substance administered (Figure 5).

**Figure 5.** Mean values of the number of acini and ducts among the groups and study periods

alcohol and irradiation. These animals showed a decreasing tendency (Friedman, *P* = 0.0055) in the number of acini over time. The control animals or the ones that received homeopathy and were irradiated showed no statistically significant differences over time, suggesting that homeopathic treatment may have reduced the effect of alcohol on the acini number.

Data analysis (Kruskal-Wallis) showed a larger number of ducts (*P* < 0.05) at 12 hours in the homeopathy group than in the other groups. In the remaining periods, there was no statistically significant difference between groups.

There was a progressive increase (Friedman, *P* = 0.0132) in the number of ducts over time in the animals receiving alcohol and irradiation. The irradiated control animals presented no statistically significant difference (*P* = 0.0666) over time, nonetheless, a progressive decrease (*P* = 0.0256) in the number of ducts could be observed in the animals receiving homeopathy. There were no statistically significant differences among the periods in non-irradiated animals, regardless of the group, except for the control group that showed an increase in the number of ducts at 3 and 10 days of study.

**DISCUSSION**

The decrease in the amount of saliva causes a significant change in the quality of life, as it has important functions in the organism. Therefore, several authors as Dirix and Nuyts, 2010; Hunter et al., 2013; Sood et al., 2014 evaluated the behavior of salivary glands when exposed to radiotherapy and verified the harmful effects of radiation. Corroborating all previous studies, this study showed a lower rate of salivation in irradiated animals when compared to animals that did not undergo irradiation, after 12 hours, 3 and 24 days.

Previous studies showed that in periods of 3-14 days after irradiation, a significant reduction in salivary flow can be noticed, agreeing with our results, that demonstrate the greatest reduction in salivary volume 3 days after irradiation. After 10 days, an increase in salivary flow was detected independently of the drug used, showing regeneration of the salivary glands beginning two weeks after radiation exposure. After 17 days, a slight decrease in flow rate was observed, which can be explained by the secondary effect of radiation on tissues.

Lag phase (time interval between pilocarpine injection and the start of secretion) showed no statistical differences comparing different times within the same group, disagreeing with the results of Vissink et al., 1990 who found a significant change in the lag phase 3 days after irradiation. Our findings also disagree with the outcomes of Coppes et al., 2000, wherein the increase and decrease of salivation time were observed 1, 3, 6 and 10 days after irradiation.

The present study was based on animal models due to its histological, physiological and secretory similarity to human salivary glands. The use of animals allowed the analysis of salivary flow as well as cellular alterations in the tissue. The radiation exposure selected was a single dose of 15 Gy, a widely used and sufficiently harmful dose to the salivary glands, as evidenced by Kaluzny et al., 2014; Vissink et al., 1990; Nagler et al., 1998.

Concerning the morphometric analysis, employing the count of the number of structures, it was observed that regardless of the sacrifice time and medication received, the number of the acini in irradiated groups significantly decreased when compared to non-irradiated animals. Nonetheless, no significant increase or decrease in the number of ducts was observed in any group. These results confirm the different responses of glandular structures when exposed to radiation varies according to the region, as reported by Redman, 2008; Anderson et al., 1981. The acini exhibited greater radiosensitivity and it is, therefore, the most susceptible part of the glandular structure, while the striated and convoluted ducts showed an intermediate radiosensitivity. Conversely, external and extra lobular ducts presented radioreistance. This finding corroborates the study of Boraks et al., 2008, in which it was concluded that ionizing radiation promotes significant changes in the glandular parenchyma. In the present research, a decreasing trend in the number of acinar cells was observed, being the group of irradiated animals the most affected by cellular reduction, as observed by Shan et al., 2008.

To minimize the deleterious effects of radiation on surrounding healthy tissues, the development of effective radioprotective substances acquired great importance and has been an object of study. Our research selected a homeopathic solution to be investigated, as this type of treatment has been effectively used in several managements, fighting the disease itself or the effects caused by the conditions. Several studies demonstrate the effectiveness of homeopathy in the treatment of multiple sclerosis; chronic prostatitis due to radiation; acute radiodermatitis; cancerous lesion; xerostomia; DNA damage, and oxidative stress; and as hepatoprotective.

In the present research, although obtaining higher salivary volumes in groups that underwent homeopathic treatment in most time points of euthanasia, only groups sacrificed at 17 days presented statistically significant differences. The difference was observed between the irradiated homeopathy group and the other irradiated groups, suggesting a delayed radioprotective effect of homeopathy in the salivary glands. These findings are in agreement with Haila et al., 2005, who attested an increased glandular function with significant improvement in xerostomia cases of syndromic patients undergoing homeopathic treatment.

When assessing the effect of homeopathy by morphometric analysis, statistically significant differences were observed concerning the number of acini in irradiated alcohol group, which tended to decrease over time. Conversely, group control and homeopathy presented no significant difference. Therefore,
a decrease in the harmful effect of alcohol by the homeopathic medication is suggested, since the vehicle of homeopathic drugs is alcohol in the same concentration of the substance administered to the animals of the alcohol group, which may suggest a radioprotective effect of the homeopathic substance in glandular acini.

Still analyzing morphometrically the glands exposed to radiation, the animals of the alcohol group showed a progressive increase in the number of ducts, contrariwise a progressive decrease in the number of ducts in animals receiving homeopathy was observed. One possible explanation for this finding is the different interaction of structures when facing x-radiation, as observed in the study of Anderson et al., 1981; Roslindo et al., 1989. The lacunar spaces left by destroyed acini tend to be occupied by existing ducts, adding up more ducts through the grades idealized in the methodology of the study.

A greater number of ducts observed in irradiated alcohol group when compared to other groups that also underwent radiotherapy may cause some weirdness. However, this increase is not converted into a higher rate of salivation, since such structures are not responsible for saliva production; it works only as conductive for saliva excretion, as stated by Anderson et al., 1981. Even though the mechanisms related to salivary tissue destruction and its greater sensitivity are still inconclusive, it is reported that acute effects begin 24 h after the start of therapy and stabilize in 72 h.

Despite the proven therapeutic action of homeopathic treatment in the management of patients undergoing X-ray radiation exposure and the suggested radioprotective effect in this study, further studies should be conducted to prove the drug as radioprotective, so it may be used safely and effectively contributing to the quality of life of patients.

**CONCLUSION**

The homeopathic treatment was effective at 17 days considering the group of irradiated animals, suggesting a delayed radioprotective effect of the homeopathic solution, when evaluated the salivary function. Regarding the parotid gland morphology in irradiated animals, the number of acini showed a significant decrease trend in the alcohol group, different from the control and homeopathy groups, suggesting a radioprotective effect of the homeopathic solution on acinar cells.

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