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AVALIAÇÃO DO EFEITO RADIOPROTETOR DA LIDOCAÍNA E DA HISTAMINA EM GLÂNDULAS PARÓTIDAS DE RATOS SUBMETIDOS À RADIOTERAPIA

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AVALIAÇÃO DO EFEITO RADIOPROTETOR DA LIDOCAÍNA E DA HISTAMINA EM GLÂNDULAS PARÓTIDAS DE RATOS SUBMETIDOS À RADIOTERAPIA

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Dissertação apresentada à Faculdade de Odontologia da Pontifícia Universidade Católica do Rio Grande do Sul como parte dos requisitos para obtenção do título de Mestre em Odontologia, área de concentração em Estomatologia Clínica.

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EPÍGRAFE

“Conheça todas as teorias, domine todas as técnicas, mas ao tocar uma alma humana seja apenas outra alma humana”

Carl G. Jung

AGRADECIMENTOS

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RESUMO

A radioterapia direcionada à região de cabeça e pescoço frequentemente envolve as glândulas salivares maiores, podendo resultar em hipossalivação e xerostomia. Disfunções salivares qualitativas e quantitativas predispõe o indivíduo a alterações na mucosa bucal e nos dentes, causam prejuízo às funções orais e impacto negativo na qualidade de vida. No primeiro artigo desta dissertação foi realizada uma revisão de literatura abordando o manejo terapêutico das disfunções salivares. Foram revisadas modalidades de tratamento já consagradas na literatura, bem como novas opções ainda em investigação. O segundo artigo descreve um estudo experimental que teve como objetivo avaliar o efeito da lidocaína e da histamina sobre alterações morfológicas induzidas pela radioterapia em glândulas parótidas de ratos. Foram utilizados 56 ratos *Wistar*, divididos em quatro grupos: Controle, Irradiado, Lidocaína e Histamina. Os animais foram submetidos à radiação ionizante, excetuando-se o grupo-controle, em sessão única de 20 Gy. No grupo lidocaína o fármaco foi administrado por via intraperitoneal na concentração de 2%, 10 minutos antes da radioterapia, na dosagem de 1mg/Kg. No grupo histamina a substância foi administrada por via subcutânea na concentração de 0,5 mg/0,5 mL e dosagem de 0,1 mg/Kg, diariamente, por sete dias, iniciando-se 24 horas antes da radioterapia. Sete e trinta dias após a radioterapia os animais foram eutanasiados e suas parótidas foram dissecadas para analise morfológica e mensuração da área nuclear das células acinares. Alterações morfológicas como desorganização acinar, vacuolação citoplasmática, alterações sugestivas de apoptose/necrose e pleomorfismo nuclear foram observadas nas glândulas dos animais irradiados, sem diferenças entre os grupos que receberam lidocaína ou histamina. A área nuclear das células acinares foi significativamente superior nos grupos Lidocaína e

Histamina. Apesar deste resultado, as glândulas parótidas dos animais irradiados apresentaram importantes alterações morfológicas, independente do tratamento com lidocaína ou histamina. A metodologia utilizada e os resultados obtidos neste estudo não suportam o efeito radioprotetor das substâncias administradas sobre a morfologia de glândulas parótidas de ratos.

Palavras-chave: Xerostomia. Hipossalivação. Glândulas Salivares. Radioterapia. Lidocaína. Histamina.

ABSTRACT

ABSTRACT

Head and neck radiotherapy often involves major salivary glands, which may result in hyposalivation and xerostomia. Qualitative and quantitative salivary dysfunctions predispose the individual to changes in the oral mucosa and teeth, cause impairment to oral functions and negative impact on quality of life. In the first article of this study we carried out a literature review addressing the therapeutic management of salivary dysfunction. Established in the literature treatment modalities were reviewed, as well as new options still under investigation. The second paper describes an experimental study, which aimed to evaluate the radioprotective effect of histamine and lidocaine on morphological changes induced by radiation in parotid glands of rats. Fifty-six Wistar rats were divided into four groups: Control, Irradiated, Lidocaine and Histamine. The experimental groups were submitted to ionizing radiation, with the exception of the control group, in a single session of 20 Gy. In the Lidocaine Group this drug was administered at a concentration of 2%, intraperitoneally 10 minutes before radiotherapy, dose of 1mg / kg. In the histamine group, the substance was administered subcutaneously at a concentration of 0.5 mg/0.5 mL and a dose of 0.1 mg/kg daily for seven days, starting 24 hours before radiotherapy. Seven and 30 days after radiotherapy the animals were euthanized and their parotids were dissected for morphological analysis and measurement of the nuclear area of acinar cells. Morphological alterations such as acinar disorganization, cytoplasmic vacuolation, suggestive alterations of apoptosis / necrosis and nuclear pleomorphism were observed in the glands of the irradiated rats, with no differences between the groups receiving lidocaine or histamine. Nuclear area of acinar cells was significantly higher in the Lidocaine and Histamine groups. Despite this result, the parotid glands of irradiated animals showed significant mophological changes, regardless of

treatment with lidocaine or histamine. The methodology used and results obtained in this study do not support the radioprotective effect of administered substances on the morphology of the rats parotid glands.

Keywords: Xerostomia, hyposalivation, salivary glands, treatments, radiotherapy, lidocaine, histamine.

EFFECT OF LIDOCAINE AND HISTAMINE ON RADIOTHERAPY-INDUCED MORPHOLOGICAL CHANGES IN PAROTIDS OF RATS

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

AQP-5	Aquaporina – 5
Ca	Cálcio
CEUA	Comitê de Ética no Uso de Animais
CGRP	*Calcitonin-gene-related-peptide*
cGy/min	Centigrays por minuto
CMC	*Carboxymethyl cellulose*
DNA	*Desoxy Ribonucleic Ácid*
Gy	Gray
HEC	*Hydroxyethyl cellulose*
HIST	*Histamine Group*
HPMC	*Hydroxypropyl Methylcellulose*
IL-1β	Interleucina-1β
IMRT	*Intensity-modulated radiatiotherapy*
IRRAD	*Irradiated Group*
LID	*Licocaine Group*
NO	*Nitric Oxide*
PGM	*Polyglyceryl Methacrylate*
ROS	*Reactive Oxigen Spécies*
RTC3D	Radioterapia Conformada 3D
SP	*Substance P*
TNF-α	*Tumor Necrosis Factor-α*
VIP	*Vasoactive-intestinal-polypeptide*

SUMÁRIO

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1 INTRODUÇÃO

O câncer de cabeça e pescoço representa cerca de 5% de todas as neoplasias malignas, correspondendo a um grupo heterogêneo de tumores localizados nos lábios, cavidade bucal, orofaringe, laringe, hipofaringe, nasofaringe, glândulas salivares, cavidade nasal, seios paranasais, meato acústico externo e ouvido médio.[1] A radioterapia é uma importante modalidade de tratamento para essas neoplasias, podendo ter indicação terapêutica primária, adjuvante à cirurgia, à quimioterapia ou como método paliativo no manejo de lesões em estágio avançado.[2]

A radioterapia consiste na utilização de doses elevadas de radiação ionizante, a qual atua sobre o DNA nuclear por meio da produção de radicais livres, levando à morte ou incapacidade de replicação celular. Apesar de efetiva no tratamento de neoplasias malignas da região de cabeça e pescoço a radioterapia apresenta uma série de efeitos adversos, que dependerão do volume e do local irradiados, da dose total de radiação, do seu fracionamento, da idade e condições clínicas do paciente, além de sua associação com outros tratamentos.[2,3]

A região de cabeça a pescoço é complexa e composta por estruturas distintas, cada uma com sua própria resposta à radiação. Algumas alterações imediatas ocasionadas pela radioterapia são observadas na mucosa oral e na pele tais como eritema, descamação e ulcerações. Apesar de apresentarem *turnover* lento, as células acinares das glândulas salivares apresentam elevada radiossensibilidade.[4] A intensidade da disfunção salivar está relaciona à dose de radiação utilizada, ao volume de tecido irradiado e a resposta individual do paciente.[3-6] As alterações glandulares iniciam-se pelo dano à membrana plasmática, que promove perda da resposta aos controles autonômicos, edema, degeneração e necrose das células acinares. Os efeitos tardios são consequência da fibrose e

atrofia dos lóbulos.[7] A saliva resultante sofre alterações qualitativas, com diminuição da atividade das amilases, da capacidade tampão e do pH, com consequente acidificação. Há elevação dos níveis de cálcio, potássio, sódio e redução na concentração de fosfato.[8]

Em tratamentos radioterápicos convencionais a xerostomia inicia-se a partir da primeira semana. Os danos passam a ser irreversíveis após doses cumulativas de 26 a 39 Gy, podendo levar os pacientes a apresentar volumes salivares inferiores a 10% daquele que apresentavam previamente à radioterapia.[7,9,10] A redução do fluxo salivar pode aumentar a quantidade de microrganismos patogênicos e estar associada ao desenvolvimento de lesões cariosas, candidíase e de outras doenças infecciosas bucais. Além disso, a hipossalivação usualmente resulta em ardência bucal, halitose, maior risco a lesões ulceradas na mucosa bucal, disfagia, disfonia entre outras alterações, que exercem impacto negativo na qualidade de vida dos pacientes.[11]

Na tentativa de contornar os efeitos adversos da radioterapia, estudos têm testado diferentes métodos de prevenção e tratamento da xerostomia. Dentre eles é possível destacar os antioxidantes, fatores de crescimento, tratamentos paliativos com saliva artificial, uso de agonistas colinérgicos muscarínicos tais como pilocarpina, cevimelina e betanecol, repovoamento com células-tronco, radiação laser de baixa potência dentre outros. [7,9,12,13]

Alguns estudos têm demonstrado o efeito da histamina em prevenir alterações morfológicas e funcionais causadas pela radiação ionizante nas glândulas salivares.[14] A histamina é uma amina endógena sintetizada no organismo a partir do aminoácido histidina e foi inicialmente caracterizada como mediadora de processos inflamatórios, porém também atua em diferentes condições patológicas e

fisiológicas. Ativa os receptores específicos H1, H2, H3 e H4, induzindo a secreção de interleucina-1β (IL-1β), fator de necrose tumoral alpha (TNF-α), IL-6, óxido nítrico (NO) e a produção de espécies reativas de oxigênio (ROS). A histamina atua na proliferação e diferenciação celulares, hematopoiese, desenvolvimento embrionário, regeneração e cicatrização de feridas.[15-17] Kim et al.[10] demonstraram em um estudo em humanos que a histamina induziu a translocação da aquaporina-5 (AQP-5) para a porção externa na membrana plasmática de células acinares. Esta organela participa do transporte de líquidos e possui papel significativo na secreção salivar.[18,19]

A lidocaína é um anestésico local e seu efeito radioprotetor sobre as glândulas salivares também tem sido sugerido. Os anestésicos locais, de um modo geral, são capazes de desempenhar um papel anti-inflamatório em diversos tipos celulares incluindo monócitos, macrófagos e neutrófilos. Além disso, demonstram *in vitro* efeito protetor sobre danos causados por endotoxinas.[20,21] Provavelmente a lidocaína atue pelo seu efeito estabilizador sobre a membrana celular, primeira estrutura a ser lesada pela radiação ionizante. Este fármaco parece proteger os neuroreceptores e os mediadores de sinalização intracelular da radiação ionizante, preservando assim a função das glândulas salivares durante a radioterapia. [22,23]

Diante do exposto, faz-se necessária a investigação de métodos terapêuticos que possam preservar a estrutura das glândulas salivares dos efeitos deletérios da radiação ionizante, minimizando os efeitos adversos agudos e tardios que resultam em hipossalivação e xerostomia. Portanto, este estudo teve como objetivo investigar os possíveis efeitos da histamina e da lidocaína sobre alterações morfológicas em glândulas parótidas de ratos submetidos à radioterapia na região de cabeça e pescoço.

2 PROPOSIÇÃO

2.1 Objetivo Geral

Avaliar o efeito da lidocaína e da histamina na prevenção de alterações induzidas pela radioterapia em glândulas parótidas de ratos.

2.2 Objetivos específicos

- Realizar uma revisão de literatura abordando as modalidades terapêuticas para a hipofunção salivar.
- Avaliar, por meio da análise histológica, o efeito da histamina e da lidocaína na morfologia das glândulas parótidas de ratos submetidos à radiação ionizante.

ARTIGO DE REVISÃO DE LITERATURA

Salivary Hypofunction: an update on therapeutic strategies

Artigo a ser submetido para avaliação no periódico: Gerodontology
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SALIVARY HYPOFUNCTION: AN UPDATE ON THERAPEUTIC STRATEGIES

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RESUMO

A saliva é de suma importância na manutenção da saúde bucal e geral, pois atua no processo digestivo, na remineralização dentária, além de apresentar propriedades antibacterianas, antifúngicas e antivirais. Disfunções salivares qualitativas e quantitativas predispõe o indivíduo a alterações na mucosa bucal e nos dentes, causam prejuízo às funções orais e impacto negativo na qualidade de vida. Nesta revisão foi abordado o manejo terapêutico para as disfunções salivares, contemplando modalidades de tratamento já consagradas na literatura, bem como novas opções ainda em investigação. Na escolha do tratamento da hipofunção salivar devem ser considerados fatores como capacidade glandular residual, doenças sistêmicas, medicamentos utilizados pelo paciente, bem como custo e efeitos adversos. As modalidades terapêuticas para a hipofunção salivar foram classificadas em sintomáticas, estimulantes e regenerativas. Substitutos salivares podem promover o alívio dos sintomas, pois mantém a lubrificação da mucosa. Nos casos de haver tecido salivar funcional, pode-se optar pelo uso dos estimulantes salivares tópicos ou sistêmicos. O transplante de células tronco é uma nova modalidade terapêutica ainda em investigação em modelos animais. A seleção da melhor modalidade terapêutica para a hipofunção salivar deve ser individualizada e a tomada de decisão realizada pelo profissional em conjunto com o paciente. Métodos terapêuticos que possibilitem o aumento do fluxo salivar podem melhorar consideravelmente a qualidade de vida dos pacientes.

Palavras-chave: xerostomia, hiposalivation, treatment, salivary gland., therapeutics.

ABSTRACT

Objective: In this study, the therapeutic management for salivary dysfunctions was addressed, including new treatment options still under investigation.

Background: Saliva has an important role in maintaining oral and general health, as it acts in the digestive process, in dental remineralization, besides presenting antibacterial, antifungal and antiviral properties. Qualitative and quantitative salivary dysfunctions predispose to changes in the oral mucosa and teeth, cause impairment to oral functions and negative impact on quality of life.

Materials and methods: A Medline/PubMed search was conducted using the terms *xerostomia, hyposalivation*, in combination with *treatment*, *salivary substitutes*, *citric acid*, *málic acid*, *chewing gum*, *acupuncture*, *pilocarpine, bethanecol, cevimeline, hyperbaric oxygen therapy* and *stem cell therapy*. Articles published in the English language from 1995 to 2016 were selected and reviewed. Suitable references from these articles were also reviewed.

Results: Salivary substitutes are able to promote symptom relief by maintaining lubrication of the mucosa. If there is functional salivary tissue, one may choose to use topical or systemic salivary stimulants. Stem cells therapy is a new option but still under investigation in animals.

Conclusions: When choosing the treatment of salivary hypofunction, factors such as residual glandular capacity, systemic diseases, medications taken by the patient, along with cost and side effects should be considered. The selection of the best therapeutic method for the salivary hypofunction should be individualized and the decision made by the professional together with the patient. Therapeutic methods that allow increased salivary flow can greatly improve patients' quality of life.

Key words: xerostomia; hyposalivation; treatment; salivary gland; therapeutics.

INTRODUCTION

Saliva has an important role in oral health, since it maintains the hypotonic environment, helps the sense of taste, regulates pH levels and ionic compositions of the oral cavity, which are necessary for the functionality of salivary proteins.[1] It takes part in the remineralization of dental enamel, besides controlling the composition of oral microflora with its antibacterial, antifungal and antiviral properties, protecting the organism from detrimental extrinsic factors. Saliva is composed almost entirely of water and electrolytes, in addition to immunoglobulins, digestive enzymes, histatins among others. [2,3] The autonomic nervous system controls salivary secretion, mainly by means of parasympathetic nervous signals.[3,4] All major salivary glands together produce about 90% of the daily salivary volume, ranging from 0.5 to 1 liter.[4,5] When resting, the submandibular glands are responsible for the production of about 65% of the saliva, which has plenty of mucins and complements the lubrication of the buccal mucosa. Under stimulation, the parotid provides 50% of the salivary volume. [3,4,6,7]

Xerostomia is defined as the subjective sensation of dry mouth and hyposalivation as the objective reduction of salivary flow and changes in its composition.[8] Among the main causes of salivary dysfunction are drugs, aging, radiotherapy, chemotherapy and systemic diseases such as rheumatological, endocrine, neurological, genetic, metabolic and infectious disorders.[5] Several drugs are capable of inducing hyposalivation and xerostomia, that usually regresse when drug use is discontinued. Among the drugs associated with salivary hypofunction are tricyclic antidepressants, antihypertensive, diuretic and antispasmodic drugs.[9,10] Another important cause of salivary changes is head and neck radiotherapy. Major salivary glands are often involved in the radiation fields because they are in the vicinity of the primary tumors and lymphatic chains of those regions. As a

consequence of radiotherapy there is a process of glandular degeneration, resulting in hyposalivation and xerostomia[11]. The severity of salivary dysfunction is related to the dose of radiation used, the volume of tissue irradiated and the patient's individual response.[11,12]

Qualitative and quantitative salivary dysfunctions cause a negative impact on the quality of life and predisposes the individual to alterations in the buccal mucosa and in the teeth.[1,4,5] There is impairment of oral functions, resulting in dysgeusia, dysphagia and dysarthria. The buccal mucosa becomes dry and atrophic, giving rise to frequent traumas.[5,8] There are also alterations in oral microflora and salivary composition, and there may be progression of caries, gingivitis, halitosis, mucositis, oropharyngeal candidiasis, maladaptation of dentures, bacterial sialadenitis among others. [13,14]

Considering the above mentioned, the present study is an updated approach of the main therapeutic options to salivary dysfunctions. A Medline/PubMed search was conducted using the terms *xerostomia, hyposalivation*, in combination with *treatment, salivary substitutes, citric acid, málic acid, chewing gum, acupuncture, pilocarpine, bethanecol, cevimeline, hyperbaric oxygen therapy* and *stem cell therapy*. Articles published in the English language from 1995 to 2016 were selected and reviewed. Suitable references from these articles were also reviewed.

THERAPEUTIC OPTIONS

Once the diagnosis of salivary dysfunction has been established, the therapeutic approach should be implemented, which depends on the cause and the

remaining glandular function. The treatments for xerostomia can be classified into: symptomatic, topical or systemic stimulants and regenerative therapies. [8,15,16]

Symptomatic Treatments

The most commonly used symptomatic approaches are increased fluid intake and the use of salivary substitutes.[17] These interventions consist in the relief of xerostomia by the use of topical agents that maintain the lubrication of the mucosa. [18] According to the selection criteria used, the search for the terms "xerostomia" and "hyposalivation" and "salivary substitutes" yielded five studies, among them two controlled clinical trials.

Oral rehydrating agents, also known as salivary substitutes, act directly on the surface of the mucosa and help patients with salivary deficiency. Salivary substitutes such as animal mucin, carboxymethyl cellulose (CMC), hydroxypropyl methylcellulose (HPMC), hydroxyethylcellulose (HEC), polyglycerylmethacrylate (PGM) have been used and some have components with similar properties to the glycoproteins and antibacterial agents present in saliva.[19,20] Gels containing HPMC, given to irradiated patients, showed a potential reduction in oral discomfort caused by xerostomia and this effect was more significant than that presented by CMC. However, HPMC also has no effect on salivary stimulation. [21]

Shahdad et al.[22] in a double-blind crossover study evaluated the use of two salivary substitutes based on HEC (Biotène Oralballance™) and PGM (BioXtra™) in xerostomic patients after head and neck radiotherapy. The substances were used for two weeks, over a break period of one week without the use of any other product. Both treatments were effective in reducing xerostomia between days 0 and 14,

indicating a clinical improvement for the patients. There were no significant differences in xerostomia scores between the two products that were used.

In addition, oral xanthan gum, mucin, linseed extract and aloe vera, all found in mouthwashes, sprays or gels can be used as oral rehydrating agents.[23] Each patient adapts to the presentation of the substance according to their daily routine.[24] Jellema et al.[24] evaluated, in a double-blind controlled study, the salivary substitute xanthan gum (Xialine™) in 30 patients with radiation-induced xerostomia. The substance was used for one week, at least four times a day, and the washout period between substance use and placebo was one week. No significant differences were observed between xanthan gum and placebo in relation to xerostomia, salivary viscosity or influence on swallowing.

As they are easily removed from the oral cavity by swallowing, salivary substitutes have a short-term effect and some are contraindicated in dentate patients due to tooth enamel demineralization they might cause. Therefore, in cases of severe hyposalivation, salivary substitutes containing calcium, phosphates and fluorides are indicated, which are able to prevent dental demineralization or even help its remineralization.[5,25]

Salivary Stimulants

Citric Acid

According to the selection criteria used, the search for the terms “citric acid,” “xerostomia,” and “hyposalivation” yielded 13 studies, among them one clinical, randomized and controlled trial, investigating citric acid effect on xerostomia.

According to Femiano et al.[26], when there is still functional salivary tissue, one of the alternatives for xerostomia and hyposalivation is 3% citric acid. This is a natural sialagogue, which stimulates the taste buds through efferent parasympathetic pathways, inducing increased salivary secretion. Citric acid (3%) was administered by 5 mL rinse for 30 seconds, four times a day for 30 days. There was immediate and significant improvement in dry mouth symptoms 15 minutes after its administration. The advantage of this substance compared to artificial saliva is that its effect remains for up to one hour. This longer lasting effect may be a consequence of simultaneous stimulation of the major and minor salivary glands. The clinical disadvantage of using citric acid is the possible induction of dental hypersensitivity and erosion, although the saliva has a buffer capacity, neutralizing its pH.

Malic Acid

The malic acid action is based on its dissociation in H+, acidifying the oral environment, which generates stimulation of salivary secretion to dilute its concentration in the oral cavity. [23] The use of acidic substances as salivary stimulants may increase the risk of cavities as a consequence of their erosive action on the dental surface. However, with the presence of xylitol and fluorides in acid salivary stimulants, the decrease in salivary pH levels does not exceed the limit of 5.5 - critical level for hydroxyapatite. [27] According to the selection criteria used, the search for the terms "malic acid," "xerostomia," and "hyposalivation" yielded three studies, among them two clinical, randomized and controlled trials.

Gómez-Moreno et al.[28] in a double-blind, randomized clinical study, found that 1% malic acid was effective for the stimulation of salivary flow and, consequently, for reduction of xerostomia. Topical sialogogue containing 1% malic acid was given to 25 patients with drug-induced xerostomia, comparing its effects with a control group

of 20 patients receiving placebo. After two weeks of treatment the non-stimulated and stimulated salivary flow increased significantly in the malic acid treated group, which did not occur in the placebo group.

In another double-blind study with 41 patients, the effect of 1% malic acid spray combined with 10% xylitol and 0.05% fluoride over the xerostomia and the salivary flow was analyzed. Among the patients who used the malic acid spray, 80.9% reported improvement on xerostomia in contrast to 15% of the patients who used placebo. After two weeks of use, there was a significant increase in non-stimulated ($p = 0.039$) and stimulated ($p = 0.034$) salivary flow in patients who used malic acid. [23]

Chewing Gum

Sugar-free chewing gums, containing xylitol, are used to stimulate salivary flow and promote transient xerostomia relief by the capability to stimulate residual glandular tissue.[20] In addition, they may inhibit the development of cariogenic bacteria, reducing the amount of caries.[10,11] They increase the salivary volume by the mechanical and taste receptors stimulation, and they are widely used because they do not cause side effects. [23] On the other hand, patients with total dentures may have reduced masticatory capacity and may not be able to use this method.[5] According to the selection criteria used, the search for the terms "chewing gum" and "xerostomia," and "hyposalivation" yielded 24 studies, among them two clinical and controlled trials.

Davies [29] compared the use of mucin-based artificial saliva spray with sugar-free chewing gum in 43 patients with xerostomia. The substances were used four times a day for five days. The results showed no significant difference between the two groups regarding reduction of xerostomia, although 69% of individuals prefer to use chewing gum.

Kaae et al.[30] evaluated the use of tasteless sugar-free chewing gum as a treatment for xerostomia in 20 patients undergoing head and neck radiotherapy. All patients were submitted to salivary collection, stimulated and unstimulated, and answered a questionnaire to evaluate xerostomia. Individuals were instructed to use chewing gum three to five times a day for 14 days. The control group consisted of 10 healthy young who did not smoke and did not use any medication. The patients were evaluated at two different times. At the first visit, 90% of the irradiated patients reported having "a little xerostomia" or "a lot of xerostomia". At the second visit, only 30% of patients reported having "a little xerostomia" and no patient reported having "too much xerostomia". The increase in salivary flow after chewing gum stimulation was significant at both visits ($p = 0.008$ and $p = 0.05$, respectively).

Acupuncture

It is assumed that the possible mechanism of action of acupuncture in the treatment of patients with xerostomia/hyposalivation is based on stimulation of the autonomic nervous system through the afferent neurons, with an increase in the activity of the parasympathetic nervous system, which increases the release of specific neuropeptides. These neuropeptides possibly have numerous trophic effects, which include increased blood flow in the salivary glands, elevated acinar cell metabolism and salivary production, and possibly tissue regeneration.[31] Johnstone et al.[17], in an uncontrolled study, investigated the effect of acupuncture on salivary flow in 18 patients who underwent radiotherapy in the head and neck region and who had discontinuation of pilocarpine therapy due to adverse effects. Three points were acupressured in the auricular region on both sides and, if no salivation was noticed in the first 20 minutes, these points were electrostimulated for 45 minutes. Two sessions were performed the first week and three or four weekly sessions were

performed subsequently, depending on the patient's response. Improvement in xerostomia has been reported in 50% of the patients, but it is necessary that residual functional glandular capacity still exists for salivary secretion to increase. According to the selection criteria used, the search for the terms "acupuncture" and "xerostomia" and "hyposalivation" yielded 46 studies, among them one clinical, randomized and controlled trial.

Cho et al.[32] in a study of 12 patients with radiation-induced xerostomia applied this technique twice a week for six weeks (in acupressure points ST-6, LI-4, ST-36 and SP-6). The control group was pressured two centimeters away from the proper points. Salivary flow was measured at the beginning of treatment, three and six weeks later. There was an increase in stimulated and unstimulated saliva levels in both groups. However, only in the treated group the elevation was significant. The study showed that acupuncture may contribute to the treatment of salivary hypofunction.

Pilocarpine

Pilocarpine is a parasympathomimetic drug, agonist for the muscarinic receptors on the surface of the salivary cells. It acts as a stimulant of secretion of exocrine glands and participates in the contraction of the smooth muscle, gastrointestinal tract, urinary tract, gallbladder, bile ducts and bronchi.[5,20,33] The administration of pilocarpine to stimulate residual glandular function after head and neck radiotherapy is widely accepted, but the effect persists only while the drug is administered. Part of its effect in patients with post-radiotherapy xerostomia is attributed to the stimulation of the minor salivary glands of the palate, which have been shown to be resistant to radiation.[18] Our research with the words "pilocarpine"

and "xerostomia" and "hyposalivation" yielded 23 articles, among them two clinical, randomized and controlled trials.

In a randomized double-blind clinical study, Taweechaisupapong et al.[34] evaluated the efficacy and safety of pilocarpine tablets in alleviating xerostomia symptoms in individuals with salivary hypofunction. Three different presentations of pilocarpine were evaluated: 5 mg tablets (Salagen™) and 3 and 5 mg drops. The patients were questioned about oral dryness, discomfort and dysphonia. As an objective evaluation, salivary collection was performed at 0, 30, 60, 90, 120, 150 and 180 minutes after the use of the substances. The improvement in xerostomia levels was 12.1%, 63.6% and 69.7% in patients who received 5 mg tablets, 3 mg drops and 5 mg drops, respectively. The use of Salagen™ tablets, 3 and 5 mg drops, significantly increased total saliva production if compared to placebo ($p = 0.0001$, $p = 0.0004$, $p = 0.000$, respectively).

Wu et al.[35] evaluated the effect of 5 mg pilocarpine tablets (Salagen™), used four times daily for 12 weeks in 44 patients with Sjögren's Syndrome. Among the patients in the pilocarpine group, 69.6% had a reduction in xerostomia and 65.2% had an increase in salivary flow, values that were significantly higher in comparison to the placebo group ($p = 0.0032$ and $p = 0.02$, respectively). The authors concluded that 5 mg pilocarpine, used four times daily, is effective, safe and tolerable for the relief from symptoms of xerostomia in patients with Sjögren's Syndrome.

Bethanecol

Bethanecol is a carbamate ester resistant to the action of cholinesterase and most of its action is linked to activity at the M3 muscarinic receptor. The effectiveness of this drug in reducing xerostomia has been tested, with promising results in the increase of salivary flow.[5,20]. According to the selection criteria used, the search for

the terms “bethanecol” and “hyposalivation” and “xerostomia” yielded three articles, among them two clinical, randomized and controlled trials.

In a randomized clinical trial, Jham et al.[36] evaluated the efficacy of bethanecol on salivary flow when used concomitantly with head and neck radiotherapy in 43 patients. One group received 25 mg oral bethanecol three times daily, and the other received artificial saliva. The drug was used during and after the end of the radiotherapy treatment. Samples of stimulated and unstimulated saliva were collected in four moments: at the beginning, between the 15th and 19th sessions of radiotherapy, immediately after and two months after the end of radiotherapy. Although the patients who received bethanecol presented higher salivary flow, no significant differences were found between the bethanecol and control groups.

The effect of bethanecol on the salivary flow was investigated by Jaguar et al.[37] in patients with oropharynx and nasopharyngeal carcinoma submitted to three-dimensional (RTC3D) or intensity-modulated radiotherapy (IMRT). They used 25 mg bethanecol tablets twice a day from the beginning of radiotherapy to one month after their completion. Total saliva samples, stimulated and unstimulated, were collected, and patients were submitted to salivary gland scintigraphy. Patients in the placebo group had significantly higher degrees of xerostomia when compared to those receiving bethanecol, and there was no significant difference between the two modalities of radiotherapy (RTC3D and IMRT). Bethanecol also preserved stimulated and unstimulated salivary flow in irradiated patients. According to the authors, the use of bethanecol during radiotherapy is safe and contributes to the increase of salivary secretion with an important impact in reducing the damage to the salivary glands.

Cevimeline

Cevimeline is an analogue of acetylcholine, with high bond to M3 muscarinic receptors located in the epithelium of the lacrimal and salivary glands.[38,39] Our research with the words "cevimeline" and "hyposalivation" and "xerostomia" yielded 25 articles, among which, were selected three clinical controlled randomized trials.

A randomized double-blind controlled cross-over study evaluated the efficacy of cevimeline in the treatment of xerostomia and xerophthalmia in patients with Sjögren's Syndrome. Forty-four patients were evaluated and used cevimeline (30 mg) or placebo three times a day during 10 weeks. Although salivary flow increased with the use of cevimeline, there was no significant difference between groups (stimulated whole saliva p=0.489, stimulated parotid saliva p=0.073). No significant difference was observed in xerostomia scores between groups.[40]

Chambers et al.[41] evaluated 570 patients with radiation-induced xerostomia. Participants took cevimeline 30 mg or placebo three times a day for twelve weeks. After the sixth week the drug dosage was increased to 45 mg. Unstimulated and stimulated salivary collection was performed, and xerostomia was evaluated at each visit by visual analogue scale. Patients were questioned about their condition during sleep, comfort regarding the use of denture, use of artificial saliva and liquid intake. Among the patients treated with cevimeline, 47.4% reported improvement in xerostomia, versus 33.3% of placebo-treated patients (p = 0.0162). There was a significant difference in the unstimulated salivary flow for the cevimeline treated group when compared to the placebo group (p = 0.0093 in the first phase and p=0.0215 in the second phase of the study). On the other hand, no significant difference was found regarding stimulated salivary flow, comfort during sleep, liquid intake or use of artificial saliva between the placebo and cevimeline groups.

Even though cevimeline to increase saliva production, the drug-response mechanism is still unknown. Suzuki et al [38], in a cross-over and controlled study, administered 30 mg of cevimeline to healthy patients to evaluate the effects of the drug on the salivary and plasma levels of the neuropeptides substance-P (SP), calcitonin-gene-related-peptide (CGRP) and vasoactive-intestinal-polypeptide (VIP). Salivary and serum samples were obtained prior to drug administration, then after 30, 60, 90, 120, 180 and 240 minutes. There was a significant increase in salivary volume between 90, 180 and 240 minutes after administration of cevimeline when compared to placebo. There was an increase in salivary SP polypeptide levels, which acts as a vasodilator and increases blood flow, which may indicate an association between this substance and the elevation of salivary secretion stimulated by the drug. On the other hand, there was no increase in plasma levels of the other peptides studied.

Hyperbaric Oxygen Therapy

Hyperbaric oxygen therapy has been used to increase the dose of oxygen in the plasma and its distribution to the tissues. This reduces hypoxia in damaged tissues, inducing local angiogenesis, which provides better tissue recovery. The regular and periodic increase of the oxygen supply in the tissues in hypoxia has been shown to be stimulating to the phagocytic action of the leukocytes, for the production of fibroblasts, increase in the formation of collagen, formation of new capillaries, inhibition of aerobic and anaerobic bacteria.[41-43,] According to the selection criteria used, the search for the terms "hyperbaric oxygen therapy" and "hyposalivation" and "xerostomia" yielded nine articles, among them two clinical, randomized and controlled trials.

Teguh et al.[44] studied the effect of hyperbaric oxygen therapy on reducing the toxicity of radiotherapy of the head and neck region in 19 patients with oral and nasopharyngeal cancer. The total radiation dose received ranged from 46 to 70 Gy and patients started hyperbaric oxygen therapy two days after completing radiotherapy sessions. The hyperbaric therapy chamber was pressurized for 10 minutes at a treatment pressure of 2.5 ata. In this pressurization 100% of oxygen was supplied through an oronasal mask, three times of 25 minutes, interval for 5 minutes of normal breathing and completed with a 15-minute oxygen therapy. This treatment was performed five times a week for six weeks. A significant difference was found in the treated group when compared to the untreated group in relation to salivary consistency and xerostomia ($p = 0.01$ and $p = 0.009$, respectively).

Tahir et al.[45], in a retrospective study, analyzed head and neck radiotherapy patients treated in the hyperbaric chamber. The mean radiation dose was 64 Gy, and hyperbaric therapy was performed with a pressure of 2.4 ata, 100% oxygenation, 70 minutes with oxygen at 100%, two pauses of 5 minutes, one session per day, seven days per week. Out of the 50 xerostomic patients, xerostomia had improved in 32 patients ($p = 0.002$).

Regenerative Therapy

The cells that constitute the salivary glands have constant basal proliferative activity, suggesting that the glandular epithelium maintains its regenerative capacity. One of the theories explaining such proliferative activity describes cell differentiation from stem cells present in the region of the intercalated ducts. The extraction of mesenchymal stem cells from the parotid and submandibular glands has been performed in some studies and those have the capacity to differentiate both in acinar cells and ductal cells.[46]

In addition to the cells of the glandular epithelium, cells of mesenchymal origin can be extracted from the bone marrow and cultured in vitro to differentiate and then implanted in salivary glands damaged by radiotherapy.[47-49] Another tissue that can be used to extract multipotent cells is the adipose tissue. Such cells are able to differentiate into strains of osteogenic, adipogenic, myogenic and chondrogenic tissues. It is easy to obtain through aspiration, which causes little discomfort to the donor and has been the focus of researches on gene therapy. [50]

Due to this being a recent field of research, studies addressing stem cell regenerative therapy in salivary glands are scarce and performed in animal models. Three studies addressing stem cell transplantation in the salivary glands of rats submitted to ionizing radiation are described below.

Sumita et al.[51] tested the regenerative capacity of stem cells from the bone marrow in the salivary glands of irradiated rats (18 Gy). The researchers administered 1 x 10^7 stem cells through the caudal vein in each animal immediately after radiotherapy, which was repeated twice a week for 6 weeks. The animals that received the stem cells showed higher salivary flow in the eighth and twenty-fourth weeks when compared to the control group ($p < 0.05$). Likewise, the weight of the salivary glands in the treated animals was higher than in the control group ($p < 0.05$). Treated animals also showed lower apoptotic activity ($p < 0.01$). According to the authors, the results demonstrated that stem cell therapy, administered intravenously, is effective in regenerating the salivary glands of irradiated animals, reestablishing glandular function.

Kojima et al.[52] evaluated the regenerative capacity of salivary glands of rats submitted to radiotherapy (10 Gy). Stem cells derived from adipose tissue were administered directly into the submandibular salivary gland ten weeks after

radiotherapy (5,000,000 cells). The animals that received the stem cells showed, after the fifth week, a 75% increase in stimulated salivary flow when compared to the irradiated group ($p < 0.05$).

Xiong et al.[50] also evaluated the regenerative capacity of adipose stem cells grafted to the salivary glands of rats irradiated with 18 Gy. The salivary flow of the animals in the irradiated group decreased 48.27% eight weeks after radiotherapy in relation to the non-irradiated group, remaining at 50% until the 24th week of the experiment ($p < 0.05$). The group that received stem cell transplantation together with radiotherapy showed recovery in the salivary flow of 71.45% at the end of 24 weeks. Stem cells of adipose origin survive in the submandibular glands and play an important role in protecting against radiation-induced apoptosis promoting angiogenic activity.

DISCUSSION

In this article, therapeutic options were discussed for patients with xerostomia and hyposalivation. In general, the studies covered in this review do not define the best option for the treatment of salivary hypofunction, since several factors such as residual glandular capacity, individual access to treatment and cost effectiveness should be taken into consideration. Understanding the mechanisms of action of the therapeutic modalities for salivary hypofunction, as well as its adverse effects, is fundamental in the selection of the best treatment for the patient.

Among the symptomatic treatments, oral sprays based on HEC and PGM can be highlighted. However, these substances act as lubricants on the surface of the mucosa, without assisting in salivary stimulation and production. On the other hand, salivary stimulants are an alternate method for the management of salivary

hypofunction when functional glandular tissue is still present. Acidic agents such as malic acid, combined with fluorides, induce acidification in the oral environment and thereby increase salivary production. Other options for salivary stimulants are sugarless chewing gum and acupuncture, which also promote a temporary xerostomia relief. Additionally, muscarinic receptor agonist drugs are highly investigated in the management of salivary hypofunction. These include pilocarpine, which must be used continuously to maintain salivary stimulation. Dose-dependent adverse effects such as sweating, diarrhea, headache, increased urinary frequency and nausea are limitations imposed by these drugs.

Hyperbaric therapy also provides significant improvement in levels of hyposalivation and xerostomia, though the need for frequent sessions and use of specific equipment makes it a very pricy treatment. Stem cell therapy seems to be a promising option for the treatment of salivary hypofunction, especially in irradiated patients. Using stem cell transplantation derived from adipose tissue, an increase of more than 70% was obtained in salivary production in irradiated animals. Such cells can be obtained from oneself, processed and transplanted directly into the affected salivary gland. However, the procedures require highly complex techniques and high cost.

Finally, the selection of the best therapeutic modality for salivary hypofunction should be individualized and the decision-making performed by the professional together with the patient. Treatments should be monitored by assessing dose/response, adverse effects, and cost-benefit. Therapeutic methods that allow increased salivary flow can greatly improve patients' quality of life.

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ARTIGO DE PESQUISA

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EFFECT OF LIDOCAINE AND HISTAMINE ON RADIOTHERAPY-INDUCED MORPHOLOGICAL CHANGES IN PAROTIDS OF RATS

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ABSTRACT

Purpose: This study evaluated the effect of lidocaine and histamine on radiotherapy (RT) induced morphological changes in parotids of rats**. Materials and methods:** Fifty-six male Wistar rats were divided into four groups: Control (saline), Irradiated (saline), Lidocaine (lidocaine 2%, intraperitoneally, 10 minutes before RT, 1mg/Kg), Histamine (0.5mg/0.5mL, subcutaneously, daily for seven days, starting 24 hours before RT, 0.1 mg/kg). The animals were irradiated on head and neck region with a single dose of 20 Gy. Seven and 30 days after RT the animals were euthanized and the parotids were dissected. **Results:** Morphological changes such as acinar disorganization, cytoplasmic vacuolation, apoptosis/necrosis, and nuclear pleomorphism were observed in the parotids glands of the Irradiated, Lidocaine and Histamine groups without significant differences. The nuclear area of the acinar cells was significantly higher in the lidocaine and histamine groups compared to irradiated group. **Conclusions:** Parotid glands of the irradiated animals presented important morphological alterations, either under treatment with lidocaine or histamine. Although the nuclei of the acinar cells of the animals treated have presented superior area, the significance of this finding still needs to be clarified. The methodology used and the results obtained in the present study do not support the radioprotective effect of substances in preserving the morphology of the irradiated salivary glands.

Keywords: xerostomia, hyposalivation, salivary gland, lidocaine, histamine, radiotherapy

1 Introduction

Radiotherapy is an important therapeutic modality for head and neck malignant neoplasms. However, it presents a series of adverse effects, which will depend on the irradiated volume and site, the total dose and fractionation of the radiation, the age and clinical conditions of the patient along with the association with other treatments (Bölling et al. 2015). The head and neck region is composed of distinct anatomical structures, each one with its own response to radiation. Some acute changes caused by radiotherapy are observed in the oral mucosa and in the skin such as erythema, desquamation and ulcerations. Despite the slow turnover, the salivary glands are extremely radiosensitive (Konings et al. 2005). Glandular alterations are initiated by the damage to the plasma membrane, with loss of response to the autonomic controls. Subsequently, there is degeneration and necrosis of acinar cells, fibrosis and glandular atrophy (Porter 2010). The resulting saliva undergoes qualitative and quantitative changes, with a decrease in amylases activity, buffer capacity and pH, with consequent acidification. There is a rise in levels of calcium, potassium, sodium and a reduction in phosphate concentration (Jham and Freire 2006).

Xerostomia can be seen from the first week after radiotherapy. Depending on the model of radiotherapy used, the salivary flow can present a reduction of 50% to 60% and reach 80% after five or six weeks of treatment (Porter et al. 2010). The damage becomes irreversible after cumulative doses of 26 to 39 Gy, resulting in salivary volumes often less than 10% in relation to the initial one (Porter et al. 2010; Vissink et al. 2010). Saliva is especially important in the defense of the oral cavity and the reduction of salivary flow may increase the amount of pathogenic

microorganisms and be associated with the development of carious lesions, candidiasis and other infectious diseases of the mouth. Patients with salivary dysfunction usually present with oral burning, halitosis, increased risk of ulcerated lesions on the oral mucosa, dysphagia, dysphonia and other alterations that have a negative impact on their quality of life (Kim et al. 2009)

In an attempt to circumvent the adverse effects of radiotherapy on the salivary glands, studies have tested different methods of xerostomia prevention and treatment. We can highlight antioxidants, growth factors, palliative treatment with artificial saliva, use of muscarinic cholinergic agonists such as pilocarpine, cevimeline and betanecol, treatment with stem cells, low-level laser radiation among others (Porter et al. 2010; Vissink et al. 2010; Su et al. 2013; Nevens and Nuyts 2016).

The effect of histamine on the prevention of morphological and functional changes caused by ionizing radiation in the salivary glands has been investigated (Medina et al. 2011). Histamine is an endogenous amine synthesized in the body from the amino acid histidine. Initially it was characterized as mediator of inflammatory processes; however, it also acts in different pathological and physiological conditions. It activates the H1, H2, H3 and H4 receptors, inducing secretion of interleukin-1β (IL-1β), tumor necrosis factor (TNF) -alpha, IL-6, nitric oxide (NO), reactive oxygen species (ROS) (Rocha et al. 2016). Histamine acts on cell proliferation and differentiation, hematopoiesis, embryonic development, regeneration and wound healing (Nishina et al. 1998; Medina et al. 2011; Carabajal et al. 2012). Kim et al. (2009) observed in human submandibular glands that histamine treatment induced intracellular calcium elevation and the consequent translocation of aquaporin-5 (AQP-5) to the outer portion of the plasma membrane of acinar cells.

Lidocaine is a local anesthetic with muscle relaxant action. In general, local anesthetics are able to play an anti-inflammatory role in various cell types, including monocytes, macrophages and neutrophils, and demonstrate in vitro, protective effect on endotoxin damage (Nishina et al. 1998; Hollmann and Durieux 2000). It is suggested that lidocaine, as a membrane stabilizing agent, acts on neuroreceptors, mediators of cell signaling, present in the plasma membrane and responsible for the mobilization of calcium from the intracellular environment, preserving the function of salivary glands during radiotherapy (Su et al. 2013).

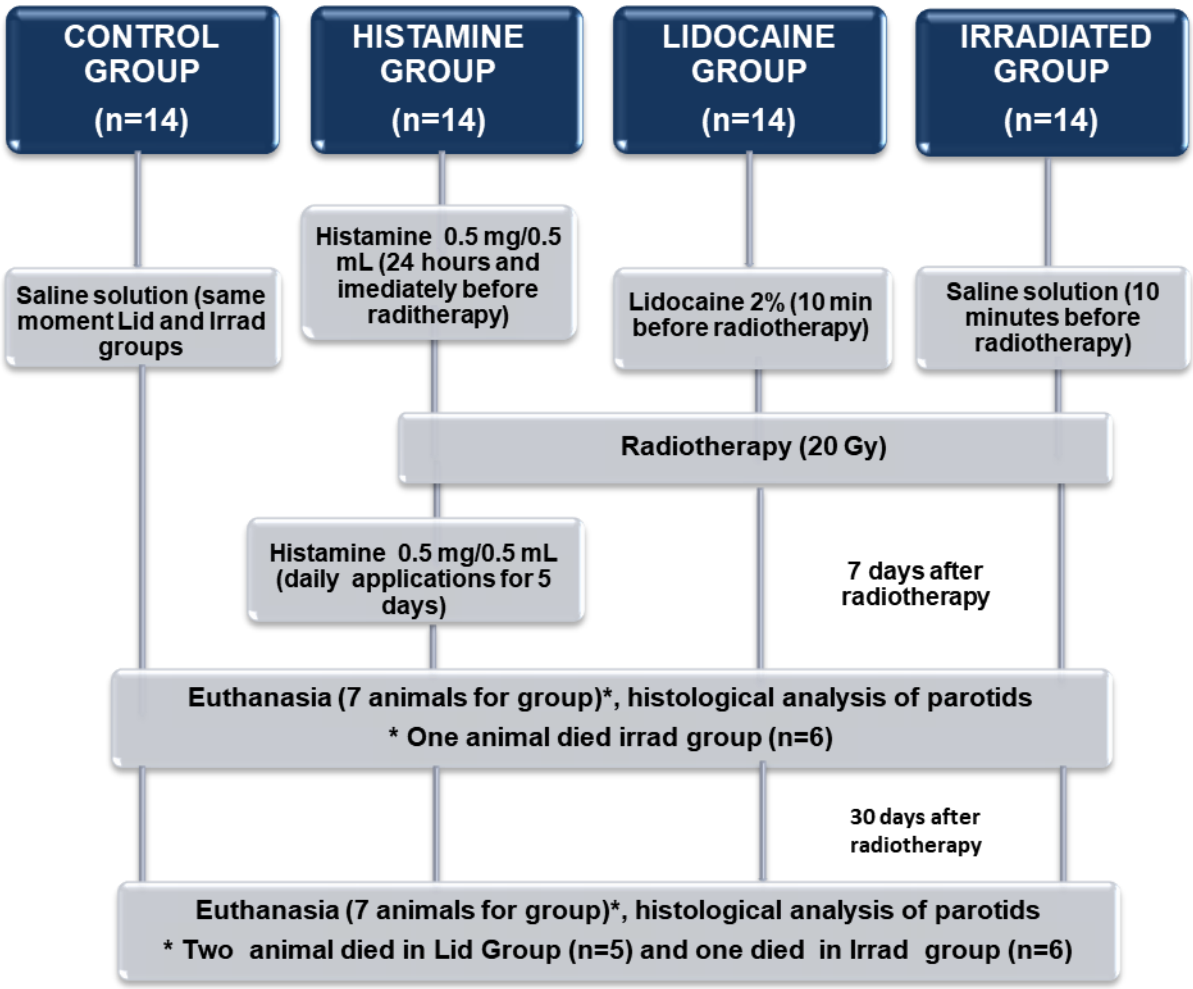
As previously mentioned, one of the main acute and late adverse effects of radiotherapy is damage to the salivary glands, leading to hyposalivation and xerostomia. Therefore, there is a need for methods that can preserve these anatomical structures from the harmful effects of ionizing radiation. The present study aimed to evaluate the effect of lidocaine and histamine on radiotherapy induced morphological changes in parotids of rats.

2 Materials and methods

The study was approved by the Ethics Committee on Animal Use (CEUA) of the Pontifical Catholic University of Rio Grande do Sul (PUCRS) under protocol number 15/00455. The sample consisted of 56 male Wistar rats, weighing 350-400g at the beginning of the experiment. Animals were kept in the Center for Experimental Biological Models of PUCRS in temperature-controlled (23 ± 1ºC) chambers equipped with input and output air filters, and with a 12 h light-dark cycle. They were housed in cages appropriate for rodents, duly identified, with a maximum of four animals per box. Autoclaved feed was provided in the form of pallets and filtered

water ad libitum. All recommendations have been followed for laboratory animal care and ethical standards for experimental animal testing in the US National Institute of Health's Guide to Laboratory Care and Use of Animals.

The animals were randomly divided into four groups (Figure 1), each one containing 14 animals: Control Group; Irradiated Group (Irrad); Lidocaine Group (Lid); Histamine Group (Hist). In the lidocaine group, lidocaine hydrochloride (Xylestesin) was administered intraperitoneally 10 minutes before the start of radiotherapy (Hakim et al. 2012; Babicová et al. 2013), at a concentration of 2% and dosage of 1 mg/Kg (Hakim et al. 2012). The substance was injected between the abdominal organs, in the posterior third, right side, with a 25 x 5 mm needle. In the histamine group, histamine dihydrochloride (Sigma-Aldrich) was administered daily for seven days subcutaneously at a concentration of 0.5mg/0.5mL and a dose of 0.1 mg/kg. The drug was injected under the distended skin, on top of the animal's head between the ears, with a 25 x 5 mm needle, starting 24 hours before radiotherapy (Medina et al. 2010). The Irrad and Control groups received single application of 1mL/kg of intraperitoneal saline.



2.1 Radiotherapy

Animals were subjected to ionizing radiation, exception of Control Group. Radiotherapy was performed in a single session in the Radiotherapy Department of São Lucas Hospital of PUCRS, of Porto Alegre. The animals were placed in the prone position, and immobilized. They were irradiated with Co60 using a teletherapy unit (Theratron Phoenix 760, Theratronics, Inc., Ottawa, Canada). The radiation dose used was 20 grays (Gy), based on the study of Choi et al. (2009). The yield of the radiation source was 56.84 cGy/min, and the distance between the emission point of the radioactive beam and the animals was 77 cm. The irradiation field corresponded to 30 cm by 30 cm, and only the head and neck region was exposed.

2.2 Euthanasia and Preparation of Tissues

Seven animals from each group were euthanized seven days after radiotherapy and the remainder 30 days after irradiation. Euthanasia was performed by inhalation of isoflurane overdose following anesthesia with the combination of ketamine chlorhydrate 100mg/kg (Dopalen) and xylasine 10mg/kg (Anasedan 2%), according to the National Experimentation Council Euthanasia Practice Guidelines Animal.

The right and left parotid glands of each animal were dissected and immersed in a flask containing 10% buffered formalin solution for 24 hours. The specimens were submitted to routine histological processing in the Pathology laboratory of the Dentistry College of PUCRS, of Porto Alegre .The processing of the pieces they were dehydrated in increasing concentrations of For alcohol and included in paraffin. From each gland, we obtained four sections of 5 μm thick stained with hematoxylin-eosin (HE).

2.3 Morphological Analysis

HE-stained slides were examined under a light microscope (Zeiss Axiolab, Berlin, Germany) qualitatively in all its extent. Then, we carried out an analysis, observing the presence of acinar disorganization, cytoplasmic vacuolation, suggestive changes of apoptosis/necrosis (presence of condensation and contraction of cellular structures, involving nuclear fragmentation) and nuclear pleomorphism. After the qualitative morphological analysis, the nuclear area of the acinar cells was measured. From each salivary gland, five equidistant fields were selected and, at

400X magnification, the images were captured by means of a capture system (Moticam 5 - System Shift - Capture, China) coupled under a microscope. Measurements of the 20-nucleus area of each image were performed using ImageJ 1.50g software (National Institutes of Health, USA). Afterwards, the five largest and the five smallest measures were excluded. With the remaining nuclei the average of the nuclear area of each slide was obtained, and then the average of the nuclear area of each group. The software was calibrated after capturing the image of the gauge ruler in the same magnification. The colouring of the image was adjusted so that the contrast of the colors of the cellular structures favored to the software a better demarcation of the limits of the nuclei. Using the wand tool the nuclei were selected and demarcated, and with the ROI manager tool the nuclear areas were measured (Figure 2).

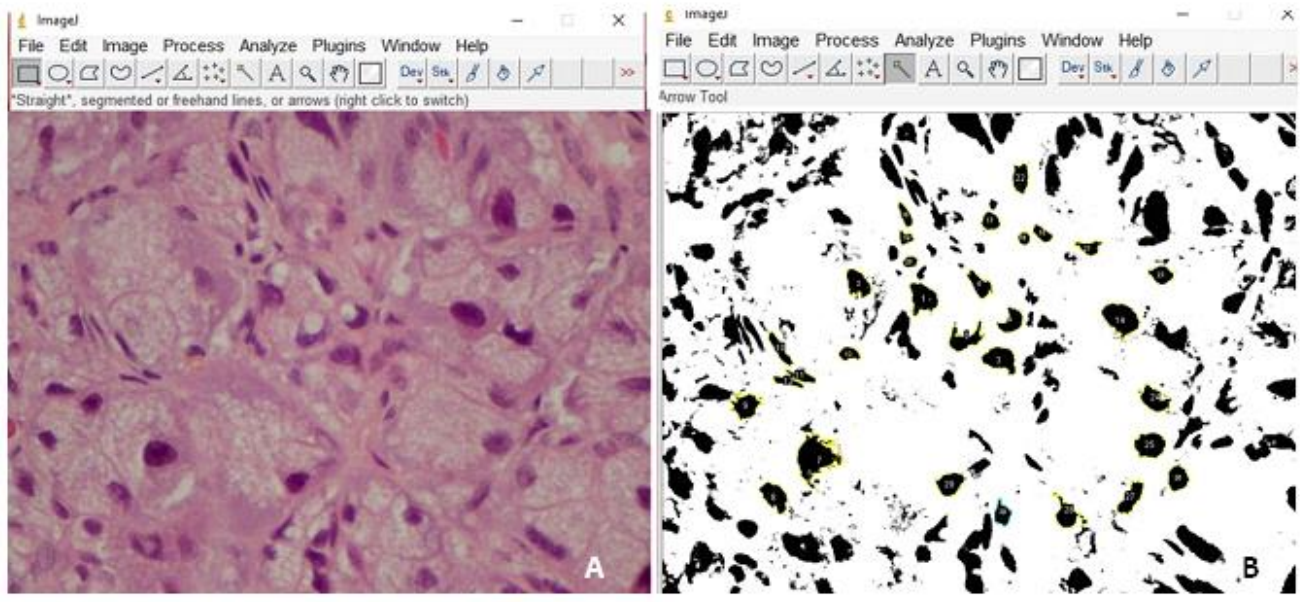


Figure 2 Original image (A), marking and measurement of the nuclear area (B).

The histological analyses were performed by a single blinded and calibrated examiner. Calibration was carried out by means of two readings of 20 slides, with interval of one week between both. The intra-examiner agreement for the analysis of

acinar disorganization, vacuolation, suggestive alterations of apoptosis/necrosis and nuclear pleomorphism was evaluated using the Kappa test, being considered satisfactory k≥0.7.

2.4 Data Analysis

Data was initially analyzed using descriptive statistics. To compare the nuclear area between the groups, the ANOVA test was used, followed by the Tukey post hoc test. The value established to reject the null hypothesis was P≤0.05. The software used to perform the statistical analysis was SPSS version 18.0.

3 Results

During the experiment, one animal from the Irrad Group died before the seven-day evaluation. In the 30-day evaluation, one animal from the Irrad Group and two from the Lid Group also deceased.

The values of the nuclear area of the acinar cells are described in Table 1. Seven days after radiotherapy, the mean nuclear area of the Irrad Group was significantly lower than the other groups (p = 0.000). The Lid (p = 0.550) and Hist (p = 0.235) groups did not differ significantly in relation to the Control Group. At 30 days of experiment, the Irrad and Control groups had significantly lower nuclear areas when compared to the Lid and Hist experimental groups (p= 0.000). There was also no significant difference between the Lid and Hist groups (p = 0.983).

Table 1: Means (± SD) of the nuclear areas (μm2) of the different groups in seven and 30 days post-radiotherapy.

	Control Group	Irradiated Group	Histamine Group	Lidocaine Group	P
7 days	128.25 ^{A} (±30.77)	90.64 ^{B} (±13,97)	141.65 ^{A} (±5.91)	147.84 ^{A} (±14.21)	**.000***
30 days	103.42 ^{B} (±7.71)	98.34 ^{B} (±13.95)	146.58 ^{A} (±14.84)	143.63 ^{A} (±18.25)	**.000***

ANOVA and Tukey´s tests, *significant at P≤0.05. Different letters in the line mean significant statistic difference.

At the seventh day of the experiment, morphological characteristics such as acinar disorganization, cytoplasmic vacuolation, suggestive alterations of apoptosis/necrosis and nuclear pleomorphism were observed in the salivary glands of the Irrad, Lid and Hist groups, with no significant differences among them. In the histological evaluation, 30 days after radiotherapy, acinar disorganization, cytoplasmic vacuolation, suggestive changes of apoptosis/necrosis and nuclear pleomorphism were also observed in the three irradiated groups, without significant differences (Figure 3).

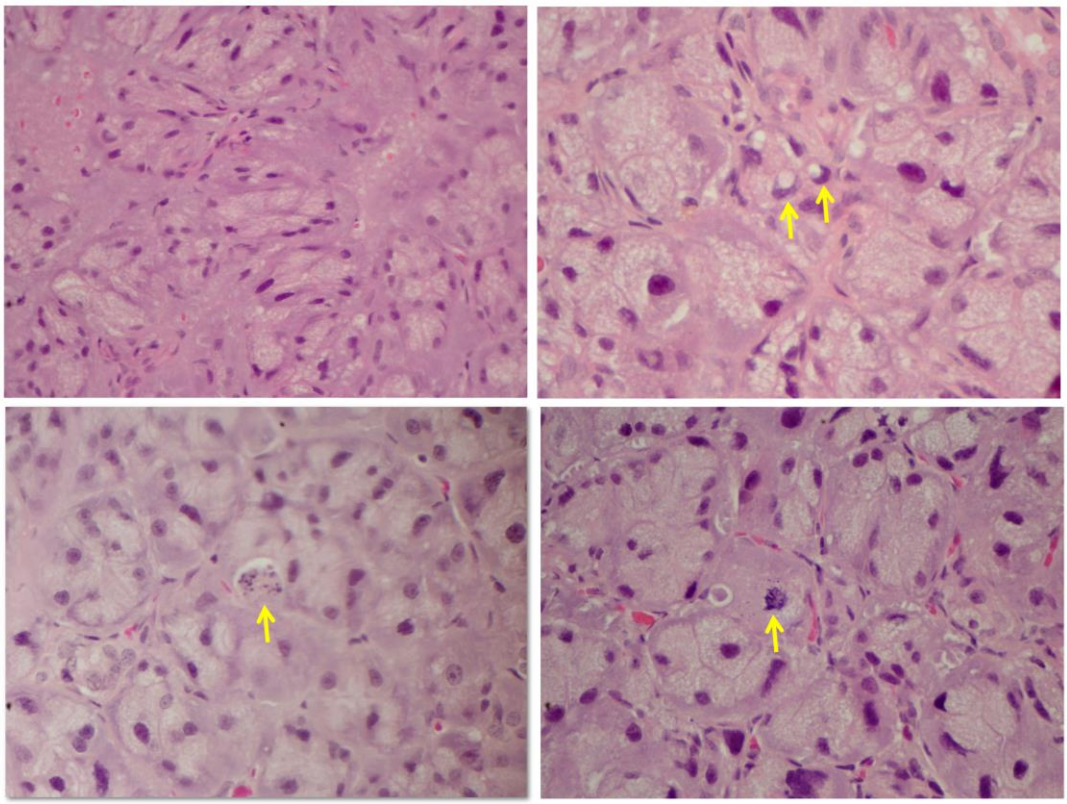


Figure 3: Histological evaluation of the parotid gland of rats demonstrating A - disorganization acinar (Irradiated Group, 30 days); B - cytoplasmic vacuolation (arrows) - (Histamine Group, 30days); C - apoptosis (Histamine Group, 7 days). D - Nuclear pleomorphism (Histamine Group, 7 days).

Discussion

The objective of this study was to evaluate and compare the effect of lidocaine and histamine on radiotherapy induced morphological changes in parotid glands of rats. Although they have different mechanisms of action, the effect of these substances in preserving the structure and function of the salivary glands submitted to ionizing radiation has been suggested in the literature (Su et al. 2013). Lidocaine, as a cell membrane stabilizing agent, acts on the neuroreceptors located on the membrane of acinar cells, which activate the protein kinase-Cα, responsible for the mobilization of calcium and excretion of water from the internal to the external medium. In addition, it inhibits the expression of tenascin-c, a protein that alters

tissue conformation and reduces its functionality (Coppes et al. 2005; Hakim et al. 2005; Yang et al. 2016). Histamine seems to protect tissues by inducing increased cell proliferation and suppressing apoptosis, vacuolation and fibrosis, regulating immune reactions triggered by ionizing radiation (Medina et al. 2010; Medina et al. 2011). Furthermore, it promotes the translocation of Aquaporin-5, the main channel that regulates the movement of water through the plasma membrane of acinar cells, promoting salivary secretion (Kim et al. 2009).

Ionizing radiation causes injury to cellular DNA, inducing the occurrence of morphological alterations in the acinar tissue, associated to the loss of glandular function (Jezkova et al. 2014). There is remodeling of the basal lamina, with modifications of acinar morphology, of nuclear shape and size (Medina et al. 2010). Moreover, there are suggestive changes of apoptosis and formation of cytoplasmic vacuoles in acinar cells, which consist of a vesicle species composed of phagocytized intracellular particles or fluids (Elmore 2007). In the present study, the histological analysis performed on glandular tissues submitted to ionizing radiation revealed the presence of these morphological alterations in all irradiated groups, with no significant difference between the animals treated with lidocaine or histamine, both in seven and 30 days after radiotherapy.

Distinct outcome was described by Medina et al. (2010) when evaluating the effect of histamine on submandibular glands of rats irradiated with a dose of 5 Gy. Three days after radiotherapy, a lower number of apoptotic cells were observed, in addition to salivary flow preservation compared to irradiated controls. In the present study, however, the dose of radiation used was 20 Gy, which may explain the results diverging from those observed by Medina et al. (2010). In addition, in our study, the salivary flow of the animals was not measured and the analysis were performed in

periods of seven and 30 days. These follow-up periods, together with the radiation dose used, allowed the evaluation of acute and chronic morphological alterations induced by radiotherapy in the glandular tissue.

Hakim et al. (2012) evaluated the radioprotective effect of lidocaine on parotid and submandibular glands of rabbits submitted to ionizing radiation at doses of 15, 25, 30 and 35 Gy. One week after radiotherapy, despite the decrease in salivary flow, the animals treated with lidocaine presented salivary function superior to the control group, evaluated by means of sialoscintigraphy and sialometry. These authors did not analyze glandular morphological alterations due to ionizing radiation, which makes it difficult to compare with the results of the present study.

In addition to the morphological evaluation previously discussed, the nuclear area of the acinar cells was measured, since the ionizing radiation induces condensation of the nuclear chromatin, resulting in changes in the shape and size of the nuclei of these cells. The nuclear area of acinar cells was significantly higher in the lidocaine and histamine groups than in the irradiated group at seven and 30 days after radiotherapy. The larger cell nuclei, therefore with less condensed chromatin, may indicate greater ease of repair to the damage caused by ionizing radiation to the acinar cells DNA (Jezková et al. 2014). An unusual and unknown result was that at 30 days of experiment the nuclear area of the lidocaine and histamine treated groups was higher than that of the non-irradiated control group.

Studies in animal models have shown that morphological and functional changes are more evident in salivary glands irradiated with a single dose between 15 and 20 Gy (Vissink et al. 1990; Hakim et al. 2005). The animals of the present study have been irradiated with a dose of 20 Gy, presented during the experiment considerable physical impairment, with loss of hair in the irradiated region and loss of

body mass. Four animals died due to the effects of radiotherapy. These data demonstrated that the methodology used in this experiment was adequate and effective in promoting glandular alterations in the parotid glands of the animal models.

In the present study we observed that the parotid glands of the irradiated animals presented important morphological alterations, either under treatment with lidocaine or histamine. Although the nuclei of the acinar cells of the animals treated with these substances have presented superior area, the significance of this finding still needs to be clarified. The methodology used and the results obtained in the present study do not support the radioprotective effect of lidocaine and histamine in preserving the morphology of the irradiated salivary glands.

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DISCUSSÃO COMPLEMENTAR

5 DISCUSSÃO COMPLEMENTAR

A saliva é de suma importância na manutenção da saúde bucal e geral. Disfunções salivares qualitativas e quantitativas predispõe o indivíduo a alterações na mucosa bucal e nos dentes, causam prejuízo às funções orais e impacto negativo na qualidade de vida.[13,24] A radioterapia da região de cabeça e pescoço é uma importante causa de hipossalivação e xerostomia, pois apesar de apresentarem turnover lento, as glândulas salivares são extremamente radiossensíveis.[13,25]

No primeiro artigo dessa dissertação foi realizada uma revisão da literatura com o objetivo de abordar opções terapêuticas para as disfunções salivares. Os sprays orais à base de HEC (Hidroxietilcelulose) e PGM (Poliglicerilmetacrilato) atuam unicamente como lubrificantes da superfície da mucosa, não estimulando a produção salivar. [26,27] Quando ainda há tecido glandular funcional, os estimulantes salivares são uma alternativa de tratamento. Os agentes ácidos, como o ácido málico, combinado com fluoretos, acidificam o meio bucal e aumentam a produção salivar.[28] Alguns fármacos agonistas dos receptores muscarínicos como a pilocarpina são utilizados para manter o estímulo à produção salivar, porém possuem efeitos adversos como sudorese, diarreia, cefaleia, aumento da frequência urinária e náuseas.[13,27] Uma opção para o tratamento da hipofunção salivar é a terapia com células-tronco derivadas do tecido adiposo do próprio indivíduo, processadas e implantadas diretamente na glândula salivar afetada pela radiação ionizante. Esta técnica ainda demanda sensibilidade técnica e custo elevados, mas pode ser uma opção promissora no tratamento da hipofunção salivar.[29] Para selecionar-se a modalidade terapêutica para a hipofunção salivar diversos fatores devem ser considerados em conjunto com o paciente. Todo tratamento deve ser

monitorado e constantemente avaliado, seus efeitos adversos observados e a relação custo-benefício ponderada.

O segundo artigo descreve um experimento em ratos, realizado com o objetivo de avaliar o efeito da lidocaína e histamina sobre alterações morfológicas induzidas pela radioterapia nas glândulas parótidas. A lidocaína, sendo um agente estabilizador de membrana, atua sobre os neuroreceptores das células acinares, ativando a proteína quinase-Cα responsável pela mobilização de cálcio e excreção de fluidos do meio intra para o extra-celular. Além disso, regula a expressão da tenascina-c, proteína que regula o comportamento, a adesão e a forma do tecido.[23,30-33] A histamina induz o aumento da proliferação celular, reduzindo os danos teciduais por meio da regulação das reações imunológicas desencadeadas pela radioterapia. Atua também nas aquaporinas presentes na membrana das células acinares, que proporcionam a secreção salivar.[10,14,34]

A análise histológica realizada nos tecidos glandulares submetidos à radiação ionizante revelou a presença de alterações morfológicas tais como desorganização acinar, vacuolação citoplasmática, alterações sugestivas de apoptose/necrose e pleomorfismo nuclear em todos os grupos irradiados, sem diferença significativa entre os animais que foram tratados com lidocaína ou histamina. A área nuclear das células acinares mostrou-se significativamente superior nos grupos tratados com lidocaína e histamina em relação ao grupo irradiado tanto aos sete, quanto aos 30 dias após a radioterapia. O núcleo celular maior, portanto com a cromatina menos condensada, pode indicar maior facilidade de reparo aos danos causados pela radiação ionizante no DNA das células acinares. [35] Apesar deste resultado, não se pode inferir que a lidocaína e a histamina tenham apresentado efeito em preservar a morfologia das glândulas salivares irradiadas.

Diversas doses de radiação e distintos períodos de acompanhamento têm sido utilizados para analisarem-se os danos causados pela radiação às glândulas salivares. Choi et al.[36] avaliaram os efeitos da radioterapia em ratos irradiados com doses únicas de 3, 10, 20 e 30 Gy. Períodos de até cinco dias após a radiação foram utilizados para estudar os efeitos agudos, e de 10, 20, 30 e 60 dias para avaliarem-se os efeitos tardios. Alterações degenerativas agudas foram observadas até o décimo dia de estudo, sendo dependentes da dose de radiação administrada. Neste mesmo estudo, sinais de degeneração e decréscimo das células acinares foram encontrados nas glândulas salivares 30 dias após a irradiação. Portanto, no presente estudo optou-se por utilizar dose única de 20 Gy e avaliar os danos celulares aos sete e 30 dias após os animais terem sido submetidos à radiação ionizante. Para a administração da lidocaína, buscaram-se na literatura as concentrações mais utilizadas,[31,32] definindo-se administrar neste modelo animal a concentração de 2%, na dosagem de 1mg/Kg, intraperitonealmente. De acordo com a natureza deste fármaco, a via intraperitoneal causa menos desconforto ao animal. Segundo Medina et al.,[34] a histamina causa menos efeitos colaterais quando administrada pela via subcutânea. Com base neste estudo, optou-se por utilizar a histamina na concentração de 0,1 mg/kg, iniciando-se 24 horas antes da radioterapia e encerrando seis dias após. Desta forma foi possível manter os níveis do fármaco no organismo durante a radioterapia e nos dias subsequentes.

Devido à alta complexidade metodológica, não foi possível realizar a avaliação do fluxo salivar dos animais. Notou-se durante o experimento que, mesmo antes da radioterapia e com os animais contidos em um plano inclinado, a quantidade de saliva expelida apresentava-se muito baixa, não havendo a possibilidade de ser coletada.

Levando-se em consideração as limitações metodológicas desta pesquisa, os resultados deste estudo sugerem que nas dosagens e vias de administração empregadas, a lidocaína e a histamina não apresentaram efeito em preservar a morfologia das glândulas salivares submetidas à radiação ionizante.

CONCLUSÕES

6 CONCLUSÕES

Com base na metodologia empregada e nos resultados obtidos, podemos concluir que:

- A lidocaína e a histamina não apresentaram efeitos em preservar a morfologia de glândulas parótidas de ratos submetidas à radiação ionizante.

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7 REFERÊNCIAS COMPLEMENTARES

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ANEXO A

APROVAÇÃO DA COMISSÃO CIENTÍFICA DA FACULDADE DE ODONTOLOGIA DA PUCRS

SIPESQ

Sistema de Pesquisas da PUCRS

Código SIPESQ: 6392 Porto Alegre, 27 de maio de 2015.

Prezado(a) Pesquisador(a),

A Comissão Científica da FACULDADE DE ODONTOLOGIA da PUCRS apreciou e aprovou o Projeto de Pesquisa "AVALIAÇÃO DO EFEITO RADIOPROTETOR DA LIDOCAÍNA E DA HISTAMINA EM GLÂNDULAS PARÓTIDAS DE RATOS" coordenado por FERNANDA GONCALVES SALUM. Caso este projeto necessite apreciação do Comitê de Ética em Pesquisa (CEP) e/ou da Comissão de Ética no Uso de Animais (CEUA), toda a documentação anexa deve ser idêntica à documentação enviada ao CEP/CEUA, juntamente com o Documento Unificado gerado pelo SIPESQ.

Atenciosamente,

Comissão Científica da FACULDADE DE ODONTOLOGIA

ANEXO B

APROVAÇÃO DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS DA PUCRS

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA, INOVAÇÃO E DESENVOLVIMENTO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Ofício 61/2015 - CEUA

Porto Alegre, 08 de setembro de 2015.

Prezado Sr(a). Pesquisador(a),

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou seu Protocolo de Pesquisa, registro CEUA 15/00455, intitulado **"Avaliação do efeito radioprotetor da lidocaína e da histamina em glândulas parótidas de ratos".**

Sua investigação, respeitando com detalhe as descrições contidas no projeto e formulários avaliados pela CEUA, está **autorizada** a partir da presente data.

Informamos que é necessário o encaminhamento de relatório final quando finalizar esta investigação. Adicionalmente, ressaltamos que conforme previsto na Lei no. 11.794, de 08 de outubro de 2008 (Lei Arouca), que regulamenta os procedimentos para o uso científico de animais, é função da CEUA zelar pelo cumprimento dos procedimentos informados, realizando inspeções periódicas nos locais de pesquisa.

Nº de Animais	Espécie	Duração do Projeto
56	Rattus novergicus	09/2015 – 04/2016

Atenciosamente,

Prof. Dr. João Batista Blessmann Weber
Coordenador da CEUA/PUCRS

Ilma. Sra.
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