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Radioprotective Effect of Sodium Selenite on Mandible of Irradiated Rats

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The purpose of this study was to test the radioprotective effect of selenium in the bone microarchitecture of irradiated rats mandibles. Forty rats were separated into 4 groups with 10 animals: control group (CG), irradiated group (IG), sodium selenite group (SSG) and sodium selenite irradiated group (SSIG). A single dose of 0.8 mg/kg sodium selenite was administered intraperitoneally in the SSG and SSIG groups. One hour later, animals of IG and SSIG groups were irradiated with 15 Gy of x-rays. Forty days after radiation a bilateral extraction of the mandibular first molars was performed. After the extraction procedure, five rats were killed after fifteen days and others five after thirty days. Micro-computed tomography was used to evaluate cortical and trabecular bone of each rat. The mean and standard deviation of each bone microarchitecture parameter were analyzed using the statistical test of two-way Analysis of Variance (ANOVA). At 15 days, the bone volume presented higher values in the CG and SSG groups ($p=0.001$). The same groups presented statistically significant higher values when bone volume fraction ($p<0.001$) and trabecular thickness ($p<0.001$) were analyzed. At 30 days, it was observed that in relation to the bone volume fraction, SSG group presented the highest value while SSIG group had the lowest value, with statistically significant difference ($p=0.016$). Sodium selenite demonstrated a median radioprotective effect in the bone microarchitecture of irradiated mandibles, which indicates the substance may be a potential radioprotective agent against chronic effects of high doses of ionizing radiation.

Key Words: radiation-protective agents, ionizing radiation, selenium.

Introduction

Malignant lesions on head and neck are usually treated by a combination of surgery and radiotherapy with/without chemotherapy (1). Despite the increased survival rate of patients who make use of these modalities of treatment, still have considerable morbidity due to the deleterious effects of radiotherapy, which uses high doses of ionizing radiation, on regular cellular components, resulting in acute and/or chronic complications in the tissues of the craniofacial region (2,3).

After radiotherapy, the oral acute complications are generally reversible. However, delayed complications promote changeless damage to irradiated regions, such as bone tissue (3). The blood vessels of the periosteum injuries cause osteoblastic and osteoclastic activity decrease, and an increase of fibrotic and adipose tissues in the bone marrow, resulting in decreased cellularity and blood vessels (4). Risk factors may potentiate the effects caused by this metabolic disorder. Among them, tooth extractions as well as any form of surgical manipulation of bone tissue, are associated with increased risk for your progress (5). This association may be caused by increased release of free radicals after the surgical procedure, boosting the oxidative stress process due release of free radicals from the effects of ionizing radiation. During the oxidative process occurs the donation of a hydrogen atom from a chain of unsaturated fatty acids of phospholipids, causing the process of lipid peroxidation and consequently damage to DNA, proteins and lipids membrane (6,7).

According to a new theory known as the "fibro-atrophic theory", osteroradionecrosis occurs because of fibro-atrophic mechanism induced by radiation. In addition, befall formation of free radicals, destruction of endothelial cells, non-specific chronic local inflammation, microvascular thrombosis, fibrosis and, finally, osseous and tissue necrosis that results in tissue hypoxia, hypocellularity and hypovascularity (8,9). Consequently, due to metabolic and homeostatic alterations of bone tissue, it occurs damage in remodeling and bone healing, besides increasing susceptibility to bone infections (3).

In order to reduce toxic effects of ionizing radiation on healthy tissues, several studies have evaluated a variety of radioprotective agents (3,6,10,11). These agents may be act by the elimination of free radicals, behave as "sweepers" or indirectly by incentive of production of antioxidant enzymes (12). Selenium, an important micronutrient for humans, protects normal tissues from damages of radiation by synthesis of selenoproteins, reducing the oxidative stress effects. Additionally, studies indicate that antioxidant enzymes, such as thioredoxin reductase and glutathione peroxidase, are composed of Se, which assigns it a radioprotective action.

Since high doses of ionizing radiation inevitably causes physiological changes to healthy tissues, as bone, and oxidative action by excessive free radicals produced is the prime factor for its development, the objective of the present study was to evaluate the damages of high doses of ionizing radiation on bone microarchitecture of rat's mandibles, besides evaluating selenium as a radioprotective agent on the bone physiology it these animals, evaluating the tooth socket repair.

Material and Methods

The local Animal Research Ethics Committee approved this work without restrictions. The sample size was calculated in Biostat software (version 5.3, Instituto de Desenvolvimento Sustentável Mamirauá, Tefé, MA, Brazil) and was based on the minimum difference between the groups studied and their standard deviation, adopting a test's power of 0.80. For this, the authors were based on the results of an earlier study with similar design (3). Thus, the sample was composed by forty male rats (*Rattus norvegicus*, Albinus Wistar) with 3-month-old, weighing approximately 350 g. The animals were kept in polycarbonate cages in a room at a controlled temperature, in a light/dark cycle of 12 h. They were feed with standard balanced food and ad libitum water.

The forty rats were randomly divided into 4 groups with 10 animals. The groups were classified as follows: control group (CG) - no sodium selenite applied and radiation; irradiated group (IG) - no sodium selenite applied, however with radiation; sodium selenite group (SSG) - sodium selenite applied and no radiation; sodium selenite irradiated group (SSIG) - sodium selenite applied and radiation. Half of animals of each group was killed at 15 days after tooth extraction (n=5), and the other half at 30 days after tooth extraction (n=5).

All the rats were anaesthetized with a single intramuscular injection containing 10 mg/kg xylazine (Rompun, Bayer S.A., São Paulo, SP, Brazil) and 100 mg/kg ketamine (Dopalen, Ceva Saúde Animal, Paulínia, SP, Brazil) with 75 days old. Then, a single dose of 0.8 mg/kg sodium selenite (Merck KgaA, Darmstadt, Germany) (10) was applied intraperitoneally to the rats of SSG and SSIG groups. The rats of IG and SSIG groups were irradiated in head and neck region one hour later with 15 Gy of X-radiation (100 cm focal length, 15x30 cm collimation field) in a linear accelerator (Clinic 6/100®, Varian Medical Systems, Palo Alto, CA, USA). With the intention of expose the rats of all groups to the same stimuli and stresses, the animals of CG and SSG groups were anaesthetized and received distilled water intraperitoneally instead of sodium selenite.

Forty days after irradiation procedure, the rats were anaesthetized again following the same protocol and a bilateral extraction of mandibular first molars was performed. For the extraction procedure, a Hollenback dental instrument (Duflex®, S.S. White, Rio de Janeiro, RJ, Brazil) was used. With fifteen days after tooth extraction, five rats were killed with an anesthesia overdose of Tanohalo (Halotano, Cristália, Itapira, SP, Brazil). The other five were killed thirty days after the extraction procedure. Before removing the hemimandibles, gingival healing and the presence of fistula were observed at the site where the mandibular first molar was removed. All the hemimandibles were immersed for 72 h in 10% buffered formalin and, after that, stored in refrigerated saline solution.

A hemimandible of each rat was scanned using a micro-CT scanner (SkyScan 1174; SkyScan, Kontich, Belgium) with the following acquisition parameters: 50 kV, 800 µA, 9.47 µm pixel size, 180° total rotation, 0.45° rotation step, 3 frames and 0.5 mm aluminum filter. In order to reconstruct the images, NRecon software (SkyScan, Kontich, Belgium) with the following parameters: 700 sections, 5 for ring artefact reduction, 1 for smoothing, 10% of beam-hardening correction and a histogram with a dynamic range of 0.01–0.12 was used.

All the images were analyzed employing CTAn software (SkyScan). The distal socket of the extracted mandibular first molar were chosen to manually define the area with alveolar socket healing. The analyses were done with a threshold varying from 25 to 65 and from 25 to 80 for the hemimandibles. After delimiting the area and standardizing the threshold, the analysis of the region of interest was done to evaluate the following parameters of the bone microarchitecture: total volume, bone volume, bone volume fraction (bone volume/total volume), trabecular number, trabecular thickness and trabecular separation, according to Bouxsein et al. (13).

The mean and standard deviation of each bone microarchitecture parameter was tabulated and the results were analyzed using the statistical test of two-way Analysis of Variance (ANOVA) and post hoc Tukey test, using IBM SPSS Statistics 22.0 (IBM Corp, Armonk, NY, USA). Statistical significance level was set with α of 5%.

Results

Before removing the hemimandibles, complete gingival healing was observed at the site where the mandibular first molar was removed. In addition, no fistulas were observed in any animals.

The mean and standard deviation of bone microarchitecture values for each group after 15 and 30 days of the tooth extraction are shown in Tables 1 and 2.

The values of bone microarchitecture after 15 days of the tooth extraction (Fig. 1) showed that there was no statistically significant difference among the groups (p=0.238) when total volume was analyzed. When the bone volume was evaluated, higher values were observed in the CG and SSG groups (p=0.001). The same groups presented statistically significant higher values when bone volume fraction (p<0.001) and trabecular thickness (p<0.001) were analyzed. The trabecular number evaluation showed a statistically significant higher value for SSIG group when compared with IG group (p=0.036). Unlike the others, trabecular separation was significantly higher in the IG and SSIG groups (p<0.001).

Table 1. Mean and standard deviation of bone microarchitecture data regarding volume analysis for each group

Group		Total volume (mm^3)	Bone volume (mm^3)	Bone volume fraction (%)
15 days	CG	1.834±0.270 a	1.376±0.183 a	75.433±7.975 a
	IG	1.891±0.086 a	0.776±0.176 c	40.803±7.651 b*
	SSG	1.761±0.196 a	1.290±0.258 ab	72.636±7.171 a
	SSIG	2.026±0.128 a	0.940±0.096 bc	46.402±3.418 b*
30 days	CG	1.574±0.086 a	1.260±0.127 a	80.155±8.009 ab
	IG	1.602±0.231 a	1.215±0.194 a	75.845±5.513 ab
	SSG	1.695±0.349 a	1.426±0.292 a	84.285±3.846 a
	SSIG	1.881±0.365 a	1.333±0.341 a	70.247±4.971 b

Control group (CG); irradiated group (IG); sodium selenite group (SSG); sodium selenite irradiated group (SSIG). Means followed by different letters in the same column are significantly different, according to ANOVA and the Tukey test. *Differ from 30 days.

Table 2. Mean and standard deviation of bone microarchitecture data regarding trabecular analysis for each group

Group		Trabecular number (1/mm)	Trabecular thickness (mm)	Trabecular separation (mm)
15 days	CG	3.753±0.218 ab	0.202±0.027 a	0.159±0.049 a
	IG	3.457±0.580 b	0.119±0.020 b*	0.331±0.037 b
	SSG	3.708±0.201 ab	0.197±0.025 a	0.175±0,041 a
	SSIG	4.395±0.600 a	0.107±0.012 b	0.310±0.082 b
30 days	CG	5.286±1.023 a	0.159±0.045 a	0.100±0.020 a
	IG	3.965±1.280 a	0.204±0.054 a	0.219±0.104 b
	SSG	4.955±0.542 a	0.171±0.013 a	0.099±0.029 a
	SSIG	4.959±2.138 a	0.161±0.065 a	0.208±0.110 ab

Control group (CG); irradiated group (IG); sodium selenite group (SSG); sodium selenite irradiated group (SSIG). Means followed by different letters in the same column are significantly different, according to ANOVA and the Tukey test. *Differ from 30 days.

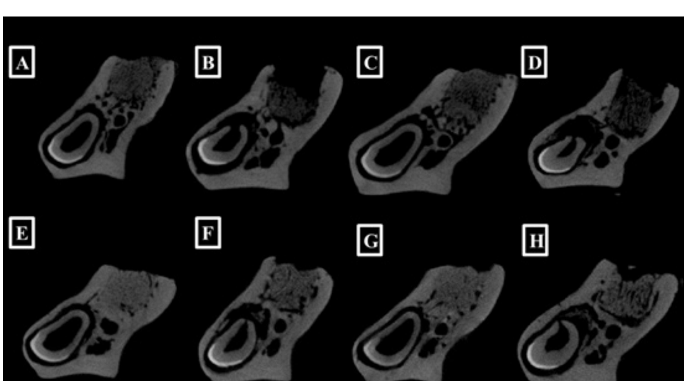

Figure 1. Region of healing in the alveolar socket at 15 days (A–D) and 30 days (E–H) after tooth extraction. A, E: Control group; B, F: irradiated group; C, G: sodium selenite group; D, H: sodium selenite irradiated group.

When the bone microarchitecture was analyzed after 30 days of the tooth extraction (Fig. 1), it was observed that in relation to the bone volume fraction, SSG group presented the highest value while SSIG group had the lowest value, with statistically significant difference (p=0.016). Furthermore, trabecular separation was statistically higher (p=0.036) in the IG group than in the CG and SSG groups. No statistical differences were observed in total volume (p=0.378), bone volume (p=0.565), trabecular number (p=0.441) and trabecular thickness (p=0.434).

Bone volume fraction showed higher values at 30 days than at 15 days in IG and SSIG (p<0.0001). Additionally, trabecular thickness significantly increased from 15 days to 30 days in IG (p=0.001).

Discussion

Radiotherapy is a conventional treatment of malignant lesions. However, despite the improvement of radiotherapy protocols, ionizing radiation still has side effects on normal cells that may have repercussions in the oral cavity. Among them, changes in bone physiology can trigger complications of greater effect, such as osteoradionecrosis. In this way, the application of experimental models using animals is a useful tool for a better understanding of these metabolic changes (14-16). Additionally, it is important to test substances as radioprotective agents in order to eliminate or decrease such side effects.

Previous studies (3,15,16-18) have sought to develop a model for induction of osteoradionecrosis in animals, through bone physiological changes with ionizing radiation; however, only a few have sought to evaluate means to prevent the development of the same. Moreover, although some experimental studies have studied the effect of radioprotective agents on the injuries of ionizing radiation (6,10,19-21), their search was made with the goal to prevent or reduce acute complications of high doses of ionizing radiation. According to our knowledge, only one study (3) was found in the literature in which it was used a substance (a non-steroidal anti-inflammatory drug) with the aim to prevent or minimize chronic complications caused by ionizing radiation. Therefore, the present study may be considered a pioneer for using a trace element essential in humans as a radioprotective agent in relation to delayed modifications in irradiated bone.

Selenium is an essential nutrient and most of its derivatives, as the sodium selenite, have their antioxidant properties largely acknowledged and their radioprotective properties has been studied in various tissues, such as salivary glands cells (22), gastrointestinal epithelium (23), kidneys (24), healthy bone (6), and osteoporotic bone (10), but all of them have evaluated acute complications. The most accepted hypothesis about the antioxidant action of selenium seems to be related to the capacity to inhibit the peroxidation, capturing free radicals and repairing damage to cellular components, since this compound is a component of peroxidase glutathione enzyme (25).Thus in this study, selenium was used to prevent or minimize the possible chronic complications on irradiated mandible bone induced by radiation.

Bone physiology, being a dynamic process, could not be directly evaluated. Thus, in this study, we used a method of indirect evaluation through the tooth socket healing capacity, which is related to clinical scenarios as possible tooth extractions. In addition, it was not found in previous studies values for parameters of trabecular bone microstructure regarding to trabecular analysis of tooth socket repair in rats associated with the use of selenium-based compounds. Therefore, the results found in the present study consist in possible references for subsequent studies.

The present study was used an external irradiation to simulate conventional protocol of radiotherapy of head and neck, in agreement with other studies (3,6,18) . As previous studies (3,6,10), it has not been used fractionated irradiation, although it is proposed in radiotherapy of head and neck of patients, due to its high complexity in experimental animal models, requiring multiple exposures to ionizing radiation and repeated anesthesia, conditions that could provide a lower survival rate of the animals. Thus, we used a single only one of 15 Gy dose. Moreover, using the equation of the linear-quadratic model to calculate a total dose in fractional irradiation treatment and according to equation of the linear-quadratic model (26) and given that the α/β radiosensitivity ratio is equals to 2 for bone tissue (5), the dose used in this study is corresponding to a total dose of approximately 64 Gy, when using multiple incremental doses of 2 Gy. Thus, it is compatible with the protocol conventionally adopted in radiotherapy of head and neck and with the minimum irradiation (60 Gy) dose required to induce osteoradionecrosis (3,15).

In order to simulate a clinical situation of a patient that was submitted to a tooth extraction after radiotherapy, the present study performed the same dental procedure before irradiation in an animal model to evaluate physiological bone changes in rats. In the present study, the macroscopic evaluation showed clinical signs compatible with the absence of osteoradionecrosis. However, despite of not being able to induce osteoradionecrosis clinically, previous studies (3, 14,15) observed microscopic alterations in the irradiated bone tissue. The present study also showed signs compatible with alterations in bone physiology due to ionizing radiation, as a substantial delay in the healing of alveolar socket in early times after dental extraction was observed.

The evaluation of total volume was similar in all groups, which means that the area of alveolar socket healing set by three-dimensional analysis on micro-CT was selected in a standardized way. This occurrence can be observed by similar values of total volume in all groups. As well as Yamazaki et al. (3), comparing control group and irradiation group, it was observed that the alveolar repair at 15 days was clearly retarded by ionizing radiation. However, the same effect is not seen 30 days after tooth extraction, indicating that irradiation has a more effectively in the intermediate period than in the final period of a alveolar socket healing. This aspect was proved by the significant increased values of bone volume fraction and the decreased values of trabecular thickness in 30 days. Furthermore, in accordance with previous studies (3,14,15), the results of this study showed the variation of values between irradiated and non-irradiated groups decrease as more time after tooth extraction. Despite the methodological differences between these studies, this result corroborates with hypothesis that the intermediary stage of the tooth socket healing is more sensitive to irradiation. Moreover, the irradiated group presented only a single parameter of the trabecular bone microstructure changed, considering that this group performed significantly less value of trabecular separation when compared with average control group even after 30 days after dental surgery procedure. Thus, this suggests the non-mineralized portion of alveolar healing is more affected than the mineralized portion in irradiated bone.

It was observed that the sodium selenite provided no prejudice in the trabecular microstructure parameters, displaying significant improvement only in relation to trabecular number to 15 days of trabecular alveolar healing. For 30 days after tooth extraction, trabecular number was positively influenced by selenium-based compound. In view of the evil effects of irradiation in bone tissue and seeking to ease them, Freitas et al. found radioprotective effect of sodium selenite on irradiated bone repair using a dose of 0.8 mg/kg. Despite of this study have used similar dose of sodium selenite to previous study, radioprotective effects obtained when evaluated the process of alveolar bone healing in mandible of rats were more discreet than bone repair in rat tibias. The use of higher doses of selenium possibly would trigger adverse effects due to in toxicity, which was confirmed by studies (10,24) that made use of high doses.

The bone microarchitecture parameters are important indicators to evaluate the bone quality in animal models. Two-dimensional evaluations using histomorphometry are traditionally used (27). However, because it is a destructive method for the sample, evaluation of bone quality through three-dimensional (3D) images has been encouraged (3).Micro-CT has been pointed out as a high resolution method that allows the 3D evaluation of microarchitecture bone, providing valuable information about the structural parameters similar to those obtained by histomorphometric analysis (27,28). Although histomorphometry was considered a reference standard in this type of evaluation, the present study used micro-CT for providing morphological and dynamic information of architecture bone, being a non-destructive method of the sample, presenting short work time and results similar to that reference method, as seen in literature (27,28). We encourage new studies that can perform complementary analyzes to the micro-CT in order to evaluate bone metabolism that can complement our results.

Due to limited number of studies that evaluated the effect of radioprotective agents on bone physiology through the evaluation of alveolar socket healing, mainly in late periods of after tooth extractions, there is a difficulty for the make a parallel of the results of the present study with other studies. About the radioprotective properties of selenium, despite the discreet results obtained by this study, the radioprotective results obtained by previous studies and the improvement of microarchitecture trabecular number in the alveolar socket healing in this study, motivate the authors of this study to believe that selenium can be an efficient agent on protection against ionizing radiation in relation to irradiated bone physiology, requiring more studies to be completed elucidated. In summary, the results of this study suggest that ionizing radiation affects physiology of irradiated bone, delaying the tooth socket repair. Sodium selenite presented a mild radioprotective action in the bone tissue of irradiated mandibles as a positive effect was observed only on the trabecular number of bone tissue microarchitecture. However, such positive effect adds to the fact that no adverse effects were observed with the dose used here indicate the substance may be a potential radioprotective agent against chronic effects of high doses of ionizing radiation.

Resumo

O propósito deste estudo foi testar o efeito radioprotetor do selênio na microarquitetura óssea de mandíbulas de ratos irradiados. Quarenta ratos foram separados em 4 grupos com 10 animais: grupo controle (GC), grupo irradiado (GI), grupo selenito de sódio (SSG) e grupo selenito de sódio irradiado (SSIG). Uma dose única de 0,8 mg/kg de selenito de sódio foi administrada intraperitonealmente nos grupos SSG e SSIG. Uma hora depois, os animais dos grupos IG e SSIG foram irradiados com 15 Gy de raios-x. Quarenta dias após a irradiação foi realizada extração bilateral dos primeiros molares inferiores. Após o procedimento de extração, cinco ratos foram mortos após quinze dias e outros cinco após trinta dias. A microtomografia computadorizada foi utilizada para avaliar o osso cortical e trabecular de cada rato. A média e o desvio padrão de cada parâmetro da microarquitetura óssea foi analisada pelo teste estatístico de Análise de Variância dois fatores (ANOVA), seguido por comparações post hoc com o teste de Tukey. Após 15 dias, o volume ósseo apresentou valores mais elevados nos grupos GC e GNS ($p=0,001$). Os mesmos grupos apresentaram valores estatisticamente significantes maiores quando se analisou fração de volume ósseo ($p<0,001$) e espessura trabecular ($p<0,001$). Após 30 dias, observou-se que, em relação à fração de volume ósseo, o grupo SSG apresentou o maior valor enquanto o grupo SSIG apresentou o menor valor, com diferença estatisticamente significante ($p=0,016$). O selenito de sódio demonstrou um efeito radioprotetor mediano na microarquitetura óssea das mandíbulas irradiadas, o que indica que a substância pode ser um potencial agente radioprotetor contra os efeitos crônicos da radioterapia.

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