Effectiveness of acidified electrolyzed water in the prevention of bronj after exodonts: experimental study in wistars rats

Eficácia da água eletrolisada acidificada na prevenção da bronj após exodontias: estudo experimental em ratos wistars.

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ABSTRACT
Bisphosphonate-related osteonecrosis of the jaws (BRONJ) is a complication associated with dental intervention and prolonged use of bisphosphonates (BF). Acidified Electrolyzed Water (AEA) is a substance that seems to improve tissue regeneration. To evaluate the effectiveness of (AEA) as an adjuvant in the treatment of BRONJ after lower molar extractions in Wistar rats. A split-mouth study was carried out in twelve Wistar rats (6 females and 6 males) received weekly intravenous injection of 0.3 mL of zoledronate (80 μg/kg in PBS) for 9 weeks and underwent tooth extraction. mandibular first and second molars in the eighth and ninth week, respectively. The Acidified Electrolyzed Water (AAG) group (right side of the mandible) was irrigated with AEA every three days while the control group (CG) (right side of the mandible) received no treatment after extractions. The animals were euthanized 6 weeks after the second extraction, the mandibles were dissected for clinical analysis with the data obtained submitted to statistical analysis with a significance level of 0.05. (Fischer's test). Two males were lost during AEA therapy, leaving 10 animals, 6 females and 4 males. Inflammation was absent in all alveoli; bone sequestration was noted in all alveoli; suppuration was present in 3 sites of the GC group, epithelialization present in 5 GAEA alveoli and 2 GC. Comparison of bone exposure did not show statistically significant results. The results obtained were slightly favorable to the use of AEA, however, without statistical significance.

Keywords: Bisphosphonates; extraction; bisphosphonate-associated osteonecrosis.

RESUMO
A osteonecrose dos maxilares relacionada ao uso de bisfosfonatos (BRONJ) é uma complicação associada à intervenção odontológica e ao uso prolongado de bifosfonatos (BF). A Água Eletrolisada Acidificada (AEA) é uma substância que parece melhorar a regeneração tecidual. Avaliar a efetividade da (AEA) como adjuvante no tratamento da BRONJ após exodontias dos molares inferiores em ratos Wistar. Foi realizado um estudo de boca dividida em doze ratos Wistars (6 fêmeas e 6 machos) receberam injeção endovenosa semanal de 0,3 mL de zoledronato (80 μg/kg em PBS) por 9 semanas e foram submetidos à exodontia dos primeiros e segundos molares inferiores na oitava e nona semana, respectivamente. O grupo da Água Eletrolisada Acidificada (GAEA) (lado direito da mandíbula) foi irrigado com AEA a cada três dias enquanto o grupo controle (GC) (lado direito da mandíbula), não recebeu tratamento após as exodontias. Os animais foram eutanasiados 6 semanas após a segunda exodontia, as mandíbulas foram dissecadas para análise clínica com os dados obtidos submetidos à análise estatística com nível de significância de 0,05. (Teste de Fischer). Dois machos foram perdidos durante a terapia com AEA, restando 10 animais, 6 fêmeas e 4 machos. A inflamação mostrou-se ausente em todos os alvéolos; notou-se sequestro ósseo em todos os alvéolos; supuração estava presente em 3 sítios do grupo GC, epitelialização presente em 5 alvéolos GAEA e 2 GC. A comparação da exposição óssea não apresentou resultados estatisticamente significantes. Os resultados obtidos foram ligeiramente favoráveis ao uso da AEA entretanto, sem significância estatística.

Palavras-chave: Bisfosfonatos; exodontia; osteonecrose associada a bisfosfonatos.
INTRODUCTION

Bisphosphonates (BFs) are antiresorptive medications with oral or intravenous dosage (LORENZO-POUSO et al., 2019), frequently used for the treatment of bone metastases, osteoporosis and other bone diseases (LORENZO-POUSO et al., 2019). The extensive use of these medications has led to bone complications in the maxilla and mandible, which have been the subject of extensive investigation in recent year (KHAN et al., 2015).

The term bisphosphonate-associated osteonecrosis of the jaw (BRONJ), “Bisphosphonate-Related Osteonecrosis of the Jaw (BRONJ)” was first described when Marx reported in 2003 that 36 patients taking pamidronate or zolendranate (ZOL) developed mandibular and/or maxillary bone lesions (MARX, 2003). Subsequently, the American Association of Oral and Maxillofacial Surgery (AAOMS) recommended the definition of the English term “BRONJ”, or BRONJ in Portuguese, as a region of exposed bone in the maxillofacial region in a patient treated with BFs and who did not receive radiotherapy in the region. craniofacial and that does not heal during the 8 weeks after its identification (RUGGIERO et al., 2009).

Recently, it was discovered that other medications besides bisphosphonates can cause this condition, such as Denosumab (an anti-resorptive agent) and Bevacizumab (an angiogenic inhibitor), which led the AAOMS to institute a new, broader term to name these lesions, "osteonecrosis of the drug-related jaws" (BRONJ), or "Bisphosphonate-Related Osteonecrosis of the Jaw (BRONJ)" (RUGGIERO et al., 2009; 2014).

The pathophysiology of BRONJ is a subject of great debate in the literature. Some hypotheses are related to inhibition of osteoclastic resorption and remodeling (REINWALD and BURR, 2008; RISTOW et al., 2014), inflammation and infection (AGHALOO et al., 2014; KHAN et al., 2015), inhibition of angiogenesis (BEZZI et al., 2003; PABST et al., 2014) dysfunction of innate or acquired immunity and soft tissue toxicity (KIKUIRI et al., 2010).

At the local level, the risk factors for this condition are mainly tooth extractions (URADE et al., 2011) and periodontal disease (SHIBAHARA et al., 2018).

Other causes of inflammation that can spread easily are those originating from dental infections such as caries and pulpitis (SHIBAHARA et al., 2018). However, tooth extraction is reported as the most common trigger for the condition occurring with a prevalence of 41% in patients using bisphosphonate (URADE et al., 2011)

Although several treatment protocols have been proposed, there is still no consensus on the therapeutic approach to BRONJ, due to the limited effectiveness of these treatments. Several new therapeutic proposals have been tested for the prevention and treatment of this condition (AZARPAZHOOH, and LIMEBACK, 2008; CEPONIS et al., 2017; INCHINGOLO et al., 2017; NØRHLAND and HARTLEV, 2016; SILVA et al., 2017; SOYDAN and UCKAN, 2014). In this context, Acid Electrolyzed Water (AEA) that has already been
investigated for wound healing and as a microbial agent (BUI et al., 2017; OKUBO, URAKAMI, TAMURA, 1999; TAMAKI et al., 2014; YAHAGI et al., 2000) may show promise as an adjunct to the prevention of BRONJ.

AEA is generated by electrolysis of water containing sodium chloride or potassium chloride in an electrolysis chamber. An exchange diaphragm separates the cathode from the anode, where AEA is formed with a pH close to 2, high oxidation reduction potential or "Oxidation Reduction Potential, (ORP)“, high concentrations of dissolved oxygen, available free chlorine or " Called Free Chlorine, (FAC)“ and hypochlorous acid, which has strong bactericidal activity and disinfection potential (KUBOTA et al., 2009).

The objective of this work was to test the use of the AEA substance as an adjuvant for the treatment of BRONJ after tooth extractions.

MATERIAL AND METHODS

This work was approved by the Ethics Committee in Research on the Use of Animals at Universidade Positivo (CEUA-UP) under protocol 503 and was carried out in accordance with the ARRIVE guidelines (KILKENNY et al., 2010).

The study was carried out in the vivarium of Universidade Positivo (UP, Curitiba, Brazil) and involved twelve rats (Rattusnorvegicus, Wistar albinus), 6 females and 6 males, six months old, weighing approximately 500 g. During the experimental period, the ambient conditions of light, temperature and humidity were controlled to maintain a photoperiod of 12 h, with a temperature of 18–22 °C and a humidity of 65%. The animals were placed in cages (n=2/cage) and provided with regular animal feed (Nuvital, Colombo/PR) and water ad libitum.

To determine the number of animals per group, the parameters obtained in the histological evaluation of bone repair from a previous study were used (HOWIE et al., 2015). The calculation was performed with a 95% two-sided confidence interval, 80% power ratio of 1:1 sample size, on the website http://www.openepi.com/SampleSize/SSMean.html.

All animals were submitted to an osteonecrosis induction model, performed according to the study by HOWIE et al. (2015), to promote bilateral mandibular osteonecrosis. Subsequently, to perform the split-mouth study, they received AEA treatment only on the right side of the mandible. Thus, the right hemimandibles made up the treatment group with acidified electrolyzed water (GAEA). The left side received no treatment, thus composing the control group (CG).

Induction of osteonecrosis

Under sedation, all animals received a weekly intravenous injection, through the tail vein, of ZOL at a dose of 80 μg/kg, in PBS (Novartis, Curitiba, Brazil) for 9 consecutive
weeks (Figure 1A). All animals were sedated by isoflurane inhalation (Cristália, Itapira, SP, Brazil) for approximately one minute, under the supervision of a veterinarian.

During the eighth week of application, the animals underwent surgery to remove the lower first molars (1MI). In the ninth week of application, the animals underwent a second surgical intervention for extraction of the lower second molars (2MI).

The extractions were performed by two experienced surgeons. The animals, in addition to being sedated with isoflurane, were anesthetized with 10% ketamine hydrochloride at a dose of 75mg/kg (Vetbrands, Paulínia, SP) and xylazine hydrochloride 2% at a dose of 5mg/kg (Vetbrands, Paulínia, SP, Brazil), by intraperitoneal injection. They were then positioned laterally and mouth opening was performed with orthodontic elastics n 5/16. The tongue was removed by suturing with 4-0 nylon thread. A surgical extractor adapted to perform the syndesmotomy and dislocation and a Mayo-Hegar needle holder for tooth avulsion were used (Figure 1B, C). After the extractions, an osteotomy was performed with a n.2 spherical carbide bur, through a straight handpiece coupled to a surgical electric motor at 15,000 revolutions per minute (RPM, Stryker, Kalamzaoo, MI, USA), with the aim of remove the root remains and also create a bone defect (Figure 1D). Thus, the procedure was comparable to a complex tooth extraction in humans.

To control postoperative pain, they were medicated with tramadol on the three days following surgery, at a dose of 5mg/kg and soft food.

The animals were observed for signs of bleeding, discomfort or lack of food intake, and were fed with crushed chow for 3 days after the extractions.

![Figure 1: Methodology for inducing osteonecrosis. A) Intravenous injection via tail vein of ZOL at a dose of 80 μg/kg in PBS. B) Extraction of lower first molars, access detail. C) After tooth dislocation, extraction of the lower first molar. D) Alveolus debrided with a spherical bur, to standardize the defect.](Image)
**AEA treatment**

Still in the eighth week of ZOL application, right after the 1MI extractions, at the same surgical moment, the BRONJ prevention protocol was started with the weekly application of AEA, the applications were carried out for 6 weeks.

AEA (Chanson Water Filter, Chanson do Brasil, São Paulo, Brazil) was used as an irrigation solution for the right mandibular alveoli, three times a week, on alternate days, in the region of the tooth extractions. The animals were sedated for one minute via inhalation with isoflurane for this procedure (Cristália, Itapira, SP, Brazil). Once anesthetized, the animals were positioned laterally and mouth opening was performed with orthodontic elastics n. 5/16. The tongue was pulled away by a molt-type peeler. During irrigation with acidified water (3 ml - pH 2.5), a surgical sucker was positioned on the lingual surface of the operated socket to prevent the irrigation solution from traveling to other areas of the oral cavity or being swallowed by the animal.

**Euthanasia and clinical analysis of osteonecrosis**

Six weeks after the second surgery, euthanasia was performed by overdose of isoflurane. Subsequently, they were submitted to photographs and a clinical analysis in the region of the extractions, each side of the mandibles was analyzed individually, grouping the data for the GAEA and GC groups.

The presence or absence of the following clinical aspects was evaluated: inflammation (erythema and edema), suppuration, bone sequestration. It was also noted whether there was partial or total epithelialization of the alveolus and the presence of exposed bone. Furthermore, exposure was measured in the mesio-distal and bucco-lingual directions with the aid of a millimeter periodontal probe (Figure 2).

After the clinical analysis of the region, the mandibles were dissected with a scalpel blade and molt detacher and were stored in 10% formalin for future analysis.
Figure 2: Demonstration of M-D and V-L measurement after mandible dissection with millimeter probe.

Source: Authors (2023)

Statistical analysis

Data were subjected to descriptive and inferential analysis using SPSS Statistics® for Windows (v. 24; IBM, Corp., New York, USA). The assumptions of normality and equality of variance were evaluated using the Shapiro-Wilk test. Numerical variables were compared between CG and GAEA groups using the Wilcoxon test, and qualitative variables using Fisher's Exact Test. A significance level of 0.05 was considered.

RESULTS

Two animals (males) were lost during the AEA therapy stage. Thus, 10 animals (six females and four males) remained for final analysis.

We can observe the differences in the clinical, animal aspect, in the GAEA groups (Figure 3A) in the GC group (Figure 3B).
Figure 3-A) Clinical aspect of the GAEA group: B) The clinical aspect of the CG group.

One of the animals presented extraoral suppuration in the area of the GC group

Table I demonstrates the comparison between the groups on the evaluated clinical parameters. No animals in both groups showed features of inflammation. No animal presented suppuration on the treatment side (GAEA), but three animals presented suppuration points on the control side (CG), and among these animals one of them also presented extraoral suppuration, however, there was no statistical difference (p = 0.211). Regarding bone sequestration, all animals presented this condition bilaterally. Regarding epithelialization, in the GAEA five animals showed partial or total epithelialization of the wound, and in the CG only 2, however this result was also not statistically significant (p = 0.350).
Table I. Comparison of clinical parameters evaluated between GAEA and CG

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>GAEA</th>
<th>GC</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inflammation</strong></td>
<td>Present n (%)</td>
<td>0 (0,0)</td>
<td>0 (0,0)</td>
</tr>
<tr>
<td>Absent</td>
<td>10 (10,0)</td>
<td>10 (10,0)</td>
<td></td>
</tr>
<tr>
<td><strong>Suppuration</strong></td>
<td>Present n (%)</td>
<td>0 (0,0)</td>
<td>3 (8,3)</td>
</tr>
<tr>
<td>Absent</td>
<td>10 (10,0)</td>
<td>7 (91,7)</td>
<td></td>
</tr>
<tr>
<td><strong>Bone sequestration</strong></td>
<td>Present n (%)</td>
<td>10 (58,3)</td>
<td>10 (83,3)</td>
</tr>
<tr>
<td>Absent</td>
<td>0 (0,0)</td>
<td>0 (0,0)</td>
<td></td>
</tr>
<tr>
<td><strong>Total or partial epithelialization</strong></td>
<td>Present n (%)</td>
<td>5 (50,0)</td>
<td>2 (20,0)</td>
</tr>
<tr>
<td>Absent</td>
<td>5 (50,0)</td>
<td>8 (80,0)</td>
<td></td>
</tr>
</tbody>
</table>

Note: Fisher's Exact Test. Significance level p < 0.05.

*It was not possible to perform statistical calculation.

Source: Authors (2023)

Regarding the measurements performed on bone exposure, it is possible to verify in table II that there was no difference between the groups

Table II. Comparison of bone exposure measurements between GAEA and GC.

<table>
<thead>
<tr>
<th></th>
<th>GAEA</th>
<th>GC</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Exposure MD</strong></td>
<td>5 (1-6)</td>
<td>5 (4-12)</td>
<td>0,205</td>
</tr>
<tr>
<td><strong>Exposure VL</strong></td>
<td>2 (1-3)</td>
<td>2 (2-3)</td>
<td>0,102</td>
</tr>
</tbody>
</table>

Wilcoxon test. Significance level p < 0.05.

Source: Authors (2023)

**DISCUSSION**

Alveolar necrotic lesions undergo a complex mechanism to repair the damage (MASI et al., 2016). Wound healing comprises four sequential but overlapping phases; homeostasis, inflammation, proliferation and remodeling (MASI et al., 2016). This entire healing process comprises the extracellular matrix, cytokines, blood cells and growth factors that activate cell proliferation through angiogenesis, myelogenesis and gene transcription (MASI et al., 2016).
Modulating inflammation and opportunistic infection can bring satisfactory results (KANG et al., 2013). With this in mind, the possibility that topical applications of AEA can control and change the local pH, causing the repair to occur faster, is promising, as a more acidic pH has been shown to stimulate angiogenesis, collagen formation, macrophage formation and chemotaxis, fibroblastic activity, keratocyte proliferation, in addition to beneficial effects on enzymatic activities (GLESER et al., 2000; KANG et al., 2013). Factors such as NF-kB, an important regulator of gene expression in several tissues, proved to be sensitive to reactive oxygen species (oxidative stress) and to the increase in intracellular calcium (GLESER et al., 2000).

In the recent past, the most elucidated action of AEA was attributed to its hypochlorous acid (HOCl) content, which is a precursor of gaseous chlorine, which has strong bactericidal activity and disinfection potential (KUBOTA et al., 2009). However, the direct beneficial effect (HOCl) regarding wound healing is unclear, but there seem to be other mechanisms related to the reactive oxygen species generated by HOCl when in contact with injured tissues (YAHAGI et al., 2000). This interpretation is supported by the known effects of free radicals on inflammation, as well as the role of inflammation in initiating healing. AEA is rich in singlet oxygen, a very reactive species, which appears to affect DNA synthesis and proliferation in fibroblasts. A low level of these species stimulates DNA synthesis and cell division, while a high level inhibits DNA synthesis (SUBIRADE et al., 1995).

In a study carried out to identify the effect of the mechanism of slightly acidic electrolyzed water on skin wounds in mice, it was observed that treatment with acidic water decreased pro-inflammatory cytokines such as interleukins IL-1β, IL-6, keratinocyte chemoattractant and tumor necrosis factor-α. Other markers strongly associated with wound healing, the metalloproteinases MMP1 and MMP9, were also up-regulated (YOU et al., 2017).

Other authors observed that the sites of infection with perforated appendicitis were lower in patients submitted to acidified water lavage (KUBOTA et al., 2009). Acidic water also provided nephroprotective effects on cisplatin-induced kidney injury in mice, probably due to increased activity of the antioxidant defense system and inhibition of lipid peroxidation (KUBOTA et al., 2009).

It seems sensible to think that the modulation exerted by reactive oxygen species, can trigger early wound healing through the migration and proliferation of fibroblasts, however discovering the appropriate therapeutic dosage, pH and ideal Oxidation Reduction potential, as well as fully understanding its way of acting can enable AEA therapy in cases of BRONJ.

This study presented, in the comparison of clinical variables, a promising result between the GAEA group compared to the CG group. Clinically, the side that composed the GAEA did not present suppuration with regard to epithelialization, although there was no statistical difference, we observed it in 3 more rats than the CG.
This result can be justified by the fact that the pH of the AEA substance used (pH2) is more acidic than some described (pH 5 to 6.5) (YOU et al., 2017). So far, few experiments have explored the correlation of the modulatory effect of AEA substance / acid pH variation [34]. It is also possible that the dosage may be changed. To better understand this scenario, further studies are needed, perhaps with a slightly acidic pH. We also believe that immunohistochemical and microtomographic analyzes initially designed would show an enriched panorama of the healing process, for which the mandibles were dissected and stored in 10% formalin for future analysis.

The absence of such confirmation is a limitation of this study. The treatment to prevent osteonecrosis of the jaws in GAEA proved to be accessible, easy to apply and low cost.

CONCLUSION

The comparison of clinical variables showed a promising result for the use of AEA. AEA has low cost, easy application and accessibility are positive characteristics. In this study, the result obtained was not statistically significant.

New studies are needed to better elucidate the mechanism underlying the effects of AEA on BRONJ healing, as well as to validate its use as a therapeutic alternative.

REFERENCES


