Aqueous-ozone irrigation of bone monocortical wounds in hyperglycemic rats

Alexandre Viana Frascino, Andrea Mantesso, Luciana Corrêa, Maria Cristina Zindel Deboni

ABSTRACT

PURPOSE: To analyze the effects of aqueous ozone irrigation over bone healing in hyperglycemia-induced rats.

METHODS: Forty-eight male Wistar rats were allocated into Group H (hyperglycemic) or Group N (control). Monocortical bone wounds were performed on femurs’ anterolateral face. Wounds were treated with a trans-operatory single irrigation of 100ml of aqueous ozone [0.004mg/ml] whereas control groups received 100ml of pure water (Milli-Q®). Histomorphological and histomorphometrical analyses were accomplished after seven, 14 and 21 days. Kruskal-Wallis and Mann-Whitney statistical tests were applied for bone neoformation quantification and assessment.

RESULTS: Aqueous ozone wounds irrigated revealed diffuse hemorrhage and increased neoformed of blood vessels number. There was no statistical significant difference in bone trabeculae neoformation. After seven and 14 days, the number of osteoclasts was higher in aqueous ozone groups than in those treated with pure water.

CONCLUSION: Independently of blood glucose levels, aqueous ozone allowed an increase in blood vessels neoformation and osteoclast migration, without affect bone trabeculae neoformation.

Key words: Bone, Ozone, Osteoclasts, Hyperglycemia, Rats.
Introduction

Bone healing comprises coordinated interaction between different cell types and release of several cytokines. Oxygen plays a pivotal role in tissue repair, mainly regarding local angiogenesis improvement and stimuli of undifferentiated cell migration.

Hyperglycemia leads to osteopenia and impairment of wound healing, which is intimately associated with unbalance between the production and the metabolism of reactive oxygen species (ROS). Also, hyperglycemia can increase the inflammatory response and the osteoclastic activity, which can compromise the bone neoformation during bone repair.

Ozone is toxic to the respiratory system but recently has been reported to be safe for a variety of medical and dental treatments. Literature describes that its dilution in water (aqueous-ozone) or ozonized-oil produces a securer and more stable vehicle when compared to gaseous ozone. The use of ozonized-oil for the treatment of bisphosphonate related jaws osteonecrosis was also described with positive results. However, there is a lack of in vivo studies in animal models to certificate the exact cellular pathways which ozone interacts with mineralized tissues and which are the safe doses for its applications.

The aim of the study was to analyze the effects of aqueous ozone irrigation on bone trabeculae neoformation and osteoclasts number in standard monocortical femoral wound of hyperglycemia-induced rats.

Methods

This research was conducted at the Animal Experimentation Laboratory of the Oral Surgery Department of School of Dentistry of the University. All the procedures were approved by the institutional Committee of Experimental Animal Research under protocol number 04/2009 and followed the recommendations of the Brazilian Society of Laboratory Animal Science (SBCAL - COBEA).

Forty-eight male rats (Rattus norvegicus Albinus, Wistar) weighing from 200g to 250g were kept in natural ventilated polypropylene cages lined with sterile wood shavings, under day/night cycle of 12/12 hours with food and water ad libitum. The animals suffered standard bone wounds and were distributed randomly into two groups: Group H (hyperglycemic) and Group N (control). Both groups were subdivided into “water” and “ozone” in accordance with the treatments of the wounds (see below).

Hyperglycemia induction

Twenty-four animals (Group H) were rented to hyperglycemia by a single intraperitoneal dose of Streptozotocin (STZ - Sigma Chemical Co. St Louis, MO, USA) (60mg/kg) using solution of sodium citrate buffer as vehicle (0.1mL). The animals of the control group (Group N) were treated with the vehicle alone. Capillary blood glucose of all animals was checked seventy-two hours after drug administration and immediately before the surgical procedures by a monitor (Accucheck - Roche®) using a blood drop from a tail tip wound. Animals showing blood glucose levels above 110 mg/dL were considered hyperglycemic.

Ozonized water production

Ozone was produced from pure oxygen as a direct result of an electrical discharge employing an ozone generator (Ozone & Life Model O&L3.0RD). Oxygen flow rate adjusted to 1/2 L/min and ozone at 41mg/L of ozone/oxygen gaseous concentration was bubbled in 2000mlMilliQ® water freshly prepared. Water ozonization was performed for 5 min to obtain a final concentration of ozonized water of 0.004mg/ml. A filter shattered ozone residual gas from the scrubber. Concentration of diluted ozone in water was checked during the experiment by the Indigo Blue method using Ozone-Chemets Kit (Chemetrics; Calverton, VA, USA).

Surgical procedures

Prophylactic antibiotic therapy was performed in all groups with benzyl penicillin G benzathine (Roche®), 150.000 UI/Kg intramuscularly. General anesthesia was achieved by intramuscular injection of hydrochloride ketamine (Dopalen® - Vetbrands) and xylazine (Rompum®- Bayer SA) a dose of 0.8 mg/kg and 0.3 mg/kg, respectively.

Antero lateral femurs faces were accessed through an incision of the skin and resection of soft tissues. Monocortical bone defects were created using trephine burs with 2 mm of diameter (Dentoflex®). Bone perforations were irrigated with 100mL of aqueous ozone in animals of Group N<sub>ozone</sub> (n=12) and animals of Group N<sub>water</sub> (n=12). Bone perforations of the twelve animals of Group H<sub>water</sub> and Group N<sub>water</sub> underwent irrigations with 100ml MilliQ® water only. The internal soft tissues were repositioned and sutured (silk thread 3.0-Ethicom, Johnson & Johnson®). Skin was realigned and also sutured (Nylon 3.0-Brasuture®).

At seven, 14 and 21 days postoperatively four animals, in each group, were euthanatized in a CO<sub>2</sub> chamber.
Histomorphological and histomorphometric analysis

The femurs were dissected and kept in 10% formaldehyde for 48 hours before decalcification in 10% EDTA (pH 7.4) for six weeks. The femurs were cut lengthly and embedded in paraffin. Some representative fragments of pancreas in the group H were also collected for β-cells analysis in the Langerhans islet. Histological sections with 5μm were stained with hematoxylin and eosin (H&E).

Two blind trained observers accomplished the histomorphological analyses. An arbitrary scale was applied to describe healing features (rates: 0 = absent, 1 = discrete, 2 = moderate, 3 = intense) in the site of the injury. Observers considered the presence of inflammatory infiltrate, edema, necrosis, and diffuse hemorrhage, presence of neoformed vessels and bone trabeculae, and hematopoietic bone marrow.

Bone trabeculae neoformation

For bone trabeculae analysis, two sections of each wound (four fields at 100x original magnification in each section) were analyzed. Digital images of each field were obtained using a CCD camera (Sony®, Tokyo, Japan) and the percentage of bone trabeculae area was calculated by means RGB subtraction using the histomorphometrical measurement software ImageLab2000®, Sao Paulo, Brazil.

Osteoclast counting

Osteoclast activity was determined through counting of osteoclastic cell line stained by Tartrate-resistant acid phosphatase (TRAP -Sigma-Aldrich, Saint Louis, MO, USA). For TRAP reaction, sections were deparaffinized, rinsed in PBS and incubated with a solution containing 50 mM sodium acetate (pH 5.2), 0.15% naphthol-AS-TR phosphate, 50 mM sodium tartrate, and 0.1% Fast Red T.R (Sigma-Aldrich, kit #387) for up to 2 h in water bath a 37°C. Subsequently, the sections were rinsed in PBS and counterstained with Gill’s hematoxylin for 1 minute at room temperature. Human blood smear was used as positive control. Specimens stained without sodium tartrate were considered as negative controls. Multinucleated giant cells TRAP-positive present in the callus region were manually counted in eight (8) fields at X400 magnification.

Statistical analysis

Histomorphological results were expressed in median and minimum/maximum values, and the data of bone trabeculae neoformation and osteoclast counting were in average and standard deviation. For histomorphometric analyses of non-parametric data, Kruskal-Wallis test was applied to the groups concomitantly, and Mann-Whitney test was for the pair of groups. Statistics were performed using Statistical Package for Social Sciences (SPSS, 17.0). The adopted significance level was 5% (0.05).

Results

It could not be verified either signs of infection or femoral fracture in any of the animals used in this study. Table 1 shows the averages capillary blood glucose observed in the groups at the moment of surgical procedure. Body weight gain was significantly lower in Group H in all studied periods (p=0.0290).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Capillary blood glucose (mg/dL)</th>
<th>Body weight gain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N_water</td>
<td>84.00 (±4.56)</td>
<td>102.47±3.46</td>
</tr>
<tr>
<td>N_ozone</td>
<td>84.25(±2.13)</td>
<td>131.62±3.32</td>
</tr>
<tr>
<td>H_water</td>
<td>440.67(±45.55)</td>
<td>93.37±6.20</td>
</tr>
<tr>
<td>H_ozone</td>
<td>446.83(±23.29)</td>
<td>99.90±4.26</td>
</tr>
<tr>
<td>P value</td>
<td>0.0373</td>
<td>0.0290</td>
</tr>
</tbody>
</table>

Kruskal-Wallis test. Significant when p<0.05.

Histomorphological and histomorphometric analysis

Pancreas fragments exhibited moderate to intense necrosis in the Langerhans islets, confirming the toxic effect of streptozotocin in the group H. Table 2 shows the median values of intensity for some tissue components observed in the different experimental periods. In general, at seven days the group N_ozone exhibited more pronounced inflammatory infiltrate, edema, and vessel neoformation than N_water (Figure 1 A and C). Regarding hyperglycemic groups, N_ozone also showed more intense vessel neoformation than H_water (Figure 1 B and D). At 14 days, the groups N_ozone and H_ozone continue to exhibit more intense edema and higher number of vessels than N_water and H_water. Diffuse hemorrhage in the groups with ozone also was more pronounced, mainly in the H_ozone (Figure 2). Edema remains present in all the groups at 21 days, but they were more intense in N_ozone in the comparison with N_water. Also vessel neoformation remains more intense in N_ozone and H_ozone. These groups (N and H ozone) exhibited remnants of blood clot at 21 days. Hematopoietic bone marrow was present in all the specimens in all periods with minimal intensity differences (Figure 3).
TABLE 2 - Median values of histomorphological analysis [Min-Max].

<table>
<thead>
<tr>
<th>Groups</th>
<th>( N_{\text{water}} )</th>
<th>( N_{\text{ozone}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7D 14D 21D</td>
<td>7D 14D 21D</td>
</tr>
<tr>
<td>Necrosis</td>
<td>0[0-0] 0[0-0] 0[0-0]</td>
<td>0[0-0] 0[0-0] 0[0-0]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Groups</th>
<th>( H_{\text{water}} )</th>
<th>( H_{\text{ozone}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7D 14D 21D</td>
<td>7D 14D 21D</td>
</tr>
<tr>
<td>Inflammatory infiltrate</td>
<td>1[0-2] 2(1-2) 2(1-2)</td>
<td>1(0-1) 2(1-2) 2(1-2)</td>
</tr>
<tr>
<td>Necrosis</td>
<td>0[0-0] 0(0-0) 0(0-0)</td>
<td>0(0-0) 0(0-0) 0(0-0)</td>
</tr>
<tr>
<td>Blood clot</td>
<td>2[2-3] 2(1-2) 1(1-1)</td>
<td>2(2-2) 3(2-3) 2(1-2)</td>
</tr>
<tr>
<td>Edema</td>
<td>3[3-3] 2(1-2) 2(1-2)</td>
<td>3[3-3] 3(2-3) 2(1-2)</td>
</tr>
<tr>
<td>Vessels presence</td>
<td>2[2-2] 2(2-2) 1(1-1)</td>
<td>3[3-3] 3(2-3) 2(2-2)</td>
</tr>
<tr>
<td>Hematopoietic tissue regen.</td>
<td>1[1-2] 2(1-2) 2(2-2)</td>
<td>2(1-2) 3(2-3) 3(2-3)</td>
</tr>
</tbody>
</table>

0 = absent; 1 = discrete; 2 = moderate; 3 = intense.

FIGURE 1 – Histological pattern of the bone defect at seven days (HE, original magnification 100x [A, B and C] and 200x [D]). In the hyperglycemic individuals, mainly those treated with ozone (D), the granulation tissue is more exuberant with intense neovascularization than in the normal individuals. Normal individuals treated with ozone (C) also show more angiogenesis than those treated with water (A).

FIGURE 2 – Histological pattern of the bone defect at 14 days [HE, original magnification 100x (A, B and C) and 200x (D)]. Blood clot was partially substituted by new-formed bone in all the groups. However in the hyperglycemic group treated with ozone (D) remnants of the clot are more pronounced. In the normal individuals treated with ozone (C), the bone trabeculae are more coalescent which may indicate that bone remodeling is more advanced in this group.
Aqueous-ozone irrigation of bone monocortical wounds in hyperglycemic rats

The mean percentages of neoformed bone trabeculae for each group are presented in Figure 4. At seven days, the lowest mean percentage of neoformed bone was in the N_{ozone}, but there were no statistically significant differences among the groups. At 14 days, the N_{ozone} showed the highest mean percentage of neoformed bone, but also there were no statistically significant differences among the groups. At 21 days, the highest mean percentage of neoformed was in the N_{water}, but the differences were not significant.

The histomorphological evaluations revealed that aqueous ozone might affect bone repair by enhancing vascular neoformation and extending osteoclast number near healing region, but was not able to stimulate bone trabeculae neoformation.

**Osteoclast counting**

Figure 5 shows TRAP-positive cells which present morphology of osteoclasts. These cells were adjacent to the new-formed trabeculae in the region of the surgical defect. In some fields it was possible to observe these cells in the Howship lacunae, which may indicate active bone resorption. The mean number of osteoclasts is depicted in Figure 6. N_{ozone} exhibited the highest number of osteoclasts at seven and 14 days, with significant difference in the comparison with the other groups (p<0.05). At 21 days the highest number was for group H_{ozone}, but without significant differences.

**Discussion**

The histomorphological evaluations revealed that aqueous ozone might affect bone repair by enhancing vascular neoformation and extending osteoclast number near healing region, but was not able to stimulate bone trabeculae neoformation. Previous investigations reported that ozone interactions with
organic fluids stimulates vascular neoformation through a ROS mediates mechanism\(^1\) and improves inflammatory response within pro-inflammatory cytokines interleukin-1 (IL-1) and factor-\(\alpha\) tumor necrosis (TNF-\(\alpha\)) release\(^12\).

The tartrate-resistant acid phosphatase (TRAP) staining revealed significant augment of osteoclasts counting nearby the majority of all defects submitted to aqueous ozone irrigation after seven and fourteen days. One of the regulatory mechanisms of osteoclast activity is reactive oxygen species (ROS)\(^13\). Therefore, we can assume that aqueous ozone boosted ROS concentrations and that it may have stimulated osteoclast migration near the wound.

Ozone toxicity for respiratory tissues is widely known. However, due to its high instability, different vehicles have been engaged for ozone delivery\(^6,\)\(^15\). Managing of water or oil as vehicle has been described to be safe and convenient. In this research 41mg/L of ozone/oxygen dilution in 2L MilliQ water was engaged for ozone delivery. Independently of blood glucose levels, aqueous ozone irrigation of Wistar rats’ standard femoral wounds improved capillary neoformation and enhanced osteoclast migration in wound healing regions without interference in bone trabeculae neoformation.

**Conclusion**

Independently of blood glucose levels, aqueous ozone irrigation of Wistar rats’ standard femoral wounds improved capillary neoformation and enhanced osteoclast migration in wound healing regions without interference in bone trabeculae neoformation.

**References**

Aqueous-ozone irrigation of bone monocortical wounds in hyperglycemic rats


Acknowledgment

To Professor Willfredo Urruchi (PhD) for technical assistance.

Correspondence:
Maria Cristina Zindel Deboni
Faculdade de Odontologia da USP
Departamento de Cirurgia, Prótese e Traumatologia Bucomaxilofaciais
Avenida Professor Lineu Prestes, 2227
05508-000 São Paulo – SP Brasil
Tel.: (55 11)3091-7832
mczdebon@usp.br

Received: January 13, 2013
Review: March 17, 2013
Accepted: April 14, 2013
Conflict of interest: none
Financial source: Coordination of Improvement for Higher Academic Staff (CAPES)

1Research performed at Division of Oral Surgery, Department of Surgery, Prosthesis and Traumatology Maxillofacial, School of Dentistry, University of Sao Paulo (USP), Brazil. Part of Master degree thesis, Dental Sciences, Oral and Maxillofacial Surgery and Traumatology. Tutor: Prof. Dra. Maria Cristina Zindel Deboni.