Journal of
PERIODONTAL RESEARCH

J Periodont Res 2015; 50: 240–247
All rights reserved

© 2014 John Wiley & Sons A/S.
Published by John Wiley & Sons Ltd
JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/jre.12201

Ozone dosing alters the biological potential and therapeutic outcomes of plasma rich in growth factors

E. Anitua, M. M. Zalduendo, M. Troya, G. Orive
Foundation Eduardo Anitua, Vitoria, Spain

Anitua E, Zalduendo MM, Troya M, Orive G. Ozone dosing alters the biological potential and therapeutic outcomes of plasma rich in growth factors. J Periodont Res 2015; 50: 240–247. © 2014 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

Background and Objective: Until now, ozone has been used in a rather empirical way. This *in-vitro* study investigates, for the first time, whether different ozone treatments of plasma rich in growth factors (PRGF) alter the biological properties and outcomes of this autologous platelet-rich plasma.

Material and Methods: Human plasma rich in growth factors was treated with ozone using one of the following protocols: a continuous-flow method; or a syringe method in which constant volumes of ozone and PRGF were mixed. In both cases, ozone was added before, during and after the addition of calcium chloride. Three ozone concentrations, of the therapeutic range 20, 40 and 80 μg/mL, were tested. Fibrin clot properties, growth factor content and the proliferative effect on primary osteoblasts and gingival fibroblasts were evaluated.

Results: Ozone treatment of PRGF using the continuous flow protocol impaired formation of the fibrin scaffold, drastically reduced the levels of growth factors and significantly decreased the proliferative potential of PRGF on primary osteoblasts and gingival fibroblasts. In contrast, treatment of PRGF with ozone using the syringe method, before, during and after the coagulation process, did not alter the biological outcomes of the autologous therapy.

Conclusion: These findings suggest that ozone dose and the way that ozone combines with PRGF may alter the biological potential and therapeutic outcomes of PRGF.

Eduardo Anitua, PhD, Instituto Eduardo Anitua, c/ Jose Maria Cagigal 19, 10007 Vitoria, Spain
Tel: +34 945160653
Fax: +34 945160657
e-mail: eduardoanitua@eduardoanitua.com
and
Gorka Orive, PhD, Instituto Eduardo Anitua, c/ Jose Maria Cagigal 19, 10007 Vitoria, Spain
Tel: +34 945160653
Fax: +34 945160657
e-mail: gorka.orive@ehu.es

Key words: growth factors; ozone; plasma rich in growth factors; platelet rich plasma

Accepted for publication April 22, 2014

Ozone behaves as a double-edged sword because of its potent oxidant properties. It can be used as a therapy (1,2) but it also may damage the respiratory tract. The most widespread approach is ozonated autohemotherapy (O_3-AHT), which consists of collecting blood from the patient, mixing it with medical ozone (O_2+O_3, where the latter represents 5% at most) and then injecting it back into the same patient. The use of ozone as a therapy is based on the belief that blood behaves as a potent antioxidant system because it contains several antioxidant molecules, including ascorbic acid, uric acid and albumin, or several enzymes, such as catalase. Ozone may enhance the antioxidant capacity of blood and therefore improve its potential to treat chronic infectious diseases or immunological or neurodegenerative disorders (3–6).

The extreme reactivity and instability of the ozone molecule is a major limitation of this approach. In fact, the ozone molecule is more reactive than oxygen and generates reactive oxygen species (ROS) such as hydrogen peroxide, leading to alterations in homeostasis (1,5,7). When ozone is dissolved in blood, it decomposes and

reacts instantly with antioxidants and polyunsaturated fatty acids, whose peroxidation involves the formation of lipid oxidation products. These lipid peroxidation products may trigger several reactions, such as the release of endogenous mediators of inflammation (2,3,5,8).

Despite the fact that ozone therapy has not yet been accepted as orthodox medicine it is used in a rather empirical way for the treatment of vascular pathologies, ulcers, and acute and chronic infectious diseases. In addition, it can be administered subcutaneously, intramuscularly, intradiscally or intracavitarily (5,9,10). In dentistry, ozone has also been used in either gaseous or aqueous form as a possible oral antiseptic agent, which relies on its antimicrobial properties (4,11–14).

Platelet-rich plasma represents a new biological therapy for use in dentistry, among other clinical specialties, through stimulating the release of endogenous growth factors for oral tissue-regeneration purposes (15–25). In relation to this, the positive effect of platelet-rich plasma on osteoblast and periodontal ligament cell function has been probed (26). Moreover, platelet-rich fibrin can improve the clinical parameters associated with human intrabony periodontal defects (27).

However, until now, little is known regarding the potential of the combination of these two therapies. In this study, we evaluated, for the first time, whether different protocols of ozone treatment of plasma rich in growth factors (PRGF) alter the biological properties and outcomes of this autologous platelet-rich plasma. To address this, two different ozonation protocols were tested and complete evaluation of the ozonated PRGF, including its composition (growth factors) and biomechanical (fibrin clot properties) and biological (proliferative effects on primary oral cells) properties, was carried out.

Material and methods

The study was performed following the principles of the Declaration of Helsinki, as revised in 2008.

Ozone treatment of PRGF

Ozone (O_2/O_3 gas mixture) was generated from medical-grade oxygen using an ozone generator (Hyper Medozon; Herrmann Apparatebau GmbH, Weinmann, Hamburg, Germany). Two protocols were performed to ozonate the plasma preparations: continuous-flow ozone treatment; and treatment with a constant volume of ozone (by syringe) (Fig. 1). Three concentrations of ozone (20, 40 and 80 μg/mL), which are within the therapeutic range, were evaluated in all experiments. The ozone-treatment period was 10 min in all cases.

Continuous-flow ozonation. After providing written informed consent, blood from four human Caucasian donors was collected into 9-mL tubes containing 3.8% (wt/vol) sodium citrate. The blood was centrifuged at 580 ***g*** for 8 min (Endoret Dentistry; BTI Biotechnology Institute, S.L., Miñano, Álava, Spain) at room temperature, then, a 2-mL volume of plasma above the buffy coat (F2) was removed from each donor sample. Thereafter, part of the plasma from two donors was ozonated before, and the other part during the addition of the activator (calcium chloride) (Endoret Dentistry). The plasma of the other two donors was ozonated after the addition of calcium chloride. To achieve this ozonation, one end of a cannula was connected to the "permanent" output of the ozone generator and the other was inserted in the tube containing the plasma using a syringe needle. Then, the desired ozone concentration was selected and the ozonation was performed continuously for 10 min. In the case of ozonation before addition of the activator, the plasma was ozonated for 10 min and calcium chloride was added immediately thereafter. Then, the plasma was divided into five aliquots: four were maintained at 37°C for measurement of coagulation time and clot retraction; and the fifth aliquot was maintained for 1 h at 37°C in order to provide a sample, after centrifugation at 3000 ***g*** for 15 min, of ozonated supernatant that was then filtered, aliquoted and stored at −80°C. The latter was used to determine the content of growth factors and to perform the cell-proliferation assay.

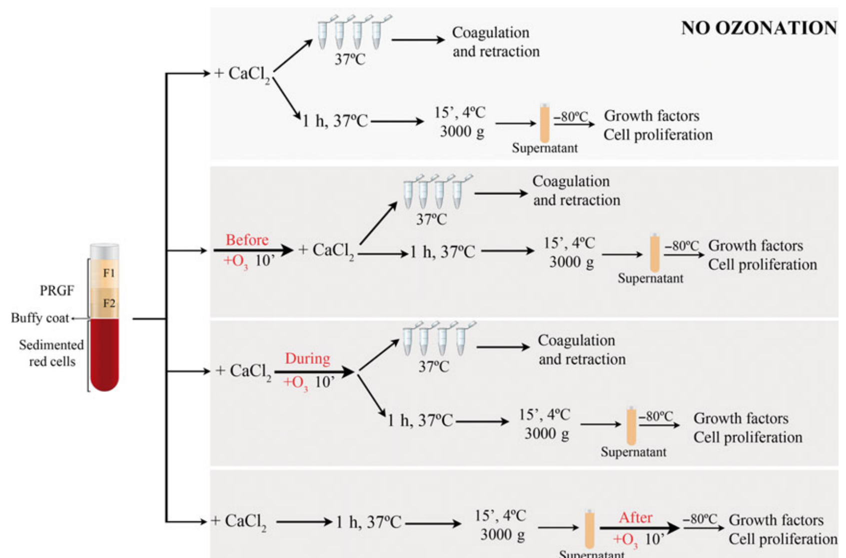

Fig. 1. Illustrative representation of the experimental procedure. Two different ozonation protocols were followed: continuous flow; and constant volume (by syringe). In both protocols ozone was added before, during and after addition of the activator (calcium chloride). All parameters evaluated were compared with nonozonated plasma rich in growth factors (PRGF).

In the case of ozonation during the addition of calcium chloride, calcium chloride was added to the plasma, which was ozonated immediately thereafter. Ten minutes later, the same protocol, as described above, was followed, dividing the ozonated plasma into different aliquots for either evaluation of the fibrin properties or to obtain supernatant for further analysis.

Finally, calcium chloride was added to the plasma of the other two donors, and this plasma was stored at 37°C for 1 h, the clot was centrifuged (at 3000 ***g***, for 15 min at 4°C) and the supernatant was obtained. Then, that supernatant was ozonated as previously described, and finally stored at −80°C to be used later.

2. *Ozonation with constant volume (by syringe)*. As in the protocol described above, blood from two human Caucasian donors was collected into 9-mL tubes containing 3.8% (wt/vol) sodium citrate, after obtaining written informed consent. After the tubes were centrifuged at 580 ***g*** for 8 min (Endoret Dentistry) at room temperature, a 2-mL volume of plasma above the buffy coat (F2) was removed from each donor sample. In this protocol, ozonation was also performed before, during and after coagulation. However, in this case a volume of ozone was collected with a syringe and immediately introduced into a second syringe, via a multidirectional stopcock, containing an identical volume of plasma. Samples were gently and continuously mixed with the gas for 10 min. Once the samples were ozonated, the experiment was continued as described for the first protocol.

Determination of the effect of plasma ozonation on coagulation and retraction

In both protocols, part of the plasma samples that were ozonated both before and during the addition of calcium chloride, as well as samples of nonozonated plasma, were divided into four aliquots of equal volume to determine the coagulation time. Once the samples had coagulated, each aliquot was used to quantify the volume of plasma released after clot retraction at the study time points 15, 30, 45 and 60 min. Samples were assayed in triplicate and the results are expressed in percentage of released volume, taking into account the initial volume of the clot.

Quantification of the content of growth factors

In order to determine the effect of ozone on the content of the main growth factors in plasma, the concentrations of platelet-derived growth factor AB, transforming growth factor beta1, hepatocyte growth factor and insulin-like growth factor 1 (R&D Systems, Minneapolis, MN, USA) were determined using ELISA kits. These growth factors were quantified in the supernatant of plasma ozonated using all procedures described earlier in the Material and methods, as well as in the supernatant of nonozonated plasma. Samples were assayed in triplicate.

Cell proliferation assay

Primary human gingival fibroblasts and primary human alveolar osteoblasts, isolated as previously described (28,29), were used to assess the effect of ozone on the ability of PRGF to induce cell proliferation.

Gingival fibroblasts and alveolar osteoblasts were seeded at a density of 5000 cells per cm^2 and 8000 cells per cm^2, respectively, on 96-well optical bottom black plates. Gingival and osteoblast cultures were maintained with a final concentration of 20% of the corresponding plasma samples in serum-free Dulbecco's modified Eagle's medium/F12 (Gibco-Invitrogen, Grand Island, NY, USA) and osteoblast basal medium without other growth supplements (Sciencell Research Laboratories, Carlsbad, CA, USA), respectively, for 72 h. Cell proliferation was evaluated using the CYQUANT cell proliferation assay (Molecular Probes-Invitrogen, Grand Island, NY, USA). Briefly, treatments were discarded and wells were washed carefully with phosphate-buffered saline. Then the microplates were frozen at −80°C until required for assay. After thawing the plates at room temperature, the wells were incubated for 1 h at room temperature with RNase A (1.35 Ku/mL) diluted in cell lysis buffer. Then, 2× GR dye/cell-lysis buffer was added to each sample well, mixed gently and incubated for 5 min at room temperature, protected from light. Sample fluorescence was measured using a fluorescence microplate reader (Twinkle LB 970; Berthold Technologies, Bad Wildbad, Germany). A DNA standard curve was included in each assay for converting sample fluorescence values into DNA concentration.

Statistical analysis

The Kruskal–Wallis test for independent samples was used to assess the potential differences among treatments for each experimental procedure. Statistical differences between groups were accepted for p-values lower than 0.05. The results are expressed as mean ± SD.

Results

Effects of ozone therapy on the PRGF fibrin scaffold

Treatment of PRGF with the continuous ozone flow method modified some properties of the fibrin scaffold, including the coagulation and retraction times. In fact, the coagulation of PRGF was impaired after ozone treatment by this method (data not shown), independently of when the ozone was applied and the concentration of ozone used.

Fibrin scaffolds of PRGF ozone treated using the syringe method were only slightly altered (Fig. 2). In particular, when 20 μg/mL of ozone was

applied to nonactivated PRGF, a significant decrease in the coagulation time of the fibrin scaffold was observed compared with fibrin scaffolds prepared from nontreated PRGF (control) and from PRGF incubated with ozone at both 40 and 80 μg/mL (Fig. 2A). In fact, the clot-formation time was reduced from 51.7 ± 9.3, 54.1 ± 12.2 and 52.5 ± 19.8 min for nontreated PRGF and for PRGF treated with 40 and 80 μg/mL of ozone, respectively, to 21.6 ± 1.8 min for PRGF treated with 20 μg/mL of ozone.

However, when 20 μg/mL of ozone was applied to freshly activated PRGF (just after calcium chloride administration), a decrease in the time of clot formation was statistically significant only in comparison with the 80-μg/mL ozone concentration, but not when compared with nonozonated plasma (Fig. 2B).

No statistically significant differences were observed in the retraction process of fibrin scaffolds obtained from PRGF when treated with ozone using the syringe method (Fig. 2C and 2D).

Growth factor content from different ozone-treated supernatants and fibrin scaffolds

Assuming that ozone treatment of PRGF using the continuous-flow method impaired the formation of fibrin scaffolds, we decided to treat a different set of supernatants with ozone in order to test the potential effects of ozone on the composition of morphogens (Fig. 3).

Ozone treatment significantly reduced the presence of growth factors in the supernatants, these differences being statistically significant in all cases when compared with nontreated PRGF. Ozone treatment markedly affected the levels of platelet-derived growth factor AB, which decreased from 14,675 ± 2,934 pg/mL to 898 ± 134, 252 ± 92 and 221 ± 34 pg/mL when treated with 20, 40 and 80 μg/mL, respectively, of ozone (Fig. 3A). The levels of transforming growth factor-beta1 and hepatocyte growth factor decreased by more than 50% when treated with ozone (Fig. 3B and 3C), whereas the concentration of insulin-like growth factor 1 was reduced from 94 ± 24 ng/mL to 22 ± 7, 10 ± 4 and 9 ± 3 ng/mL when treated with 20, 40 and 80 μg/mL, respectively, of ozone (Fig. 3D).

Conversely, ozone treatment of PRGF using the syringe method did not alter the composition of morphogens released from the fibrin scaffold. These results were observed when ozone was applied before, during or after the coagulation process of PRGF (Fig. 4).

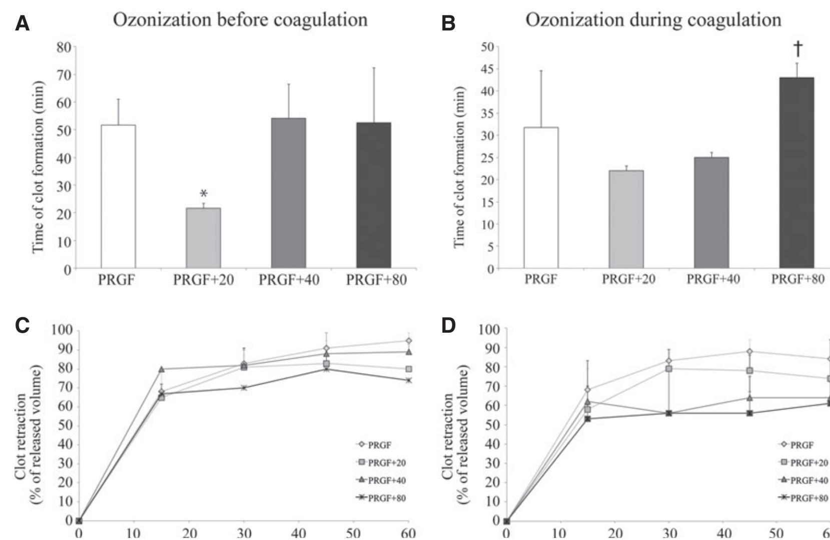

Fig. 2. Effect of ozonation, applied using the constant volume protocol, on fibrin clot properties. (A) Effect of ozonation before coagulation on the time taken for clot formation. *$p < 0.05$, statistically significant differences among plasma samples ozonated with 20 μg/mL of ozone and the rest of the plasma samples. (B) Effect of ozonation during coagulation on the time taken for clot formation. $^{\dagger}p < 0.05$, statistically significant differences between samples ozonated with 20 μg/mL of ozone and plasma samples treated with 80 μg/mL of ozone. (C) Effect, on clot retraction, of ozonation before the addition of calcium chloride. (D) Effect, on clot retraction, of ozonation during the addition of calcium chloride. PRGF, plasma rich in growth factors; PRGF-20, plasma rich in growth factors treated with 20 μg/mL of ozone; PRGF-40, plasma rich in growth factors treated with 40 μg/mL of ozone; PRGF-80, plasma rich in growth factors treated with 80 μg/mL of ozone.

Effects of ozone-treated PRGF on cell proliferation

Human primary gingival fibroblasts and alveolar osteoblasts were cultured with ozone-treated PRGF using the continuous-flow method. The results showed that the number of cells was dramatically reduced in both cell phenotypes after treatment with any concentration of ozone. Most of the cells were detached and floating and almost no cells were adherent to the cell culture surface, which indicated a high cell-death rate. In fact, cell DNA concentration was reduced in more than 95% after culture with ozone-treated PRGF using the continuous flow method (Fig. 5).

Once again, ozone treatment using the syringe method did not modify the biological potential of PRGF in terms of cell proliferation (Fig. 6), regardless or whether ozone was applied before, during or after coagulation of PRGF.

Discussion

Platelets are able to produce ROS, which might play an important role in the mechanism of platelet activation and aggregation. Oxygen-reactive species, such as hydrogen peroxide, nitric oxide and lipid oxidation products,

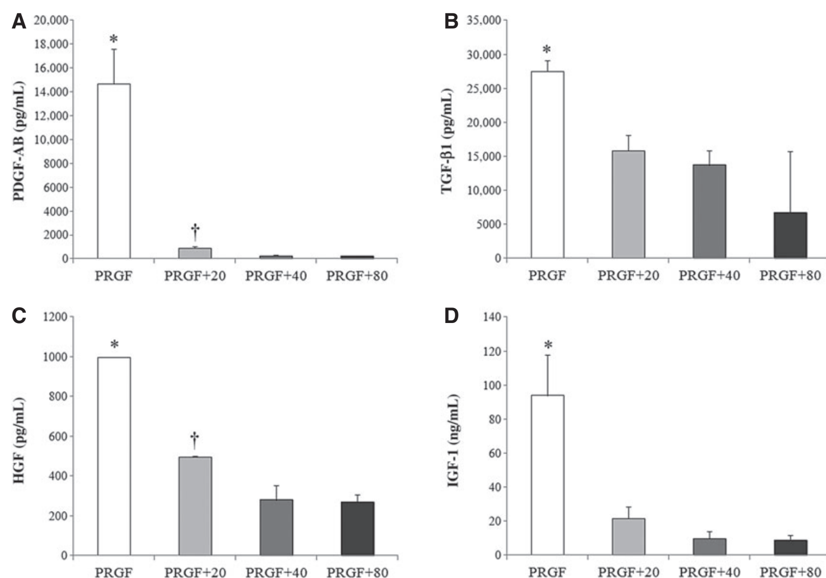

Fig. 3. Continuous flow ozonation. Effect of ozonation, after coagulation, on the content of growth factors in the plasma supernatant. (A) Platelet-derived growth factor AB (PDGF-AB). (B) Transforming growth factor-beta1 (TGF-β1). (C) Hepatocyte growth factor (HGF). (D) Insulin-like growth factor 1 (IGF-1). *$p < 0.05$, statistically significant differences among nonozonated plasma and the rest of the treated samples. †$p < 0.05$, statistically significant differences among plasma samples treated with 20 μg/mL of ozone and plasma samples treated with 40 and 80 μg/mL of ozone. PRGF, plasma rich in growth factors; PRGF-20, plasma rich in growth factors treated with 20 μg/mL of ozone; PRGF-40, plasma rich in growth factors treated with 40 μg/mL of ozone; PRGF-80, plasma rich in growth factors treated with 80 μg/mL of ozone.

are present at physiological concentrations during the coagulation process (30). The negative effect of these radicals is neutralized by diverse oxidant scavengers, Fe^{2+} and Cu^{+} chelators and a large series of antioxidant enzymes (31). The maintenance of an optimal balance between the oxygen reactive species and the corresponding protective molecules is critical for cell survival and the preservation of healthy tissues (32).

Recent evidence supports the idea that ozone might be used to increase the antioxidant capacity of blood and its derivates. Thus, it could be used to enhance the potential of these biological approaches in the treatment of several chronic infectious diseases, or immunological or neurodegenerative disorders. However, little is known about how the ozone treatment method and its concentration impinge on the properties of biological therapy and its outcomes.

In this study, we report, for the first time, how the method by which ozone is applied to PRGF drastically affects both its composition and biological properties. Ozone treatment of PRGF using the continuous flow method altered its general properties, inhibiting the coagulation process and the formation of the fibrin scaffold. The long duration of this method may have resulted in large numbers of ozone molecules reacting with polyunsaturated fatty acids, albumin and antioxidants, such as ascorbic acid and uric acid, among others (33). Carbohydrates, enzymes, DNA and RNA can also be affected by ozone overdose. The high ozone concentration may have altered platelet structure and the configuration of some key proteins involved in the plasma coagulation process, resulting in its disruption (32).

In the second ozone-treatment method, a therapeutic ozone concentration in a determined volume was mixed with an identical volume of PRGF. Under these conditions, the amount of ROS delivered was counteracted by the antioxidants present in the plasma formulation. As a consequence, the biological potential of PRGF was not altered. Neither the coagulation process nor the fibrin retraction process was modified by the ozone treatment.

Lipids are the major target of ozone-induced damage; however, proteins may also be affected. In fact, we observed that ozone treatment using the continuous flow method drastically reduced the amount of growth factors present in the PRGF. Protein oxidation processes induced by O_3 molecules may explain this negative effect. It has been previously described that ozone treatment affects the functional properties of proteins as a result of structural changes by oxidation (34). Nevertheless, the antioxidant agents present in plasma or within platelets were able to counteract the ROS generated when therapeutic ozone doses were applied (using the syringe method) before, during and after coagulation (35).

As expected, no proliferation of primary gingival fibroblasts and alveolar osteoblasts, as a result of treatment with ozone-treated PRGF using the continuous flow method, was detected. In fact, a great number of dead cells was observed in those cultures. This cytotoxic effect might be caused by the presence of unreacted ozone molecules in the supernatants. It has been shown that ozone may induce the synthesis of high concentrations of radical oxygen species in cell cultures (36), resulting in modifications of cell proliferation, metabolism and motility (11,37) as well as in cell death.

By contrast, treatment of PRGF with ozone using the syringe method, before, during and after the coagulation process, did not alter the biological outcomes of the autologous therapy.

There is not a generalized consensus to accept ozone treatment as a therapeutic technique. Orthodox medicine remains skeptical because few controlled clinical trials are available and the results are poor (10). Further preclinical and clinical studies are therefore necessary to address these issues and to determine the potential

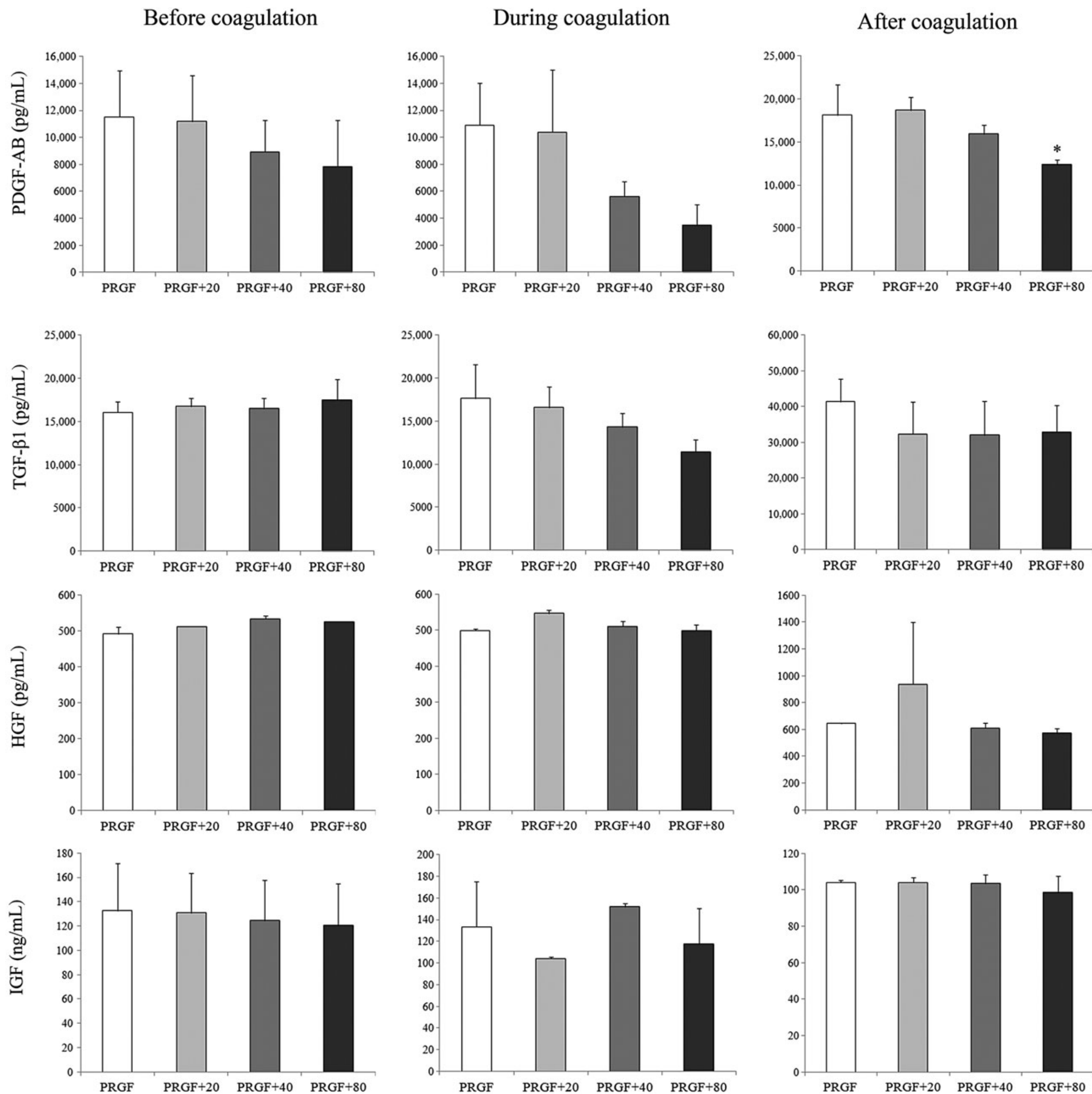

Fig. 4. Ozonation performed using the constant volume protocol. Effect of ozonation before, during and after the addition of calcium chloride, on the content of several growth factors in plasma. *$p < 0.05$, statistically significant differences among samples treated with 80 μg/mL of ozone and plasma samples treated with 20 and 40 μg/mL of ozone. HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor 1; PDGF-AB, platelet-derived growth factor AB; PRGF, plasma rich in growth factors; PRGF-20, plasma rich in growth factors treated with 20 μg/mL of ozone; PRGF-40, plasma rich in growth factors treated with 40 μg/mL of ozone; PRGF-80, plasma rich in growth factors treated with 80 μg/mL of ozone; TGF-β1, transforming growth factor-beta1.

usefulness of ozone in medicine. This study aimed to shed some light on the uncontrolled use of ozone when combined with platelet-rich plasma therapies. Our data suggest that low ozone doses do not modify the properties and outcomes of PRGF, while higher doses alter the coagulation process of the fibrin and induce a destructive effect on morphogens and growth factors, reducing or inhibiting its biological potential.

Acknowledgements

We wish to thank Virginia Cuadrado Vega for her valuable contribution with the English grammar. We also thank Foundation Eduardo Anitua for its support in PRGF research.

Conflict of interest

EA, MMZ and MT are scientists at BTI Biotechnology Institute. This biotechnology company has developed the technology of PRGF.

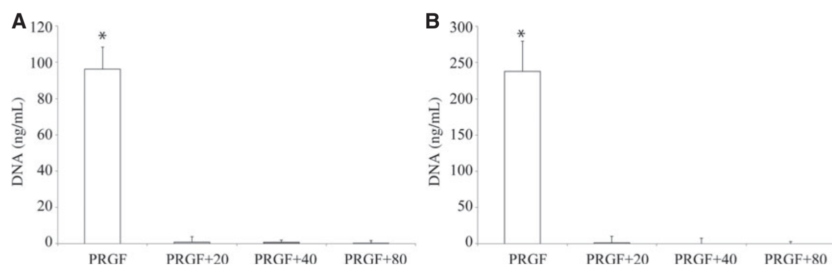

Fig. 5. Continuous flow protocol. Effect of plasma supernatant ozonation after the addition of calcium chloride on the proliferation of (A) gingival fibroblasts and (B) alveolar osteoblasts. $*p < 0.05$, statistically significant differences among nonozonated plasma and the rest of the treated samples. PRGF, plasma rich in growth factors; PRGF-20, plasma rich in growth factors treated with 20 μg/mL of ozone; PRGF-40, plasma rich in growth factors treated with 40 μg/mL of ozone; PRGF-80, plasma rich in growth factors treated with 80 μg/mL of ozone.

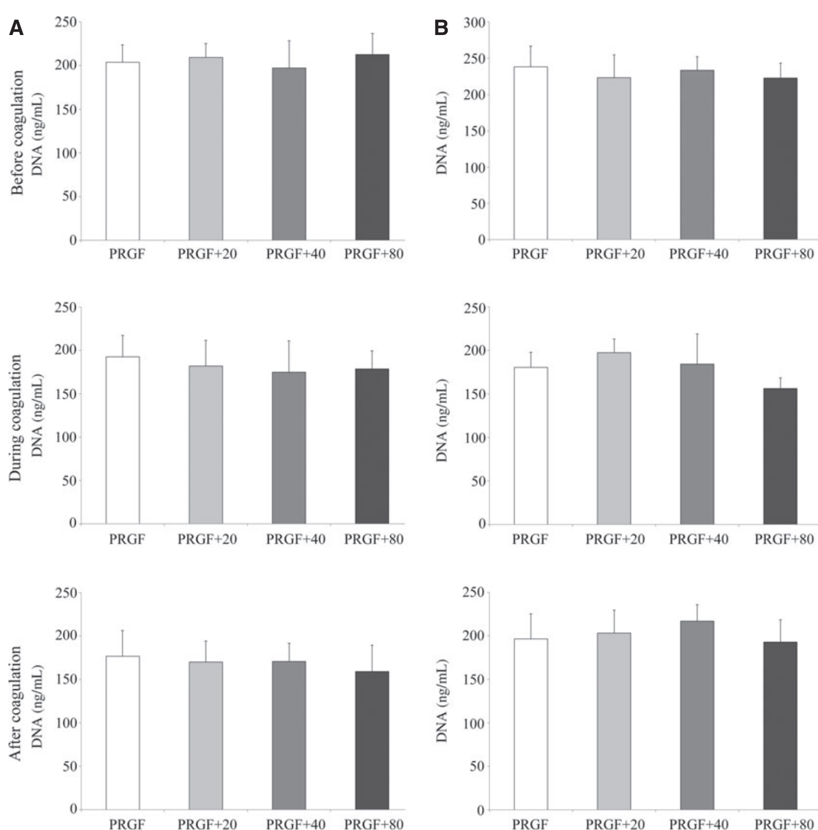

Fig. 6. Ozonation performed using the constant volume protocol. Effect of ozonation when applied before, during and after the addition of calcium chloride on the proliferation of (A) gingival fibroblasts and (B) alveolar osteoblasts. PRGF, plasma rich in growth factors; PRGF-20, plasma rich in growth factors treated with 20 μg/mL of ozone; PRGF-40, plasma rich in growth factors treated with 40 μg/mL of ozone; PRGF-80, plasma rich in growth factors treated with 80 μg/mL of ozone.

References

1. Bocci V, Valacchi G, Corradeschi F, Fanetti G. Studies on the biological effects of ozone: 8. Effects on the total antioxidant status and on interleukin-8 production. *Mediators Inflamm* 1998;**7**: 313–317.
2. Diaz-Llera S, Gonzalez-Hernandez Y, Prieto-Gonzalez EA, Azoy A. Genotoxic effect of ozone in human peripheral blood leukocytes. *Mutat Res* 2002; **517**:13–20.
3. Bocci VA. Scientific and medical aspects of ozone therapy. State of the art. *Arch Med Res* 2006;**37**:425–435.
4. Huth KC, Saugel B, Jakob FM *et al*. Effect of aqueous ozone on the NF-kappaB system. *J Dent Res* 2007;**86**:451–456.
5. Ciborowski M, Lipska A, Godzien J *et al*. Combination of LC-MS- and GC-MS-based metabolomics to study the effect of ozonated autohemotherapy on human blood. *J Proteome Res* 2012;**11**: 6231–6241.
6. Bocci VA, Zanardi I, Travagli V. Ozone acting on human blood yields a hormetic dose-response relationship. *J Transl Med* 2011;**9**:66.
7. Travagli V, Zanardi I, Silvietti A, Bocci V. A physicochemical investigation on the effects of ozone on blood. *Int J Biol Macromol* 2007;**41**:504–511.
8. Bocci V, Aldinucci C, Mosci F, Carraro F, Valacchi G. Ozonation of human blood induces a remarkable upregulation of heme oxygenase-1 and heat stress protein-70. *Mediators Inflamm* 2007;**2007**: 26785.
9. Travagli V, Zanardi I, Bernini P, Nepi S, Tenori L, Bocci V. Effects of ozone blood treatment on the metabolite profile of human blood. *Int J Toxicol* 2010; **29**:165–174.
10. Bocci V. Ozone as Janus: this controversial gas can be either toxic or medically useful. *Mediators Inflamm* 2004;**13**:3–11.
11. Huth KC, Jakob FM, Saugel B *et al*. Effect of ozone on oral cells compared with established antimicrobials. *Eur J Oral Sci* 2006;**114**:435–440.
12. Hauser-Gerspach I, Vadaszan J, Deronjic I *et al*. Influence of gaseous ozone in peri-implantitis: bactericidal efficacy and cellular response. An in vitro study using titanium and zirconia. *Clin Oral Invest* 2012;**16**:1049–1059.
13. Eick S, Tigan M, Sculean A. Effect of ozone on periodontopathogenic species– an in vitro study. *Clin Oral Invest* 2012; **16**:537–544.
14. Azarpazhooh A, Limeback H. The application of ozone in dentistry: a systematic review of literature. *J Dent* 2008;**36**: 104–116.
15. Rodrigues M, Griffith LG, Wells A. Growth factor regulation of proliferation and survival of multipotential stromal cells. *Stem Cell Res Ther* 2010;**1**:32.
16. Dereka XE, Markopoulou CE, Vrotsos IA. Role of growth factors on periodontal repair. *Growth Factors* 2006;**24**:260–267.
17. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. *Wound Repair Regen* 2008;**16**:585–601.

18. Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. *Physiol Rev* 2003;**83**:835–870.
19. Nurden AT. Platelets, inflammation and tissue regeneration. *Thromb Haemost* 2011;**105**(suppl 1):S13–S33.
20. Chen FM, Jin Y. Periodontal tissue engineering and regeneration: current approaches and expanding opportunities. *Tissue Eng Part B Rev* 2010;**16**:219–255.
21. Chen FM, An Y, Zhang R, Zhang M. New insights into and novel applications of release technology for periodontal reconstructive therapies. *J Control Release* 2011;**149**:92–110.
22. Anitua E, Orive G. Endogenous regenerative technology using plasma- and platelet-derived growth factors. *J Control Release* 2012;**157**:317–320.
23. Anitua E, Sanchez M, Prado R, Orive G. Plasma rich in growth factors: the pioneering autologous technology for tissue regeneration. *J Biomed Mater Res, Part A* 2011;**97**:536.
24. Anitua E, Prado R, Sánchez M, Orive G. Platelet-rich plasma: preparation and formulation. *Oper Tech Orthop* 2012;**22**: 25–32.
25. Anitua E, Alkhraisat MH, Orive G. Perspectives and challenges in regenerative medicine using plasma rich in growth factors. *J Control Release* 2012;**157**:29–38.
26. Creeper F, Lichanska AM, Marshall RI, Seymour GJ, Ivanovski S. The effect of platelet-rich plasma on osteoblast and periodontal ligament cell migration, proliferation and differentiation. *J Periodont Res* 2009;**44**:258–265.
27. Lekovic V, Milinkovic I, Aleksic Z *et al.* Platelet-rich fibrin and bovine porous bone mineral vs. platelet-rich fibrin in the treatment of intrabony periodontal defects. *J Periodont Res* 2012;**47**:409–417.
28. Anitua E, Troya M, Orive G. Plasma rich in growth factors promote gingival tissue regeneration by stimulating fibroblast proliferation and migration and by blocking transforming growth factor-beta1-induced myodifferentiation. *J Periodontol* 2012;**83**:1028–1037.
29. Anitua E, Tejero R, Zalduendo MM, Orive G. Plasma rich in growth factors (PRGF-Endoret) promotes bone tissue regeneration by stimulating proliferation, migration and autocrine secretion on primary human osteoblasts. *J Periodontol* 2013;**84**:1180–1190.
30. Davi G, Patrono C. Platelet activation and atherothrombosis. *N Engl J Med* 2007;**357**:2482–2494.
31. Anraku M, Chuang VT, Maruyama T, Otagiri M. Redox properties of serum albumin. *Biochim Biophys Acta* 2013; **1830**:5465–5472.
32. Ferroni P, Vazzana N, Riondino S, Cuccurullo C, Guadagni F, Davi G. Platelet function in health and disease: from molecular mechanisms, redox considerations to novel therapeutic opportunities. *Antioxid Redox Signal* 2012;**17**:1447–1485.
33. Bocci V, Borrelli E, Travagli V, Zanardi I. The ozone paradox: ozone is a strong oxidant as well as a medical drug. *Med Res Rev* 2009;**29**:646–682.
34. Uzun H, Ibanoglu E, Catal H, Ibanoglu S. Effects of ozone on functional properties of proteins. *Food Chem* 2012;**134**: 647–654.
35. Sagai M, Bocci V. Mechanisms of action involved in ozone therapy: is healing induced via a mild oxidative stress? *Med Gas Res* 2011;**1**:29.
36. Valacchi G, Bocci V. Studies on the biological effects of ozone: 11. Release of factors from human endothelial cells. *Mediators Inflamm* 2000;**9**:271–276.
37. Laval-Gilly P, Falla J, Klestadt D, Henryon M. A new approach to evaluate toxicity of gases on mobile cells in culture. *J Pharmacol Toxicol Methods* 2000;**44**:483–488.