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Ozonetherapy protects from in-stent coronary neointimal proliferation. Role of redoxins

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ABSTRACT

Background: In-stent restenosis and poor re-endothelization usually follow percutaneous transluminal coronary angioplasty, even using drug-eluting stents, due to inflammation and oxidative stress. Medical ozone has antioxidant and anti-inflammatory properties and has not been evaluated in this context.
Objectives: To evaluate whether ozonotherapy might reduce restenosis following bare metal stents implantation in relation to the redoxin system in pigs.
Methods: Twelve male Landrace pigs (51 ± 9 kg) underwent percutaneous transluminal circumflex coronary arteries bare metal stent implantation under heparine infusion and fluoroscopical guidance, using standard techniques. Pigs were randomized to ozonetherapy (n = 6) or placebo (n = 6) treatment. Before stenting (24 h) and twice a week for 30 days post-stenting, venous blood was collected, ozonized and reinfused. Same procedure was performed in placebo group except for ozonation. Both groups received antiplatelet treatment. Histopathology and immunohistochemistry studies were performed.
Results: Severe inflammatory reaction and restenosis with increase in the immunohistochemical expression of thioredoxin-1 were observed in placebo group 30 days after surgery. Oppositely, ozonetherapy drastically reduced inflammatory reaction and restenosis, and showed no increase in the Trx-1 immunohistochemical expression 30 days after surgery. Immunolabeling for Prx-2 was negative in both groups. Ozonated autohemotherapy strikingly reduced restenosis 30 days following PTCA with BMS implantation in pigs.
Conclusions: Stimulation of the redoxin system by ozone pretreatment might neutralize oxidative damage from the start and increase antioxidative buffering capacity post-injury, reducing further damage and so the demand for antioxidant enzymes. Our interpretation agrees with the ozone oxidative preconditioning mechanism, extensively investigated.

1. Introduction

Percutaneous transluminal coronary angioplasty (PTCA) with stenting reduced restenosis to 10–20% overcoming limitations of balloon angioplasty. Restenosis results from elastic recoil and intimal hyperplasia as a response to vascular injury and may be as high as 40–50% by 6 months following dilatation of complex lesions [1]. While PTCA with stenting is currently the most popular treatment for coronary artery disease, in-stent restenosis remains its main limitation. Unfortunately, inhibition of neointimal hyperplasia with sirolimus-coated stents led to poor endothelialization [2].

Inflammation and oxidative stress are the basic mechanisms causing neointimal hyperplasia and are both involved in the pathogenesis of arterial diseases. Medical ozone, a 95% O_2–5% O_3 mixture, reverses oxidative stress following chronic inflammation [3], scavenges superoxides due to activation of the redoxins superfamily and enhances peroxide detoxification in coronary artery disease [4]. Ozone up-regulates intracellular anti-oxidant enzymes, ameliorates ischemia–reperfusion injury and reduces antioxidant status and inflammation in kidneys [5] and ovaries [6], and exerts critical systemic anti-inflammatory effects [7].

Extracorporeal blood oxygenation and ozonation has been used with the aim of amplifying the results observed with ozone autohemotherapy since 1990 [8]. Ozone autohemotherapy has showed therapeutic efficacy in patients with atherosclerosis without side-effects, being recognized as worthy of testing in various diseases [8].

However, ozonetherapy potential to reduce neointimal hyperplasia after PCTA-stenting has not been evaluated.

The coronary arteries of domestic crossbred pigs respond similar to human coronary arteries after injury [9–11].

The purpose of this prospective, randomized, single-blinded study was to evaluate whether ozonotherapy might reduce restenosis following bare metal stents (BMS) implantation in relation to the redoxin system in pigs.

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2. Methods

2.1. Animals

Twelve male Landrace pigs (51 ± 9 kg) underwent percutaneous transluminal circumflex coronary arteries (CCA) BMS implantation (BMS Coroflex Delta, B. Braun, Melsungen, Germany), under heparine infusion (100 UI/kg) and fluoroscopical guidance, using standard techniques. One stent (30 ± 3 mm × 3.7 ± 0.2 mm) was impacted at high pressure in proximal CCA. Cinecoronariography was performed to evaluate coronary arterial tree during and post BMS implantation. Coronary flow was categorized in accordance with TIMI trial grades.

Pigs were randomized to ozonetherapy (n = 6) or placebo (n = 6) treatment. Before stenting (24 h) and twice a week for 30 days post-stenting, venous blood (100 mL) was collected, bubbled with an ozone-oxygen mixture to 20 μg/mL ozone final concentration, and reinfused through the jugular vein (100 mL of ozonized blood containing 2 mg ozone). Same procedure was performed in placebo group except for the ozonation step. Both groups received antiplatelet treatment: aspirin 300 mg/day and clopidogrel 300 mg (24 h pre-stenting and 75 mg/day during follow-up). Animals were housed in an indoor facility and, 30 days after implantation, were deeply anesthetized with ketamine and euthanized by exsanguination. Immediately after, hearts were excised. Segments containing the stented coronary arteries were cut using a diamond disk saw. Stents were carefully removed and after 40-h fixation in 10% phosphate-buffered formaldehyde solution pH = 7.0, and segments were processed for histology. Serial sections (3 μm thick) were stained with H&E and Masson trichrome. Histomorphometric analysis and planimetry were performed using image analysis software (Image-Pro Plus 4.5 for Windows). For immunohistochemistry, conventional streptavidin-biotin technique and primary polyclonal antibodies against CD68 (monocytes/macrophages), CD34 (endothelial cells), α-actin (smooth muscle cells, SMCs), thioredoxin (Trx)-1 and peroxiredoxin (Prx)-2 were used. Negative control sections were incubated with non-immune normal rabbit serum antibodies. Procedures followed the guidelines of the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.2. Statistical analysis

Two-tailed unpaired t test (parametric variables) and Mann–Whitney test (non-parametric variables) were used for between-treatment data comparison and a p value <0.05 was considered statistically significant (SPSS™ 18.0, SPSS Inc., Chicago, Illinois).

3. Results

Coronary flow did not change after either stent implantation or intracoronary administration of ozonized solutions. TIMI 3 flow was observed in both groups. Compared with placebo, ozonetherapy reduced neointimal thickness (μm) 127 ± 87 vs 648 ± 270 ($p < 0.03$), neointimal area (mm^2) 1.08 ± 0.68 vs 5.03 ± 0.17 ($p < 0.01$) and stenosis (%) 16.63 ± 11.17 vs 61.55 ± 19.90 ($p < 0.03$) and showed a trend to increase lumen area (mm^2) 5.52 ± 0.11 vs 3.10 ± 0.15 ($p < 0.06$, N.S.). Neointimal SMCs proliferation (α-actin immunopositivity), collagen deposition and extracellular matrix increase were the most striking lesions associated with mild neovascularization (Fig. 1).

In placebo group, two neointimal area layers were observed (Fig. 1). One deep layer showed globular SMCs, abundant extracellular matrix and no fibrosis, mild histiocytic infiltration and evident neovascularization encircling struts (hypocellular phase of inflammation). The other, superficial, layer showed abundant spindle or fusiform SMCs, fibromuscular stroma with scarce inflammatory cells (end stage of inflammation). Parietal wall showed variable mild inflammatory infiltration surrounding struts, comprising pale histiocytic cells near the stents and scarce foreign body-

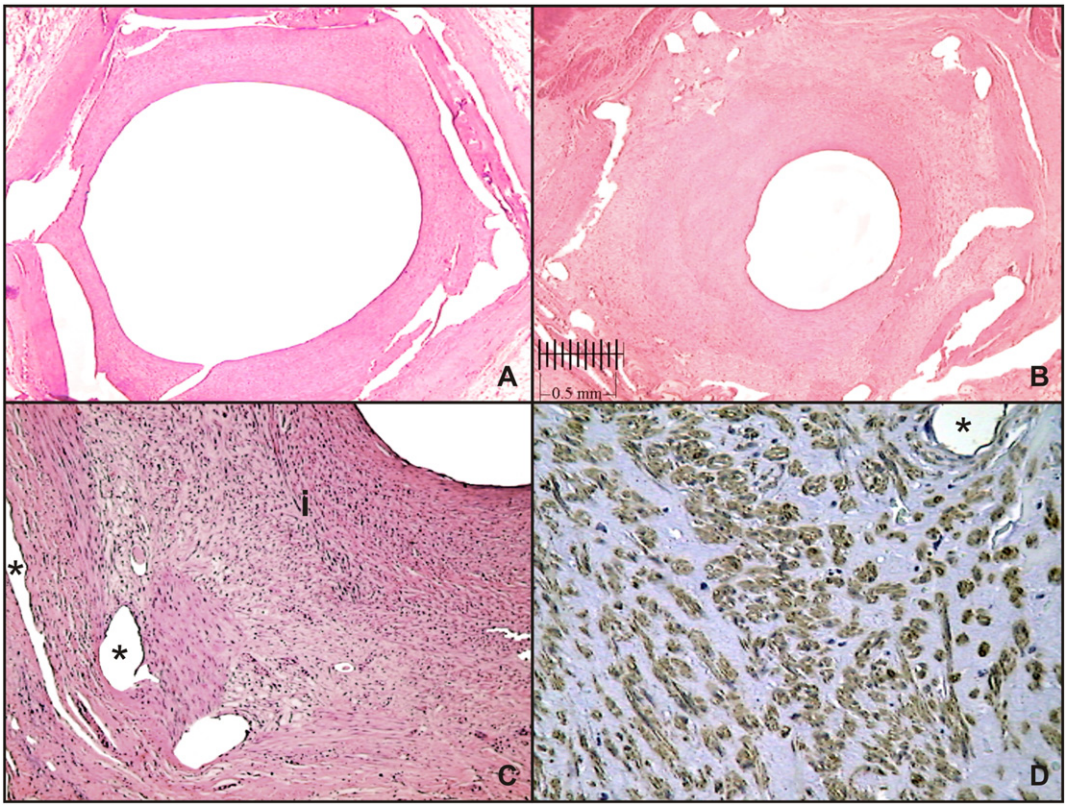

Fig. 1. Histology and morphology 30 days after stent implantation. A. Low-power image of mild–moderate neointimal thickening of a circumflex coronary artery 30 days after bare metal stent (BMS) implantation in an ozone-treated group. Lumen is fairly preserved. Optical negative spaces were formerly occupied by stent struts. H&E 4×. B. Low-power image of severe neointimal thickening of a circumflex coronary artery 30 days after BMS placement in placebo group. A marked concentric luminal stenosis is observed. H&E 4×. C. High-power image of a stented artery in placebo group showing severe neointimal healing response and marked concentric stenosis. Two layers can be clearly depicted: one internal (i) with marked collagen deposition, smooth muscle cells, scarce inflammatory infiltration and no vascularization, with preserved endoluminal surface, and the other, external, with striking smooth muscle cell proliferation, extracellular matrix increase and neovascularization. * indicates former occupation by stent strut. H&E 200×. D. High-power image of a stented artery in placebo group depicting two well-defined neointimal layers: one deep (external, left) with globular smooth muscle cells, absent/little fibrosis and abundant extracellular matrix limiting with the struts (*), and the other superficial (internal, right), with abundant spindle or fusiform smooth muscle cells, a fibromuscular stroma with scarce inflammatory cells and no neovascularization. Anti-alpha actin. 400×.

multinucleated giant cells. Severe inflammatory reaction was observed reaching adventitia, in discontinuities of the internal elastic membrane caused by stents pressing towards the muscular layer. Figs. 2 and 3 show typical features of restenosis, neovascularization and SMC s proliferation in particular, observed in placebo group.

In contrast, ozone group showed large attenuation of restenotic lesions, re-endothelization (anti-CD34 immunopositivity) and absence of macrophages infiltration (negative anti-CD68 immunolabeling).

Placebo group showed strong Trx-1 immunopositivity (Fig. 4). In contrast, less intense Trx-1 immunolabeling was found in neointimal areas and endothelial cells in ozone group. Immunolabeling for Prx-2 was negative in both groups. (See Fig. 5.)

4. Discussion

In our study, ozonetherapy strikingly reduced restenosis 30 days following percutaneous transluminal coronary angioplasty with BMS implantation in pigs. Pre-injury stimulation of the redoxin system by ozone might neutralize oxidative damage from the start and increase antioxidative buffering capacity post-injury, reducing further damage and so the demand for antioxidant enzymes.

Post-angioplasty restenosis is a multifactorial process that depends on two basic mechanisms: intimal hyperplasia and arterial remodeling [12–14]. Implantation of sirolimus- or paclitaxel-eluting stents led to inhibition of neointimal hyperplasia [2]. However, the procedure resulted in poor endothelialization of the left anterior descending coronary arteries of pigs 28 days after surgery [2]. In our study, ozonized autotransfusion succeeded in drastically reducing neointimal hyperplasia along with re-endothelialization as well.

Traditionally, intimal hyperplasia after BMS implantation has been considered stable, with an early peak between 6 months and 1 year and a late quiescent period thereafter [15]. Kimura et al. reported an early peak of intimal growth, followed by intimal regression with luminal enlargement in a clinical study with 3-year follow-up by angiography [16]. Follow-up coronary angiography of 137 lesions at six months, 114 lesions at one year, and 72 lesions at 3 years revealed a decrease in minimal luminal diameter from 2.54 ± 0.44 mm immediately after stent implantation to 1.87 ± 0.56 mm at six months [16].

By now, 20 years following Kimura et al. study, bare metal stents (BMS) continue to be widely used in patients with coronary artery disease undergoing percutaneous revascularization. Unfortunately however, late after BMS implantation, progressive luminal restenosis has also been reported resulting in a significant rate of stent failure events [17].

Our interpretation agrees with the ozone oxidative preconditioning mechanism, extensively investigated [5,8–10] and the induction of numerous antioxidant enzymes via direct activation of the transcription factor Nrf2, triggering antioxidant response [18–20].

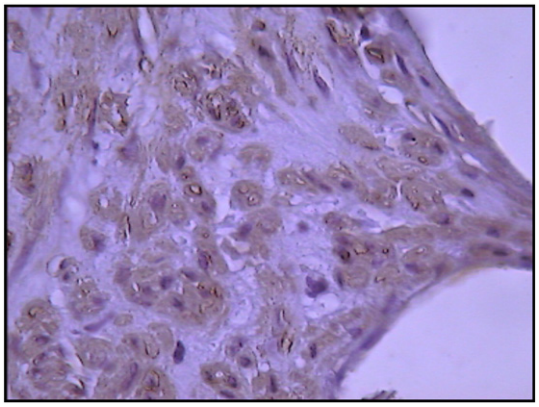

Fig. 3. Smooth muscle cell proliferation in placebo group. A proliferation of smooth muscle cells is shown surrounded by matrix increase, close to two struts. Anti-alpha actin 400×.

Ozone is extremely reactive and it must be used in micrograms. The rationale for its use is that a small acute oxidative stress will induce a positive antioxidant response [18]. Indeed, from the pharmacological viewpoint, ozone acts in a hormetic fashion according an inverted V shape curve [18,21,22]. Other reported hormetic stressors include moderate exercise, calorie restriction, hyperbaric oxygen therapy and pro-oxidant exposure [18]. Exercise-induced ROS production is involved in the induction of antioxidants, DNA repair and protein degrading enzymes, resulting in decreases in the incidence of oxidative stress-related diseases such as cardiovascular disease and type 2 diabetes, and retardation of the aging process [18].

Increased glycolysis rate, stimulation of NO synthesis and induction of growth factors reported for ozone, may concurrently contribute to explain the reduction of neointimal growth and improved re-endothelialization presently observed [22,23].

Ozone causes an increase in the red blood cell glycolysis rate, and glycolytic enzymes enhance cell proliferation [21,24]. Ozone increases endothelial nitric oxide synthase expression/activity and consequent endothelial progenitor cells' recruitment thus protecting from acute myocardial infarction [25]. Prostacyclin, a vasodilator, is also induced by ozone [26].

Induction of moderate oxidative stress due to ozone reactivity and interaction with several biological components might account for the beneficial effect of ozone therapy in reducing neointimal hyperplasia post-stenting. Nevertheless, the clinical outcome depends on the strength of oxidative stress which determines the critical balance between effectiveness and toxicity of ozone [21]. As with other hormetic stressors, moderation is good, whereas excess is harmful.

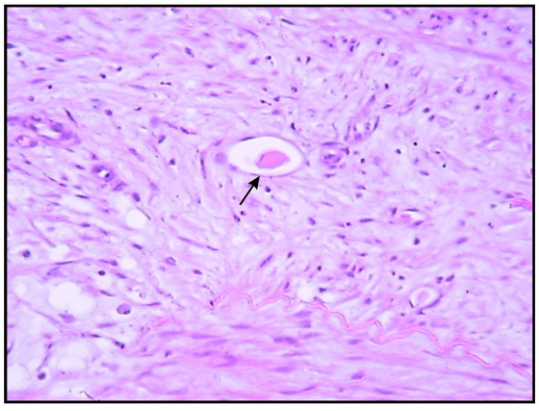

Fig. 2. Typical image of restenosis observed in placebo group. A neoformed vessel, surrounded by soft connective tissue is pointed out by an arrow. H&E 200×.

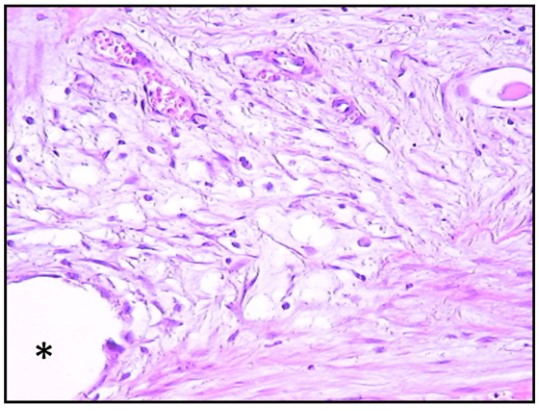

Fig. 4. Soft connective tissue in the vecinity of a strut. The asterisk (*) indicates a strut. H&E × 100.

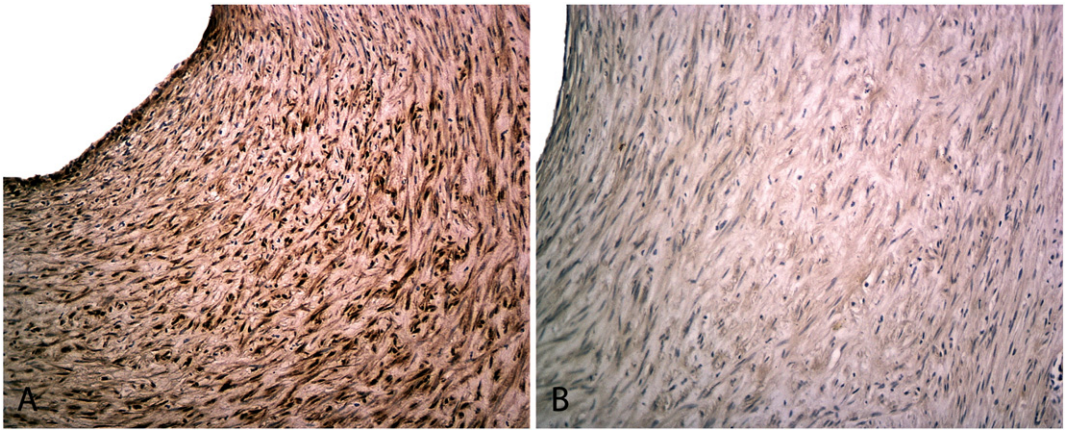

Fig. 5. Immunohistochemical staining for Trx-1 and Prx-2. A. Intense immunohistochemical staining for Trx-1 was found in neointimal areas and endothelial cells in placebo group. Anti-Trx-1. 400×. B. No positive staining for Prx-2 was observed in placebo group. Anti-Prx-2. 400×.

5. Conclusion

We suggest that ozone autohemotransfusion prevented post-stenting neointima hyperplasia and inflammatory reaction, via preinjury upregulation of the innate detoxifying and antioxidant system, involving thioredoxins. Prevention of the oxidative-inflammatory milieu might also favor later re-endothelization.

Conflict of interest

The authors report no relationships that could be construed as a conflict of interest. All authors have approved the manuscript.

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References

[1] H. Hamid, J. Coltart, 'Miracle stents'—a future without restenosis, McGill J. Med. 10 (2007) 105–111.
[2] S.Y. Lim, M.H. Jeong, S.J. Hong, et al., Inflammation and delayed endothelialization with overlapping drug-eluting stents in a porcine model of in-stent restenosis, Circ. J. 72 (2008) 463–468.
[3] V. Bocci, How a calculated oxidative stress can yield multiple therapeutic effects, Free Radic. Res. 46 (2012) 1068–1075.
[4] G. Martínez-Sánchez, L. Delgado-Roche, A. Díaz-Batista, et al., Effects of ozone therapy on haemostatic and oxidative stress index in coronary artery disease, Eur. J. Pharmacol. 691 (2012) 156–162.
[5] E.B. Sancak, H. Turkön, S. Çukur, et al., Major ozonated autohemotherapy preconditioning ameliorates kidney ischemia–reperfusion injury, Inflammation 19 (2015 Aug) 1–9.
[6] I. Sayar, S. Bicer, C. Gursul, et al., Protective effects of ellagic acid and ozone on rat ovaries with an ischemia/reperfusion injury, J. Obstet. Gynaecol . Res. 42 (2016) 52–58.
[7] V. Bocci, I. Zanardia, G. Valacchi, et al., Validity of oxygen-ozone therapy as integrated medication form in chronic inflammatory diseases, Cardiovasc. Hematol. Disord. Drug Targets 15 (2015) 127–138.
[8] N. Di Paolo, V. Bocci, D.P. Salvo, et al., Extracorporeal blood oxygenation and ozonation (EBOO): a controlled trial in patients with peripheral artery disease, Int. J. Artif. Organs. 28 (2005) 1039–1050.
[9] R.S. Schwartz, N.A. Chronos, R. Virmani, Preclinical restenosis models and drug-eluting stents: still important, still much to learn, J. Am. Coll. Cardiol. 44 (2004) 1373–1385.
[10] R.S. Schwartz, J.G. Murphy, W.D. Edwards, et al., Restenosis after balloon angioplasty: a practical proliferative model in porcine coronary arteries, Circulation 82 (1990) 2190–2200.
[11] R. Schwartz, K. Huber, J. Murphy, et al., Restenosis and the proportional neointimal response to coronary artery injury: results in a porcine model, J. Am. Coll. Cardiol. 19 (1992) 267–274.
[12] J.L. Elesbão, A.H. Pereira, M.A. Grüdtner, F. Meyer, Morphometric analysis of swine carotid artery angioplasty with or without cobalt-chromium stent implantation, J. Vasc. Br. 9 (2010) 40–46.
[13] F. Versaci, A. Gaspardone, Prevention of restenosis after stenting: the emerging role of inflammation, Coron. Artery Dis. 15 (2004) 307–311.
[14] K. Ouriel, Peripheral arterial disease, Lancet 358 (2001) 1257–1264.
[15] S.J. Park, S.J. Kang, R. Virmani, et al., In-stent neoatherosclerosis: a final common pathway of late stent failure, J. Am. Coll. Cardiol. 59 (2012) 2051–2057.
[16] T. Kimura, H. Yokoi, Y. Nakagawa, et al., Three-year follow-up after implantation of metallic coronary-artery stents, N. Engl. J. Med. 334 (1996) 561–566.
[17] S. Mowakeaa, A. Iskandar, N. Kakouros, Neoatherosclerosis in very late stenosis of bare metal stent by optical coherence tomography, Case Rep. Cardiol. 2016 (2016) 1652065.
[18] V. Bocci, G. Valacchi, Nrf2 activation as target to implement therapeutic treatments, Front Chem. 3 (2015) 4.
[19] L. Re, G. Martínez-Sánchez, M. Bordicchia, et al., Is ozone pre-conditioning effect linked to Nrf2/EpRE activation pathway in vivo? A preliminary result, Eur. J. Pharmacol. 742 (2014) 158–162.
[20] A. Pecorelli, V. Bocci, A. Acquaviva, et al., NRF2 activation is involved in ozonated human serum upregulation of HO-1 in endothelial cells, Toxicol. Appl. Pharmacol. 267 (2013) 30–40.
[21] M. Sagai, V. Bocci, Mechanisms of action involved in ozone therapy: is healing induced via a mild oxidative stress? Med. Gas Res. 1 (2011) 29.
[22] E.J. Calabrese, Hormetic mechanisms, Crit. Rev. Toxicol. 43 (2013) 580–606.
[23] J. Zhang, M. Guan, C. Xie, et al., Increased growth factors play a role in wound healing promoted by noninvasive oxygen-ozone therapy in diabetic patients with foot ulcers, Oxidative Med. Cell. Longev. 2014 (2014) 273475.
[24] A.M. Elvis, J.S. Ekta, Ozone therapy: a clinical review, J. Nat. Sci. Biol. Med. 2 (2011) 66–70.
[25] C. Di Filippo, M. Luongo, R. Marfella, et al., Oxygen/ozone protects the heart from acute myocardial infarction through local increase of eNOS activity and endothelial progenitor cells recruitment, Naunyn Schmiedeberg's Arch. Pharmacol. 382 (2010) 287–291.
[26] S. Schulz, S. Ninke, B. Watzer, R.M. Nüsing, Ozone induces synthesis of systemic prostacyclin by cyclooxygenase-2 dependent mechanism in vivo, Biochem. Pharmacol. 83 (2012) 506–513.