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artículo original

# *Ozone therapy improves the antioxidant status of high-density lipoproteins and reduces lipid peroxidation in coronary artery disease patients.*

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## Keywords

*Ozone therapy*
*coronary artery disease*
*oxidative stress*
*high density lipoprotein*
*paraoxonase.*

## Abstract

*Coronary artery disease (CAD) is the most common cause of death in western societies. Epidemiological studies have found that plasma concentration of high density lipoproteins (HDL) correlates inversely with the incidence of CAD. The quality and functionality of HDL, more than quantity, appears to be an important predictor of antiatherogenic properties of these particles. Epidemiological evidence demonstrates that low HDL-paraoxonase activity is associated with increased risk of cardiovascular disease. Evidence that antioxidant enzymes, and other subcellular activities could be modulated by low doses of ozone is now proven and support itsclinical application. The aim of the present study was to evaluate the effect of ozone therapy on paraoxonase 1 lactonase activity and lipid damage in CAD patients. We included 52 patients in the clinical study. The first group (n=26) received 20 sessions of ozone (40µg/mL; 200 mL) by rectal insufflation, meanwhile the second one was treated with oxygen only (n=26, control group). At the end of the study we determined spectrophotometrically the paraoxonase-lactonase activity, and the LDL and serum susceptibility to lipid peroxidation. The results showed that ozone therapy was able to reduce the malondialdehyde levels in treated patients at the same time that paraoxonase 1 lactonase activity was significantly ($p<0.05$) increased. Our results suggestthat ozone may be used in combination with the conventional drugs for CAD therapy. Nevertheless, future clinical trials will be necessary to establish how long HDL antioxidant status is maintained after therapy and how often it will be necessary to repeat the ozone treatment.*

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## Introduction

Coronary artery disease (CAD) is the most common cause of death in western societies.[1] Prior to the Framingham study, there was relatively little appreciation of the risk factors for cardiovascular disease.The Framingham study also provided insight into the relationship between high-density lipoprotein cholesterol (HDL-C) levels and CAD incidence.[2]

High density-lipoprotein HDL is a mixture of lipoprotein particles with densities ranging from 1.063 to 1.21 g/mL.The main protein component of HDL is apolipoprotein AI (apoA-I) which plays a key role in the biogenesis and functions of HDL.[3] Human high-density lipoproteins (HDL) play an important role against the development of atherosclerosis and cardiovascular diseases.[4]

Following a number of epidemiological studies, it was found that plasma concentration of HDL correlates inversely with the incidence of CAD.[5-8] However, more recent findings have suggested that the relationship between HDL and cardiovascular risk is more complex and extends beyond the levels of HDL in plasma.[9] The quality and functionality of HDL, more than quantity, appears to be an important predictor of antiatherogenic properties of these particles. Emerging data suggest that HDL quality and function may also be significantly reduced by atherosclerosis and other inflammatory diseases.[10]

Beside their key role in the regulation of cholesterol homeostasis, HDL exhibit antioxidant and anti-inflammatory properties that participate to their general antiatherogenic effect.[4] It is likely that the antioxidant function of HDL depends on their apoprotein moiety, and/or HDL-associated proteins.[11] Paraoxonase (PON) is an HDL-associated calcium-dependent enzyme, able to hydrolyze oxidized fatty acids from phospholipids and to reduce the accumulation of oxidized lipids in low-density lipoproteins (LDL), there by inhibiting their proinflammatory response.[1] Epidemiological evidence demonstrates that low PON1 activity is associated with increased risk of cardiovascular events and cardiovascular disease.[12]

Therapeutic strategies, designed to preserve or increase the HDL-associated PONmay be identified as an approach for atherosclerosis treatment. Evidence that antioxidant enzymes, nitric oxide pathways and other subcellular activities could be modulated by low doses of ozone is now proven and could support the effects of ozonetherapy in many pathological conditions such as CAD.[13,14] In the present study we hypothesized that ozone therapy improvesthe antioxidant state of HDL by an increase of PON activity, contributing to reduce lipoproteins oxidation.

## Materials and methods

Study design.

The protocol of the controlled clinical trialwas reviewed and approved by the Institutional Committee for Research on Human Subjects, and the procedures were in accordance with principles of the Declaration of Helsinki.[15] All patients gave their informed consent to be enrolled after receiving adequate information about the study (characteristics of the study, benefits and possible side-effects). Medical personnel were instructed to report all adverse experiences whether or not described for the medication used. Adult patients of both genders and different ethnicity with a diagnosis of CAD who attended in the Hospital “Ivan Portuondo” (Artemisa,

Cuba) from January 2010 to February 2012 were eligible to participate in the study.

Exclusion criteria were: severe septic conditions, hypersensitivity to the medication to be used, hepatic dysfunction, renal failure (serum creatinine level >1.32 µmol/L), pregnancy, cancer or other serious disease, inability to cooperate with the requirements of the study, recent history of alcohol or drug abuse, current therapy with any immunosuppressive agent or anticonvulsant, concurrent participation in another clinical study, or current treatment with an investigational drug.

For the calculation of the sample size,the Gn* Power 3 system (version3.1) was used.[16]
The type 1 error was and the type2 error was, with a minimal difference between effect rates no higher than 25%.The target level ofen roll ment was determined to be 23 patients per group. Assuming that 10% of study patients would be lost to follow-up, 26 patients per group were studied.

The patients were randomly distributed in two different groups:
(1): patients were treated with 200 mL of O3/O2 containing 40µg/mL of ozone during 20 sessions in alternated days;
(2): the control patients were similar treated with 200 mL of medical oxygen only.
Insufflation procedur.

Nelaton Robinson catheter 40 cm was introduced 10/15 cm by rectal way to deliver the gas and was place in site for 5min. The patients were encouraged to empty his or her blad derandbowels before the procedure. The insufflation was done immediately after a colonic enema incase of constipation.

Medical ozone generation.

Ozone was generated by OZOMED equipment manufactured by the Ozone Research Center (Havana, Cuba) and was administered by rectal insufflation. Ozone was obtained from medical grade oxygen, and was used immediately upon generation and represented only about 3% of thegas mixture (O3/O2). The ozone concentration was measured by using a built-in UV spectrophotometer set at 254 nm.

LDL and HDL isolation

LDL and HDL were prepared from the serum of fasted patients by discontinuous density gradient ultracentrifugation.[17] The LDL/HDL were then dialyzed against 150 mM NaCl and 1 mM CaCl2 (pH 7.4), and their protein content was determined as described previously.[18]
PON1- Lactonase activity

Five microliters of HDL diluted 1:10 (v/v) were taken for a total reaction volume of 200 µL. Lactonase activity was measured using 5-(thiobutyl) butyrolactone (TBBL) as the substrate. [19] Initial rates of hydrolysis were determined spectrophotometrically at 405 nm. The assay mixture included 1 mM TBBL and 1 mM CaCl2 in 50 mM Tris-HCl, pH 8.0. Nonenzymatic hydrolysis of TBBL was subtracted from the total rate of hydrolysis. One unit of lactonase activity was equal to 1 µmol of TBBL hydrolyzed/min mL as reported previously.[19]

LDL oxidation assays.

LDL oxidationwas carried out as previously described[20] with minor modifications. LDL oxidation reaction was initiated by adding 4 µM copper sulfate (in a double-distilled water) solution and incubating at 37°C during 6 h. Then, malondialdehyde (MDA) concentration was determined at 587 nm using the method described previously.[21] Standard solutions of MDAbis (dimethyl acetal) were employed in order to calculate MDA concentrations.

Serum susceptibility to lipid peroxidation assay.

The susceptibility to lipid peroxidation was determined by the peroxidation potential (PP). Serum samples were incubated with a solution of 2 mMcopper sulfate at 37 ºC for 24 h and then MDA concentration was determined.[22]

Statistical analysis.

Data were analyzed by one-way analysis of variance (ANOVA) followed by a homogeneity variance test (Bartlett-Box). Inaddition, a multiple comparison test was used (Duncantest). Results are presented as mean ± standard error of mean (SEM). The level of statistical significance employed was $P<0.05$.

## Results.

General characteristics of the patients involved in the study

In relation to the baseline characteristics (Table 1), both groups were similar at randomization ($P>0.05$). More than 40% of patients in both groups were older than 60 years and males were the majority. Their medical history was characterized mainly by hypertension and ischemic cardiopathy.

| | Characteristics | Control group (n=26) | | Ozone group (n=26) | |
|---|---|---|---|---|---|
| | | n | % | n | % |
| Age (years) | 50-60 | 10 | 38.46 | 9 | 34.62 |
| | 61-70 | 11 | 42.31 | 13 | 50.00 |
| | 71-80 | 5 | 19.23 | 4 | 15.38 |
| Gender | Female | 20 | 76.92 | 17 | 65.38 |
| | Male | 6 | 23.08 | 9 | 34.62 |
| Previous History | Hypertension [a] | 23 | 88.46 | 19 | 73.08 |
| | Myocardial stroke | 8 | 30.77 | 1 | 3.85 |
| | Ischemic Cardiopathy | 24 | 92.31 | 20 | 76.92 |
| Risk Factors | Hypertension | 26 | 100 | 21 | 80.77 |
| | Hypercholesterolemia [b] | 17 | 65.38 | 13 | 50.00 |
| | Obesity [c] | 8 | 30.77 | 2 | 7.69 |
| | Smoking | 19 | 73.08 | 21 | 80.77 |
| Complementary diagnosis criteria | TC (mM) | 8.23 ± 0.87 | | 7.99 ± 0.94 | |
| | BMI (kg/m$^2$) | 30.23 ± 4.85 | | 32.71 ± 5.02 | |

Biochemical determinations

Data shown in figure 1 demonstrated that PON1-lactonase activity was significantly increased ($p<0.05$) by ozone therapyin comparison with oxygen-treated patients. Meanwhile, the LDL and serum susceptibility to lipid peroxidation were significantly lower ($p<0.05$) in ozonized patients than in controls (insufflated with oxygen only).

## Discussion

Cardiovascular diseases are considered as major cause of mortality around the worldand contribute to more than one-third of deaths in the USA.[23] Consequently, developing a treatment regimen that can slow or even reverse atherosclerosisis imperative. In this context ozone therapy, simultaneously applied with regular drugs, may represent a promising approach for correction oxidative stress and improving the uncertain prognosis of many patients.

The critical importance of PON1 is underscored by findings that HDL particles isolated from mice that overexpress the gene for PON1 are highly resistant to lipid hydroperoxide formation induced by copper.[24] Conversely, decreased PON1 activity is associated with dyslipidemia and insulin resistance in leptin-deficient and LDL receptor-deficient mice and diabetic humans.[25,26] By virtue of its capacity to prevent lipid oxidation, HDL reduces the atherogenicity ofapoB-containing lipoproteins.[27] In addition to reducing oxidized LDL-induced inflammatory responses, HDL is also an effective inhibitor of endothelial cell adhesion molecule binding sites for circulating monocytes.[28] Furthermore, the hydrolytic activities of PON1 lactonase, arylesterase, and paraoxonase are all inactivated under OS-associated diseases, such as atherosclerosis.[29] The therapeutic strategies to increase HDL functionality are considered a promissory alternative for atherosclerosis control. In this context, ozone therapy represents a plausible intervention.

Now a days, scientific evidence connected the modulation of antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) as a consequence of applying low ozone doses.[30] On the other hand, Ajamieh and coworkers demonstrated that the protective mechanism mediated by Ozone-oxidative preconditioning involves protein synthesis.[31] Elevated ROS concentrations induce gene expression in many cells, whose products exhibit antioxidant activity. A major mechanism of redox homeostasis is based on the ROS-mediated induction of redox-sensitive signal cascades that lead to increased expression of antioxidants.[32]

These above mentioned facts support some of the current clinical applications of ozone therapy (33) and permit to explain, in part, the fact that PON1 lactonase activity was increased in ozone-treated CAD patients. Recently, Tavori and coworkers demonstrated that lactonase activity is important for antiatherogenic functions of HDL. They also suggest that antiatherosclerotic properties of PON1 could be, at least, associated with the removal of plaque-oxidized lipids from human atherosclerotic plaque.[29]

Oxidative modification of LDL is a key event during early atherogenesis, contributing to cholesterol and oxysterolaccumulation in the arterial wall, foam cell formation, and plaque development.[34] Biomarkers of ROS-induced damage have the potential not only to determine the extent of oxidative injury, but also to predict the potential efficiency of therapeutic strategies aimed at reducing such an OS.[13] In line with the increase in PON1 lactonase activity there was

a reduction of lipid peroxidation in both, LDL and sera from ozonized patients. We have previously report that rectal insufflation of low ozone dose induces a reduction of systemic MDA and lipid hydroperoxides in CAD.[13,14]

## Conclusions

Repeated rectal insufflations of ozone contributed to enhance the HDL-PON1 lactonase activity, meanwhile reduces the oxidative injury of lipids. These observations suggest that ozone may be used in combination with the conventional drugs for CAD therapy. Future clinical trials will be necessary to establish how long HDL antioxidant status is maintained after therapy and how often it will be necessary to repeat the ozone treatment.

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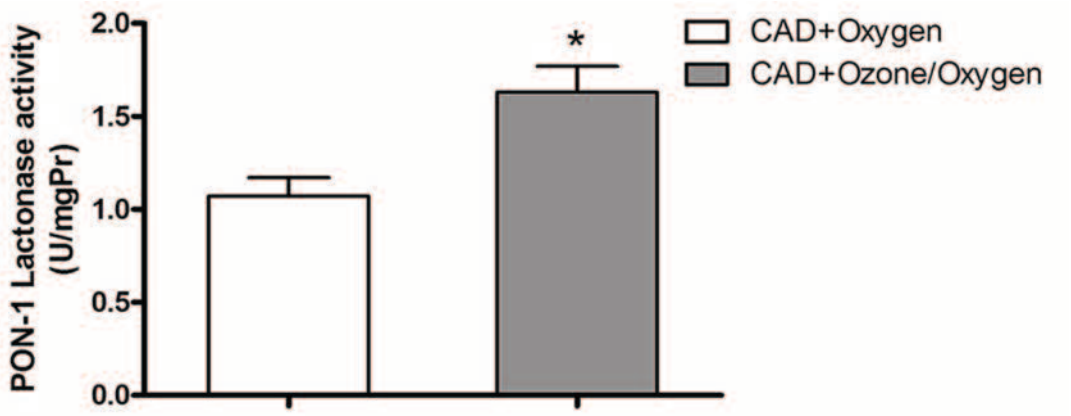
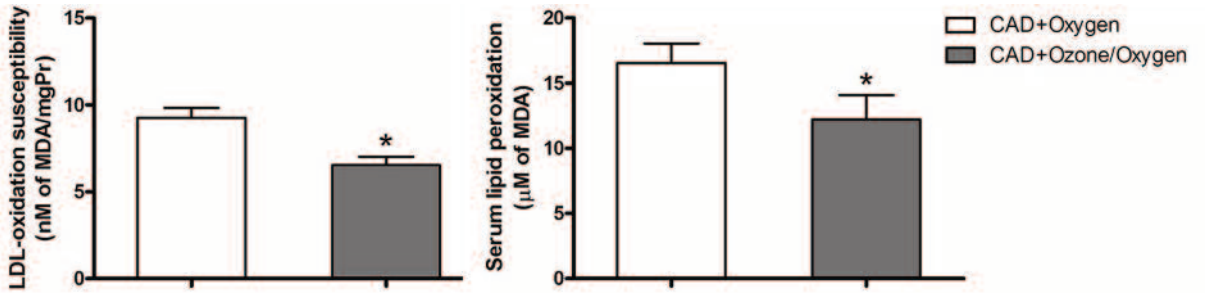
Caption for figures

Figure 1.




Effect of ozone therapy on PON1 lactonase activity.Asterisk represents statistical differences ($p<0.05$) between groups. The lactonase activity was reported relative to HDL protein concentration (mgPr).

Figure 2.




Effect of ozone therapy on low-density lipoprotein (LDL) and serum lipid oxidation. Asterisks represent statistical differences ($p<0.05$) between groups. Malondialdehyde (MDA) concentration, determined in LDL fraction, was reported relative to LDL protein concentration (mgPr).

## References

1. TsompanidiEM, Brinkmeier MS, Fatiadou EH, Giakoumi SM, KypreosKE. HDL biogenesis and functions: Role of HDL quality and quantity in atherosclerosis. Atherosclerosis 2010; 208:3-9.

2. Gordon T, Kannel WB, Castelli WP, Dawber TR. Lipoproteins, cardiovascular disease, and death. The Framingham study. Arch Intern Med 1981; 141:1128-1131.

3. Zannis VI, Kypreos KE, Chroni A, Kardassis D, Zanni EE. In: Loscalzo J, editor. Molecular mechanisms of atherosclerosis. New York, NY: Taylor & Francis; 2004. p. 111-74.

4. Negre-Salvaire A, Dousset N, Ferretti G, Bacchetti T, Curatola G, Salvayre R. Antioxidant and cytoprotective properties of high-density lipoproteins in vascular cells. Free Radic Biol Med 2006; 41:1031-1040.

5. Miller NE, Thelle DS, Forde OH, Mjos OD. The Tromso heart-study. Highdensity lipoprotein and coronary heart-disease: a prospective case-control study. Lancet 1977; 1:965-968.
6. Wilson PW, Abbott RD, Castelli WP. High density lipoprotein cholesterol and mortality. The Framingham Heart Study. Arteriosclerosis 1988; 8:737-741.

7. Gordon DJ, ProbstfieldJL, Garrison RJ. High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. Circulation 1989; 79:8-15.

8. Shah PK, Amin J. Low high density lipoprotein level is associated with increased restenosis rate after coronary angioplasty. Circulation 1992; 85:1279-1285.

9. Sviridov D, Mukhamedova N, Remaley AT. Antiatherogenic functionality of high density lipoprotein: howmuch versus howgood. JAtheroscler Thromb 2008; 15:52-62.

10. White CR, Datta G, Zhang Z, Gupta H, Garber DW, Mishra VK, et al. HDL Therapy for cardiovascular diseases: the road to HDL mimetics. Cur Atheroscler Rep 2008; 10:405-412.

11. Von Eckardstein A, Hersberger M, Rohrer L. Current understanding of the metabolism and biological actions of HDL. Curr OpinClinNutrMetab Care 2005; 8:147-152.
12. Jarvik GP, RozekLS, BrophyVH, HatsukamiTS, Richter RJ, Schellenberg GD,et al. Paraoxonase (PON1) phenotype is a better predictor of vascular disease than is PON1(192) or PON1(55) genotype. Arterioscler Thromb Vasc Biol 2000; 20:2441-2447.

13. Delgado-Roche L, Martínez-Sánchez G, Díaz A, Re L. Effects of ozone therapy on oxidative stress biomarkers in Coronary Artery Disease patients. Int J Ozone Therap 2011; 10:99-104.

14. Martínez-Sánchez G, Delgado-Roche L, Díaz A, Pérez-Davison G, Re L. Effects ofozonetherapyonhaemostaticandoxidativestressindexin coronaryarterydisease. Eur J Pharmacol 2012; 691:156-162.

15. WMA 2004.World medical associationDeclarationofHelsinki.Ethical principles for medical research involving human subjects. J IntBioethique. Adoptedbythe 18thWMAGeneralAssembly,Helsinki,Finland,June1964, pp.124-129.

16. Faul F,Erdfelder E, Lang AG, Buchner A.GnPower 3:aflexiblestatisticalpower analysisprogramforthesocial,behavioral,andbiomedicalsciences.Behav Res Methods 2007; 39:175-191.

17. Aviram M. Plasma lipoprotein separation by discontinuous density gradient ultracentrifugation in hyperlipoproteinemic patients. Biochem Med 1983; 30:111-118.

18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J BiolChem1951; 193:265-275.

19. Khersonsky O, Tawfik DS. Chromogenic and fluorogenic assays for the lactonase activity of serum paraoxonases. Chembiochem 2006; 7:49-53.

20. Belinky PA, Aviram M, Fuhrman B, Rosenblat M, Vaya J. The antioxidative effects of the isoflavanglabridin on endogenous constituents of LDL during its oxidation. Atherosclerosis 1998; 137:49-61.

21. Erdelmeier I, Gerard D, YadanJC, Chaudiere J. Reactions of N-methyl-2-phenyl-indole with malondialdehyde and 4-hydroxy-alkenals. Mechanistic aspects of the colorimetric assay of lipid peroxidation. Chem Res Toxicol 1998; 11: 1184-94.

22. Ozdemirler G, Mehmetcik G, Oztezcan S, Toker G, Sivas A, Uysal M. Peroxidation potential and antioxidant activity of serum in patients with diabetes mellitus and myocardial infarction. Metab Res 1995; 271: 194-6.

23. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Betty JD, Borden WB, et al. Heart Disease

and Stroke Statistics-2013 Update: A report from the American Heart Association. Circulation 2013; 127:e6-e245.

24. Oda MN, Bielicki JK, Ho TT, et al. Paraoxonase 1 overexpression in mice and its effect on high-density lipoproteins. BiochemBiophys Res Commun 2002, 290:921-927.

25. Mertens A, Verhamme P, BielickiJK, et al. Increased low-density lipoprotein oxidation and impaired high density lipoprotein antioxidant defense are associated with increased macrophage homing and atherosclerosis in dyslipidemic obese mice: LCAT gene transfer decreasesatherosclerosis. Circulation 2003, 107:1640-1646.

26. Sanguinetti SM, BritesFD, Fasulo V, et al. HDL oxidability and its protective effect against LDL oxidation in type 2 diabetic patients. Diabetes NutrMetabClinExper 2001; 14:27-36.

27. Barter PJ, Nicholls S, Rye KA, et al. Antiinflammatory properties of HDL. Circ Res 2004, 95:764-772.

28. AshbyDT, Rye KA, Clay MA, et al. Factors influencing the ability of HDL to inhibit expression of vascular cell adhesion molecule-1 in endothelial cells. Arterioscler Thromb Vasc Biol 1998, 18:1450-1455.

29. Tavori H, Aviram M, Khatib S, Musa R, Mannheim D, Karmeli R, et al. Human carotid lesion linoleic acid hydroperoxide inhibits paraoxonase 1 (PON1) activity via reaction with PON1 free sulfhydryl cysteine 284. Free Radic Biol Med 2011; 50:148-156.

30. Re L, Mawsouf MN, Menéndez S, León OS, Martínez-Sánchez G, Hernández F. Ozone therapy: clinical and basic evidences of its therapeutic Potential. Arch Med Res 2008; 39:17-26.

31. Ajamieh H, Merino N, Candelario-Jalil E, Menéndez S, Martínez-Sánchez G, Re L, et al. Similar protective effect of ischaemic and ozone oxidative preconditionings in liver ischaemia/reperfusion injury. Pharmacol Res 2002;45:333-339.

32. Dröge W. Free radicals in the physiological control of cell function. Physiol Rev 2002; 82:47-95.

33. Steppan J, Meaders T, Muto M, Murphy KJ. A metaanalysis of the effectiveness and safety of ozone treatments for herniated lumbar discs. J Vasc IntervRadiol 2010;21:534-548.

34. BadimonJJ, Fuster V, ChesebroJH, Badimon L. Coronary atherosclerosis. A multifactorial disease. Circulation 1993; 87:II3-II16.