·Clinical Research·

Effects of major ozonated autohemotherapy in the treatment of dry age related macular degeneration: a randomized controlled clinical study

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Abstract

• AIM: To evaluate the effect of systemic ozonated major autohaemotherapy (O_3-AHT) in patients affected by dry age related macular degeneration (AMD).

• METHODS: This study was a randomized, controlled clinical study. One hundred and forty patients with the diagnosis of AMD in both eyes, with the study eye presenting dry AMD and soft drusen, were randomly assigned in a 1:1 ratio to either receive 27 major ozonated autohemotherapy treatments during 12-month period, or a standardized multi-vitamin therapy. Primary outcome was the change in best corrected visual acuity (mean logMar change) between the baseline and 6 and 12 months, end point of the study. In addition, to investigate the safety of prolonged ozonated autohaemotherapy, we measured the routine haemato-chemical parameters and biochemical oxidative stress values at baseline and after 12 months treatment time.

• RESULTS: The mean baseline best corrected visual acuity in study eyes was 0.36 in the treatment group and 0.38 in the control group (difference not statistically significant). At the primary endpoint, 6 months post-baseline, the mean logMAR change in the treated group improved by 0.1 and the values of the control group at the same time impaired by 0.2 respect to the baseline. Four percent and twenty-five percent of eyes in the group treated with O_3-AHT gained 1 or more lines after 6 and 12 months respectively compared to 0% in the eyes which received no treatment (P <0.05 at 12 months). None of the treated patients experienced a loss in visual acuity in their study eye at 6 and 12 months, compared to 16% and 40 % of patients in the control group who lost 2 lines or more at 6 months and 12 months respectively (P <0.05 treated *vs* control group)). Major ozonated autohemotherapy was shown to be safe and well-tolerated by the patients. Moreover, the haematochemical parameters showed a decrease in the Reactive Oxygen Metabolites (300±10.1 UCARR at 12 months compared to a baseline value of 380±10.4 UCARR, P<0.05) and an increase in Biological Antioxidant Potential plasma values (2100±34.8 micromoles/ C vitamin after 12 months compared to the baseline value of 1610±36.2, P<0.05) in the treated patients when compared to the control group. This data suggests that major ozonated autohaemotherapy may exert a role in reducing oxidative stress by endogenously stimulating the production of antioxidant molecules.

• CONCLUSION: The results of this study suggests that major ozonated autohaemotherapy could be a safe and effective therapeutic option for high-risk patients with dry AMD, and that a series of such treatments could improve the natural course of AMD.

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INTRODUCTION

Age-related macular degeneration (AMD) represents one of the most important causes of irreversible visual loss in developed countries, affecting 20%-30% of people over the age 65 and it is associated with significant reduction in the quality of life, daily functioning and independence [1,2]. AMD is categorized into two main forms: a dry, non exudative, atrophic form and a wet exudative form comprinsing neovascular formations. In the non-exudative form of AMD, which account for 80%-90% of cases, there is a pattern of clinical features which include drusen, pigment clumping and/or retinal pigment epithelium (RPE) drop-out and geographic atrophy. Patients who have dry AMD typically develop gradual, insidious visual loss over

months or years with central or peri-central visual scotomas[3]. Moreover, the non-exudative form of AMD can progress into the wet form of disease. The wet form of AMD is characterized by choroidal neovascular detachment of the RPE and fibrovascular disciform scarring, and is associated with very poor visual prognosis. Therapeutic approaches have been aimed at reducing the neovascularisation: laser photocoagulation, photodynamic therapy, several medical treatments such as low dose radiation therapy and intravitreally administered drugs that specifically target the vascular endothelial growth factor; subretinal surgery to remove the offending neovascular membrane is attempted at times. All the aforementioned therapies attempt to halt the natural course of the disease, or at least to slow it down, but the lost visual activity is not recovered; in addition, these therapies may have significant side-effects[3,4]. As for the dry, pre-angiogenic form of AMD, there are no conventional therapeutic approaches and treatment remains controversial. The current recommendations for patients affected by dry AMD are oral supplements containing high doses of antioxidants and zinc, as documented by the National Eye Institute in a large, multi-center trial [5]. This antioxidant and mineral therapy was shown to slow the progression of dry AMD form from the intermediate stage to the advanced stage in a number of cases. The beneficial effect of AREDS vitamins has become clinically evident after an average treatment period of 6 years. The pathogenesis of AMD is not yet fully understood. There are environmental and genetic risk factors, such as pigmentation, dietary factors, a positive family history of AMD, high blood pressure and smoking in addition to advanced age. A growing body of evidence shows that the pathophysiological aspects of dry AMD are at least in part attributable to a retinal microcirculatory disorder, with a particular involvement of the interface of retinal pigmented epithelium and Bruch's membrane[6,7].

Oxygen - ozone therapy can yeld positive effects in the treatment of dry AMD related to antioxidant and haemoreological factors and changes in blood flow.The so-called Major Ozonated AutoHaemo Therapy (O_3-AHT) was first described in 1954 by Wehrli and Steinbart [8] and since then, after Wolff's modification [9], it has been carried out worldwide millions of times without side-effects and with therapeutic results, albeit poorly documented, in the late stage of lower limb ischemia and in AMD[10,11]. Through both *in vitro* and *in vivo*, it is able to activate erythrocyte metabolism and oxygen delivery to hypoxic tissues, partly by enhancing the formation of 2,3- diphosphoglyceric acid (2,3-DPG), and by up-regulating the expression of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) thus leading to the amelioration of an endogenous oxidative stress [12-15]. These biological effects can be elicited by a brief exposure of blood to a precisely- controlled mixture of gas composed of medical oxygen ozone (O_2-O_3), which is then re-infused into the subject.

Due to the lack of effective treatments for the dry form of AMD, we used the major ozonated autohaemotherapy in the treatment of AMD patients in order to investigate the feasibility and safety of the method and the clinical value of this approach compared to the standard therapy.

MATERIALS AND METHODS

Materials The study was a single-center, randomized, open, controlled trial. The primary efficacy end- point for the study was the mean change in log-MAR best corrected visual acuity (BCVA) in study eyes, comparing the ozonetherapy-treated group to the control group at 6 and 12 months after enrolment. Secondary efficacy outcome included proportioning of eyes with best-corrected ETDRS acuity loss or gain.

The study was approved by the Siena University Hospital Ethical Committee, and it was conducted in accordance to the ethical consideration of the World Medical Association (Helsinki Declaration).

All the patients provided written informed consent for study participation. One hundred and forty patients were randomly assigned in a 1:1 ratio to the O_3-AHT treatment or to the control group. In the control group, patients received an oral supplementation consisting of zinc and a high dose of vitamins and antioxidants, as previously reported [5].

A randomization list was computer-generated and kept by a physician who had no involvement in the study. Neither the clinical investigator nor the patient knew beforehand into which study group the patient would be randomized. Whenever a patient fulfilled the criteria for inclusion and had signed an informed consent, the principal investigator contacted the physician responsible for the randomization list, and the investigator was subsequently informed into which group the patient had been randomized. The patients and the investigator were not blinded.

Patients were recruited from the Ophthalmological Unit of the Siena University Hospital and from an ophthalmologist with private practice in Siena. Patients were enrolled in the study from January 2008 to October 2011.

The ophthalmologist was responsible for enrolling patients and determining their follow-up ophthalmologic eligibility criteria and supervising efficiency of the assessments. For inclusion in the study, patients had to be at least 59 years old and not older than 82; patients had to have diagnosis of AMD in both eyes, and have dry AMD in the study eye confirmed by fluorescein angiography and fundus photography. The study eye was supposed to have a diagnosis of non-exudative dry AMD with > 10 large, soft, semisoft and/or confluent drusen within 3mm of the foveal center and a best corrected visual acuity with the ETDRS chart between 20/32 and 20/125 inclusive. In addition, study eye was not supposed to have conditions limiting the view of

the fundus. Exclusion criteria were a study eye with concomitant retinal or choroidal disorder other than AMD, optic nerve pathology, glaucoma and bleeding.

Methods

Oxygen ozonetherapy procedure O_3-AHT was carried out as follows: 225mL of blood were drawn by vacuum from an antecubital vein into a sterile glass bottle (Ozonosan, Iffezeim, Germany) in which 25mL of 3.8% Na citrate solution (Galenica Senese Industries, Siena, Italy) as an anticoagulant, had been previously added so that the blood/citrate volume ratio was 9:1. After blood withdrawal, the bottle was momentarily disconnected leaving the venous access open by a saline infusion. The use of 225mL of blood was unrelated to the subject's body mass, rather, it was related to the dROM values in plasma and to previously-reported protocols [12]. A corresponding volume (225mL) of gas was immediately added with an O_3 concentration of 50 micrograms/mL gas. Thus the total dose of ozone was equivalent to 11.25mg. Ozone was produced by an Ozonline 80 E generator (Medica srl, Bologna, Italy), in which O_3 concentration was measured photometrically in real time and checked by iodometric titration according to the rules established by the International Ozone Association.

The gas was immediately and continuously mixed with the blood in the bottle for at least 5 minutes and with gentle rotating movement to avoid foaming. Due to the blood viscosity, the gas mixture does not instantaneously come into contact with the whole blood mass, thus this mixing time is necessary. Indeed, the pO_2 value only after this mixing period usually reaches a plateau level (of about 500mmHg). During these 5 minutes of mixing the ozone totally reacted with both the potent antioxidants of plasma and the unsaturated lipids bound to albumin, generating a small amount of hydrogen peroxide and alkenals. These two messengers were responsible for eliciting crucial biochemical reactions on both erythrocytes and within cells when the hyper-oxygenated ozonated blood was re-infused into the patient. At this point, the hyper-oxygenated ozonated blood was re-infused by promptly substituting the saline infusion with it. Reinfusion was accomplished in about 15-20 minutes and the whole procedure was carried out in approximately 40 minutes.

O_3-AHT was carried out in an out-patient setting twice weekly (on Tuesday and on Friday) for the first 7 weeks; twice monthly for a further three months and then monthly until the 12^{th} month.

Ophtalmological examination All the patients had been identified as affected by bilateral dry preangiogenic AMD as shown by fluorescein angiography before taking part in the study and, for each patient, only one eye, the functionally worse one with the lowest best corrected visual acuity, was considered. Subjects whose visual loss may have been secondary to media opacities or any other ocular disease besides AMD were excluded. The experimental group consisted of 70 dry AMD patients and the control group consisted of 70 dry AMD patients who assumed the standard multivitaminic therapy. Ophthalmologic examination of all the patients included the best corrected visual acuity (BCVA) measurement. These data were specifically recorded for each eye, at baseline and after 6 and 12 months, for both groups.

Laboratory analysis A series of hematochemical parameters (blood cells count, plasma lipids, coagulation and fibrinolysis tests) were performed in each patient of the two groups at baseline, 6 and 12 months after start of the study. Antecubital fasting whole-blood samples were drawn from a peripheral vein avoiding hemolysis into standard evacuated tubes for the blood cell count, for the hematochemical and coagulation tests and for oxidant/antioxidant plasma value determinations.

For the evaluation of the oxidative balance, samples were centrifuged at 3000xg for 10 minutes at 4℃ and plasma was collected and kept at -70℃ until the time of the analysis. Plasma was used to measure thiobarbituric acid reactive substances (TBARS), according to Yagi method [16] and to perform either the dROM test, a colorimetric determination of reactive oxygen metabolites in blood plasma, according to Vassalle method[17], or the BAP test (biological anti-oxidant potential) according to Kakita method [18].

Results for reactive oxidative metabolites were expressed as Carratelli Units (normal values for healthy subjects with a same age range of the study population : 250-300) The BAP test measures the decoloration intensity of a ferric chloride solution mixed with a thiocyanate derivative mixed by the added plasma sample photometrically at 505nm, which is proportional to the ability to reduce ferric ions by the amount of oxidants in plasma (normal values for healthy population : >2200 micromol/L).

Statistical Analysis Sample size and power calculation were based on the primary efficacy endpoint. The number of patients required for statistical significance was determined on the basis of a 1:1 randomization ratio. It was estimated that the enrolment of 70 patients would provide the study with a statistical power of 1-beta= 80% and a 2-sided alpha= 0.05 to detect a difference between the two study groups. Statistical analyses were performed using statistical package SPSS and the Student *t*-test for the difference between laboratory parameters (TBARS, dROM and BAP in treated *vs.* control group at 6 and 12 months,significativity $P<0.05$). Frequency distribution of changes in BCVA (proportion analysis) from baseline using various threshold categories (gain/loss>1 line, or more lines) were expressed in percentage. A non parametric Fisher test was used to compare the percentage between treated and control group and a $P<0.05$ was considered significant.

RESULTS

The baseline characteristics of the ozonetherapy treatment and control groups with regard to age, sex and mean baseline log-MAR acuity were not significantly different (Table 1). Mean visual acuity was 20/46 for the treatment group and 20/48 for the control group.

Table 2 shows laboratory values for the treatment and control groups at baseline, 6 and 12 months after start of the study. Treatments with ozone therapy do not cause significant modifications of critical parameters measured at baseline, 6 and 12 months.

Proportional changes in logMar acuity at 6 and 12 months are summarized in Table 3a. At 6 and 12 months, ozone treated eyes showed a average acuity change of- 0.1 and of- 0.2 logMAR respectively, while control group eyes had a change of 0.2 and of 0.3 respectively. As far as BCVA changes over time, they are summarized in Table 3b. At 6 months, none of the eyes treated with ozone had a deterioration in BCVA > 2 lines. At the same interval, 16% of the control group eyes had a > 2 lines loss, 25% had a > 3 lines loss (P<0.05). As far as line improvements, at 6 months 4% of the treated group eyes had an improvement of 1 line compared to 0% of the control group patients.

Again, at 12 months none of the treated group eyes showed a line loss > 2 or 3 lines in BCVA compared with a > 2 line loss of 40% and a >3 line loss of 38% in the control group eyes (P<0.05).

dROM and TBARS values were significantly decreased after the treatment, and BAP were increased (Table 4). These data suggest the role of oxygen ozone therapy in the oxidative balance modification.

Side Effects and Compliance We have observed only temporary face redness in a small percentage of the patients (3%) during the treatment with major ozonated autohaemo-therapy. Patients reported an improvement of their general conditions, particularly in terms of increased efficiency, mental concentration and memory. This improvement was assessed by the administration of a National Eye Visual Function Questionnaire (NEI-VFQ) before and after the end of the study in all the patients of group 1 and 2 (data not reported).

Table 1 Demographic characteristic of the patients enrolled in the study

Characteristics	O_3-aht treated	Control
Patient number	70	70
Age (Years±SD)	70.6±6.4	71.4±7
Age range (Years)	59-80	62-81
Sex (M/F)	53/17	59/11
Mean LogMAR ± SD	0.36±0.12	0.38±0.18
Mean visual acuity	20/46	20/48

SD = standard deviation; Control = control patients; O3- AHT Treated = patients treated with major ozonated autohemotherapy P> 0.05 (NS) for all comparison between group.

Table 2 Laboratory values: baseline, 6 and 12 months $\bar{x} \pm s$

Parameter	Time	Treated n=70	Control n=70
RBC ($\times 10^6/\mu L$)	Baseline	4.6±0.7	4.5±0.6
	6 months	4.5±0.6	4.5±0.8
	12 months	4.7±0.5	4.6±0.6
WBC($\times 10^3/\mu L$)	Baseline	6.1±0.8	6.2±0.5
	6 months	6.3±0.7	6.3±0.7
	12 months	6.4±0.8	6.3±0.8
PLT($\times 10^3/\mu L$)	Baseline	232.4±50	230±55.1
	6 months	237.3±52.2	232±54.3
	12 months	234.2±53	233±55
Hb (g/dL)	Baseline	14 ±1.2	14.2±1.28
	6 months	13.7±1.1	14±1
	12 months	14.1±1.2	14.1±1.2
Ht (%)	Baseline	41.7±3.6	41.5±3.1
	6 months	40.6±3.4	41±3.1
	12 months	41.3±3	41.1±3.4
Fibrinogen(mg/dL)	Baseline	293.6±80	294±88.1
	6 months	315.4±83.3	299.6± 84.4
	12 months	320.4±83	296.6± 87.7
ATIII (%)	Baseline	100.6±2.1	100.4±2.8
	6 months	100.7±2	100.5±1.4
	12 months	100.6±2.2	100.3±2.1
PT (%)	Baseline	96.2±3.3	96.1±3
	6 months	96.1±3	96.3±3.4
	12 months	96.3±3.4	96.2±3
Tot. CholesteroL(mg%)	Baseline	210±23.3	204±27
	6 months	200±31.1	205±30
	12 months	201±28	206±31.1
HDL (mg%)	Baseline	60.2±10.1	59±14
	6 months	62.2±12.4	60±13.3
	12 months	61.1±10.4	60.3±11.1

P >0.05 (Not significant) for all intergroup and intragroup comparison. RBC=red blood cells; WBC=white blood cells; PLT=platelets; Hb= Haemoglobin; Ht=hematocrit; AT III=antithrombin III; PT=protrombin time; HDL=high density lipoprotein.

DISCUSSION

At the present time, there have been considerable advances in the management of wet AMD, but there is still no established treatment for the most prevalent dry form of AMD. Left untreated, patients with dry AMD are at risk for substantial vision loss and progression to wet AMD[19].

This study suggests that the treatment with major ozonated autohaemotherapy can lead to a stabilisation or an improvement of visual acuity compared to the course of the disease under gold standard therapy (with multi-vitamins). Eyes in the control group had a mean loss of 0.2 ETDRS lines within 6 months and 0.3 EDTRS lines at 12 months, whereas treated eyes showed a mean improvement of 0.1 and 0.3 ETDRS lines at 6 and 12 months respectively None of the treated eyes deteriorated in BCVA compared with 16% of the control eyes losing 10 letters or more and 15% losing 15 letters or more at 6 months, and 40% losing 10 letters and 38% losing 15 letters or more at 12 months. The clinical relevance of a treatment effect consisting in a difference of 1 EDTRS line, in the average, may seem questionable. However, in patients with high risk dry AMD and no available therapeutic treatment, even a benefit like

Table 3a Logmar changes *vs* baseline $\bar{x} \pm s$

Patients	Treated	Control
6 months	-0.1±0.02	0.2±0.02
12 months	-0.2±0.01	0.3±0.01

$P > 0.05$ (NS) for all intergroup and intragroup comparison.

Table 3b Changes in BCVA from baseline over time

Patients	Treated	Control	Treated	Control
Loss of lines (% of patients)				
Time (*vs* Baseline)	6 months	6 months	12 months	12 months
Loss > 2 Lines	0	16%	0	40%
Loss > 3 Lines	0[a]	25%	0[a]	38%
Gain of lines (% of patients)				
Time (*vs* Baseline)	6 months	6 months	12 months	12 months
Gain > 1 Lines	4%	0	25%[a]	0

[a]$P<0.05$ *vs* control group, $P > 0.05$ (not significant) for the other intragroup and intergroup comparison.

Table 4 Time course of tbars, drom and bap tests in treated and control values $\bar{x} \pm s$

Parameter	O_3-aht treated	Control
Tbars (μmol/L)		
Baseline	36.6±8.4	37±7.4
6 months	26.3±7.1[c]	36.4±8.3[a]
12 months	27.1±8.1[c]	37±8.2[a]
dROM (U CARR)		
Baseline	380±10.4	378±11.1
6 months	326±8.7	385±10.3
12 months	300±10.1[c]	384±10.1[a]
BAP (μmol/vit C)		
Baseline	1610±36.2	1620±30.1
6 months	1840±40.1	1635±27.8
12 months	2100±34.8[c]	1631±28.9[a]

[a]$P<0.05$ control *vs* treated; [c]$P<0.05$ intragroup comparison 6 or 12 months *vs* baseline; $P>0.05$ (NS) for the other intragroup and intergroup comparison.

that could be very important. Avoiding the development of advanced AMD can have a major effect on life quality for an individual [20].

A probable mechanism of action for the oxygen ozone therapy could be an improvement in the choroidal-retinal circulatory network and an increased oxygen concentration in ischemic tissues. In patients with dry AMD and large, soft drusen the choroidal blood flow and volume were one-third lower than in age matched control subjects [6]. A systematic decrease in choroidal circulatory parameters was observed in combination with an increase in the severity of AMD features associated with risk for the development of choroidal neovascularisation, supporting the role of ischemia in the development of choroidal neovascularisation. Oxygen ozone therapy brings about several effects such as: Improvement of blood rheology [21]; Improvement of the glycolytic pathway on erythrocytes [22]; Activation of the hexose-mpnophosphate shunt on erythrocytes with an increased levels of ATP and 2,3-DPG levels [23]; Enhanced oxygen availability and delivery to hypoxic tissues due to a shift to the right of the HbO_2 dissociation curve [24]; Vasodilation by increased release of NO, CO and prostacyclin [25]; Upregulation of the enzymatic antioxidant system, phase II enzymes and Heme-oxygenase-1 due to the generalized reaction of alkenals with Nrf2-Keap1 protein. This transcriptional factor, enters into the nucleus and bind to the Antioxidant Response Element (ARE), is able to enhance the synthesis of very protective enzymes[26,27].

The feeling of wellness reported by the majority of patients could be explained with a possible activation of the neuroendocrine system [28]. Most of the biochemical and molecular steps of ozone therapy are now well within orthodox medicine [29]. There is consequently a possible link between the current evidence in AMD pathogenesis and therapeutic effects of the major ozonated autohaemotherapy at molecular and cellular levels. In the AMD the specific insult that triggers and perpetuates the disease is unknown; however it may be the result of repetitive oxidative injuries [30]. The Macula Lutea, a prime environment for ROS generation, is very prone to lipid peroxidation if the antioxidants activity is decreased. Moreover the foveola, which has the highest concentrations of cones and is responsible for the visual acuity, depends entirely on the choriocapillaris circulation because there are no retinal vessels and, among the various tissues of the body, has the highest consumption of oxygen.

One of the most important weak points of this study was the absence of the data concerning the improvement of the fundus or FFA or autofluorescein before and after treatment. Nevertheless, the functional improvement of the vision was important at least for the quality of life of the patients.

In conclusion, AMD remains a major public health issue in the elderly population. The results of this study provided further evidence that major ozonated autohemotherapy can have a positive influence on visual acuity of patients with high risk dry AMD . The treatment was safe and well tolerated. Although this study demonstrates the efficacy of O_3 AHT in a population of patients, a large and controlled clinical trial confirming the efficacy of O_3 AHT for AMD is urgently warranted. However, based on the available data, it is reasonable to consider the major ozonated autohaemo-therapy as a therapeutic option for the AMD in clinical practice.

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