

Induction of oxidative stress in human aqueous and vitreous humors by Nd:YAG laser posterior capsulotomy

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Received: 2018-01-06 Accepted: 2018-04-25

Abstract

• **AIM:** To evaluate whether the Q-switched Nd:YAG laser treatment applied in routine capsulotomy elicits oxidative stress in aqueous and vitreous humors.

• **METHODS:** Thirty-six patients who had to undergo a 25 gauge pars plana vitrectomy due to vitreoretinal disorders were enrolled, 15 of them underwent a Q-switched Nd:YAG laser capsulotomy 7d before vitrectomy due to posterior capsule opacification (PCO) (Nd:YAG laser group) while the remaining 21 patients were not laser treated before vitrectomy (no Nd:YAG laser group). Samples of the aqueous and vitreous humors were collected during vitrectomy from all patients for the assessment of oxidative parameters which were compared between the Nd:YAG laser group and no Nd:YAG laser group. Thiobarbituric acid reactive substances (TBARS), a product of membrane lipid peroxidation, nitrite levels, the antioxidative activities of SOD and catalase, the 4-HNE-protein conjugate formation, indicating structural modifications in proteins due to lipoperoxidation, were assessed in aqueous and vitreous samples.

• **RESULTS:** In the human vitreous humor TBARS levels are significantly higher in the Nd:YAG laser group compared to the no Nd:YAG laser group and importantly, there is a significant correlation between the TBARS levels and the total energy of Nd:YAG laser used during capsulotomy.

Moreover the anti-oxidative activities of SOD and catalase were significantly decreased by Nd:YAG laser treatment, both in aqueous and vitreous humors. In accordance with the TBARS data and anti-oxidative enzyme activities, significantly higher levels of proteins were conjugated with the lipoperoxidation product 4-HNE in the aqueous and vitreous humors in the Nd:YAG laser-treated group in comparison to no Nd:YAG laser group.

• **CONCLUSION:** These data, clearly suggest that any change that Q-switched Nd:YAG photo disruption may cause in the aqueous and vitreous compartments, resulting in a higher level of oxidative damage might be of considerable clinical significance particularly by accelerating the aging of the anterior and posterior segments of the eye and by worsening the intraocular pressure, the uveal, the retinal (especially macular) pathologies.

• **KEYWORDS:** posterior capsule opacification; Q-switched Nd:YAG laser; oxidative stress; vitrectomy

DOI:10.18240/ijo.2018.07.12

Citation: Bergandi L, Skorokhod OA, Franzone F, La Grotta R, Schwarzer E, Nuzzi R. Induction of oxidative stress in human aqueous and vitreous humors by Nd:YAG laser posterior capsulotomy. *Int J Ophthalmol* 2018;11(7):1145-1151

INTRODUCTION

Age-related cataract is the principal cause of treatable blindness and visual impairment in the world^[1]. Phacoemulsification is the most frequent technique applied in cataract surgery in economically and socially advanced countries. Despite substantial improvements in cataract surgery techniques and intraocular lenses (IOLs), months or years after surgery, lens epithelial cells spared by surgical abrasion can colonise the previously cell-free posterior capsule causing the development of posterior capsule opacification (PCO) with glare or vision deterioration^[2]. For this reason PCO continues to be the most frequent delayed complication of cataract surgery^[3].

The first-line treatment option is the posterior capsulotomy performed by Nd:YAG laser application presented by Aron-Rosa and Fankhauser in 1980, that is an ablation of the central posterior capsule to restore transparency^[4-6]. Although

Nd:YAG laser capsulotomy is accepted as standard treatment for PCO and it has been found to improve visual acuity and to be safe and effective, it is not without complications, which can be detected by routine examination such as IOL injury, iritis, vitritis, cystoid macular edema (CME), macular hole, macular hemorrhage, vitreous hemorrhage, changes in refraction and intraocular pressure (IOP), and endophthalmitis^[7]. Moreover, some changes can not be detected by routine biomicroscopy examination, such as a decrease of retinal thickness and a change in anterior chamber depth and angle, whose measurements and numerical monitoring require the optic coherence tomography (OCT), or a changed capillary endothelial cell count monitored by using specular microscopy^[8]. Many of the complications are caused by an acute and/or persisting local inflammation, the plausible response to local oxidative stress (OS), which might be provoked by the high energy transfer into the eye by laser treatment.

An imbalance between free radical production and antioxidant defense produces OS. The most important oxidants are reactive oxygen species (ROS) and reactive nitrogen species (RNS) that have been implicated as causative factors in many diseases because of their potential to cause oxidative deterioration of DNA, proteins, and lipids, resulting in functional impairment, damage and inflammation^[9].

Although literature is rich in clinical case reports concerning the therapeutic ophthalmic potential offered by Nd:YAG laser and its possible macroscopic complications^[7-8], there are no *ex-vivo* studies about the impact of Q-switched Nd:YAG laser capsulotomy in terms of OS in human aqueous and vitreous humors. For this reason, the present study was aimed to assess the effect of Nd:YAG laser on OS status in aqueous and vitreous humors in patients undergoing the 25 gauge pars plana vitrectomy for vitreoretinal diseases 7d after capsulotomy.

SUBJECTS AND METHODS

Patients The study was carried out in accordance with the Declaration of Helsinki for medical research involving human subjects in the period from February 2016 to June 2017 and was authorized by the local Ethical Committee (ref. number: 0021632). Signed, written consent was obtained from all patients accepting to be included in this study. All patients went through a baseline ophthalmological examination before laser capsulotomy including measurements of best corrected visual acuity (BCVA), Goldmann applanation tonometry and the fundus examination.

Thirty-six otherwise healthy patients with a history of phacoemulsification and IOL implantation at least 12mo ago, were randomly chosen among patients that had to undergo a 25 gauge pars plana vitrectomy from the central vitreous cavity, for vitreoretinal disorders. The absence or presence of PCO was verified in all patients by the same examiner using

the slit-lamp exam. The mean age of the patients with and without PCO was 71.8 ± 1.4 and 69.6 ± 1.7 y, respectively. The indications for vitrectomy were epimacular membrane in 5 PCO and 9 non-PCO cases, macular hole in 6 PCO and 9 non-PCO cases and retinal detachment in 4 PCO and 3 non-PCO cases.

The patients with PCO ($n=15$) underwent Nd:YAG laser (OptoYag, OPTOTEK medical, Ljubljana, Slovenia) capsulotomy by the same surgeon, 7d before vitrectomy. Light exposure after capsulotomy was limited by dark glasses that patients wore after the intervention. The patients without PCO ($n=21$), obviously didn't receive laser therapy and were enrolled as a control group. Patients did not receive pre- or postoperative medications.

The following clinical characteristics were recorded, and patients presenting with one or more of the criteria were excluded from the study: presence of vitreous hemorrhages or hemovitreous, proliferative diabetic retinopathy, assumption of antioxidant integrators, impossibility of classifying vitreous degeneration or any of the other relevant parameters, infections, malignant neoplasias, or renal or hepatic failure, having media opacities other than PCO or were not suitable for capsulotomy treatment. The levels of glicemia and fibrinogen were normal in both groups and patients presenting non physiological levels of glicemia and fibrinogen were excluded from the study.

Nd:YAG Laser Treatment Capsulotomy was performed employing a Q-switched Nd:YAG laser (Opto YAG, Optotek Medical, Ljubljana, Slovenia) emitting pulses from 0.5 to 10 mJ. A capsulotomy contact lens, which was a 12 mm Ocular Abraham Capsulotomy lens (Ocular Instruments, WA, USA), was used in all the cases after instilling 2-3 drops of 0.4% benoxinate. Single pulse mode Nd:YAG laser was used at the minimal amount of energy necessary to rupture and breakdown the capsule and/or vitreous bridges. A typical capsule can be opened by using a 1 to 2 mJ/pulse to create a cruciate opening. The initial energy of a 1.0 mJ/pulse was constant and independent on PCO types, then maintained or increased to obtain an adequate central capsular opening, directly proportional to thickness and intensity of the opacification of the posterior lens capsule. Mean total energy levels were 215 ± 16 mJ. In case of occurrence of vitreous bridges which provoke stretching on the retina, these were cut with the Nd:YAG laser. A large capsulotomy wasn't made because there was no advantage: every capsulotomy was only slightly larger than the size of the pupil in a darkened room and was sufficient, without damage to the flexible IOL.

Vitrectomy and Preparation of Aqueous and Vitreous Samples From each patient, an aqueous (100-150 μ L) and a vitreous (500-800 μ L) sample were collected at the beginning of the 25 gauge pars plana mini invasival vitrectomy with non

contact technique (BIOM) from the central vitreous cavity^[10-11]. Undiluted samples were immediately placed in cryotubes, and stored at -80°C until assayed 2wk after sampling at the latest. After collecting the samples, any type of contamination or exposure to light sources can be excluded. The aqueous and vitreous samples from each patient were thawed and centrifuged at 12 000 rpm for 10min. Cellular component and debris were discarded, whereas the supernatant was immediately used for biochemical measurements. The protein content of the supernatant was assessed using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA).

Measurement of Thiobarbituric Acid Reactive Substances

Production Following the indication by Yano, the thiobarbituric acid reactive substances (TBARS) assay was used to quantify the presence of lipid peroxidation products only in vitreous samples due to material restrictions of the aqueous sample^[12]. About 600 µL of the thawed clear vitreous supernatant was sonicated with a 10s burst using a Bandelin Sonoplus HD 2070 sonicator with a titanium micro-tip MS73 at 30% of maximal potency (corresponding to 60 W), centrifuged at 18 928 g for 5min and 500 µL was added to the TBA solution. TBARS generation by sonication was excluded. Samples were boiled for 20min, then rapidly cooled in ice, and centrifuged for 5min at 16 099 g. Absorbance on 300 µL of the sample was measured at 532 nm with a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT, USA). Results are expressed as nmol TBARS/mL of supernatant. For TBARS measurements, each test was performed once per patient due to sample quantity.

Measurement of Nitric Oxide Production The concentration of nitrite, a stable product of nitric oxide (NO) synthesis, was measured with the Griess method, as previously described, only in vitreous humor. The resulting concentration was expressed as nmol nitrite/mL of supernatant^[13].

Superoxide Dismutase and Catalase Activity As the levels of ROS in vitreous and aqueous samples resulted to be poorly detectable in preliminary experiments, we measured the activities of SOD and catalase as representative enzymes for local antioxidative defense, as previously described, in 50 µL aqueous and vitreous supernatants. Measurements of SOD and catalase activity for each samples were performed in duplicate. Cu, Zn-SOD and catalase activities were expressed as units per mL of supernatant^[14].

The 4-HNE-protein Conjugate Assay The accumulation of 4-HNE-protein conjugates represents the local oxidative status and lipoperoxidation process. The 4-HNE is bioactive and a high concentration toxic end-product of lipoperoxides reported as important pathomechanistic factor in many diseases^[15-16]. As to its exceptional reactivity with proteins, its conjugates with proteins were assessed by specific antibodies in Western blot, as previously described^[14,17-18].

Proteins were extracted from 30 µL of vitreous and 30 µL aqueous humor supernatant with SDS-containing, modified Laemmli buffer (final concentrations of TRIS-HCl 60 mmol/L, EDTA 1 mmol/L, 5% glycerol, SDS 2%), pH 6.8, thawed in presence of a protease inhibitor cocktail (Complete[®], Roche Diagnostics S.p.A., Milano, Italy) in order to cover a broad spectrum of serine, cysteine-, and metallo-protease-, and calpain-activities) and boiled at 95°C for 5min. Aliquots were kept at -20°C prior to use and β-mercaptoethanol (5% v/v, final concentration) was added to protein samples before loading to the SDS-PAGE. The extracted proteins were quantified, loaded at 20 µg/lane and separated in a 10% polyacrylamide/acrylamide (w/v) SDS-PAGE. Separated proteins were transferred onto a nitrocellulose membrane (Amersham Biosciences, Fairfield, Connecticut) and equality of loaded and transferred protein amounts and of protein pattern of all sample lanes was verified by Ponceau S staining. Membranes were blocked with bovine serum albumin [BSA; dissolved at 5% (w/v) in phosphate-buffered saline containing 0.1% (v/v) Tween] for 1h and subjected to the mouse monoclonal anti-4-HNE-conjugate antibody at 1:2000 dilution (clone HNEJ-2, Abcam, Cambridge, UK) overnight at 4°C. Then the membranes were washed with PBS-Tween 0.1% and incubated with the secondary anti-mouse horseradish peroxidase-conjugated antibody (Amersham, Bucks, UK) at 1:20 000 dilution for 1h at room temperature. The PBS-Tween 0.1%-washed membranes were analysed for antibody-positive bands which were visualized by enhanced chemiluminescence (ECL), acquired and quantified with Chemidoc MP equipment (Bio-Rad Laboratories, Hercules, CA, USA), using the PDQuest software (Bio-Rad, version 7.2) according to the manufacturer's instructions. The 4-HNE-modified human serum albumin (4-HNE-HSA) was prepared as reference sample for specificity control and normalization of sample conjugate values. For this, 0.1 µg 4-HNE-HSA was run in parallel with samples in each gel. For densitometry analysis protein band intensity quantification and comparison of lanes were performed using ImageJ software (version 1.46, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA); the values obtained for all 4-HNE-positive bands of a sample were referred to the reference 4-HNE-HSA band, summarized and expressed as 4-HNE arbitrary units.

Statistical Analysis Data were presented as mean±standard error (SEM), and the results were checked for normal distribution and analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. Correlations between these variables were assessed by Spearman rank correlation coefficient. Statistical significance level was set at $P=0.05$.

RESULTS

In the human vitreous humor TBARS levels are significantly higher when PCO treatment by Nd:YAG laser had been

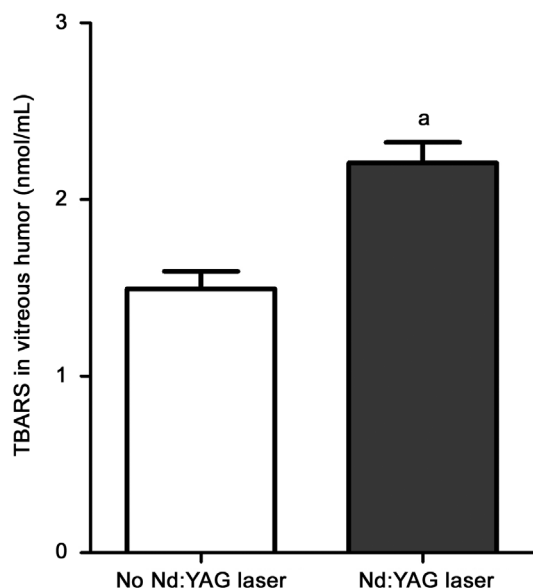


Figure 1 TBARS levels in vitreous humor after Nd:YAG laser therapy for PCO treatment. Each test was performed once per patient. ^a $P < 0.001$ Nd:YAG laser treatment, 7d before vitrectomy ($n=15$) vs no Nd:YAG laser treatment ($n=15$).

performed 7d before vitrectomy (Nd:YAG laser group), compared to TBARS levels in patients who underwent pars plana vitrectomy for a variety of vitreoretinal disorders without any precedent laser treatment (no Nd:YAG laser group) (Figure 1). As shown in Figure 2, we observed a significant correlation ($P < 0.001$) between the TBARS levels and the total energy of Nd:YAG laser used during capsulotomy. Moreover we observed that the anti-oxidative activities of SOD (Figure 3), that degrades superoxide anions, and catalase (Figure 4), that catalyzes the decomposition of hydrogen peroxide, were significantly decreased by Nd:YAG laser treatment. No correlation between the decrease of SOD and catalase activities and the total energy was detectable. The enzyme activities differed between aqueous and vitreous humors. Furthermore, in both groups, SOD and catalase activities were significantly lower in aqueous versus vitreous humors.

In accordance with the TBARS data and antioxidative enzyme activities, significantly higher levels of proteins that were conjugated with the lipoperoxidation product 4-HNE were detectable in supernatants in the Nd:YAG laser-treated group and also the level of 4-HNE-protein conjugates was significantly higher in aqueous versus vitreous humors in both groups (Figure 5). However, we did not observe any change in the nitrite levels in the vitreous humor in PCO patients by laser treatment (3.86 ± 2.08 nmol/mL vs 3.03 ± 1.74 nmol/mL with treatment) and in patients not affected by PCO (data not shown).

DISCUSSION

YAG laser capsulotomy is the treatment of choice for PCO and has been accepted as standard treatment as it rapidly improves the visual acuity^[19]. Though a routine procedure, it may sometimes cause macroscopic complications, such as IOL

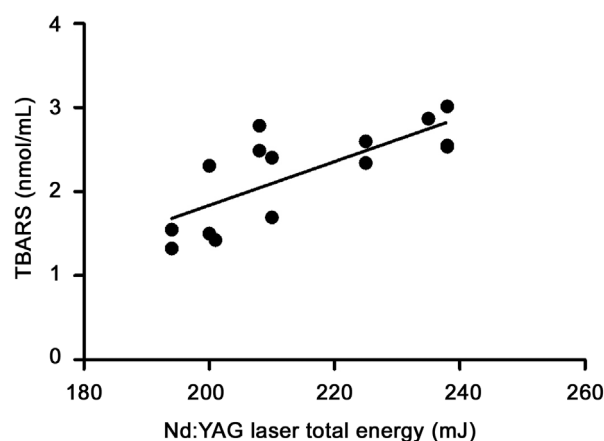


Figure 2 Relationship between Nd:YAG laser total energy and TBARS levels in vitreous humor. The figure shows a strong positive correlation ($r^2=0.7705$, $P < 0.001$) between the total energy and thiobarbituric acid-reactive substances levels in the vitreous of the 15 patients received Nd:YAG laser treatment.

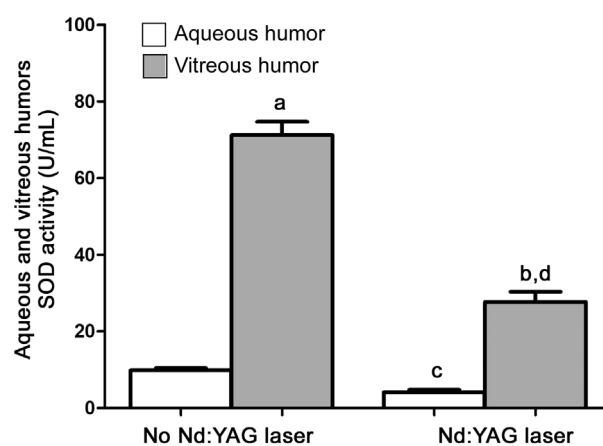


Figure 3 SOD activity in aqueous and vitreous humors after Nd:YAG laser treatment for POC treatment. ^a $P < 0.001$ vitreous humor vs aqueous humor in no Nd:YAG laser patient group; ^b $P < 0.001$ vitreous humor vs aqueous humor in Nd:YAG laser patient group; ^c $P < 0.01$ aqueous humor Nd:YAG laser patient group vs aqueous humor in no Nd:YAG laser patient group; ^d $P < 0.01$ vitreous humor in Nd:YAG laser patient group vs vitreous humor in no Nd:YAG laser patient group.

pitting, transient IOP elevation, CME and secondary retinal detachment^[20-21]. Although numerous clinical cases are reported regarding Nd:YAG application in capsulotomy there are no *ex vivo* studies yet on the impact of Q-switched Nd:YAG laser capsulotomy on human aqueous and vitreous humors in terms of OS^[7].

Our study has demonstrated that in patients undergoing the 25 gauge pars plana vitrectomy for vitreoretinal diseases 7d after capsulotomy, the OS status both in aqueous and vitreous humors was increased by the Q-switched Nd:YAG laser treatment. Indeed, Nd:YAG capsulotomy, in addition to causing photodisruption of the posterior capsule, has caused a decrease of SOD and catalase activities, the local antioxidant defense that naturally occurs within mammalian tissues to

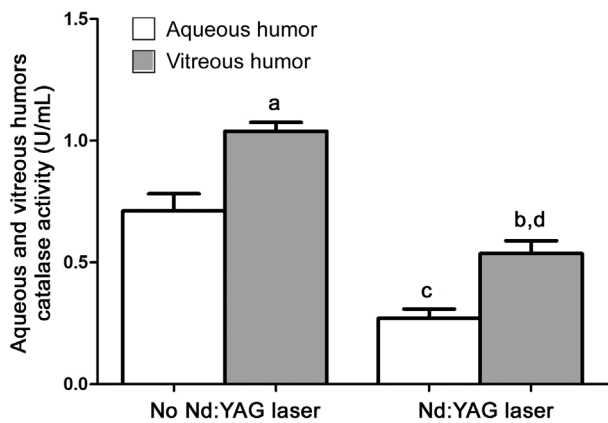


Figure 4 Catalase activity in vitreous and aqueous humors after of Nd:YAG laser treatment for PCO treatment ^a $P < 0.01$ vitreous humor vs aqueous humor in no Nd:YAG laser patient group; ^b $P < 0.001$ vitreous humor vs aqueous humor in Nd:YAG laser patient group; ^c $P < 0.01$ aqueous humor Nd:YAG laser patient group vs aqueous humor in no Nd:YAG laser patient group; ^d $P < 0.01$ vitreous humor in Nd:YAG laser patient group vs vitreous humor in no Nd:YAG laser patient group.

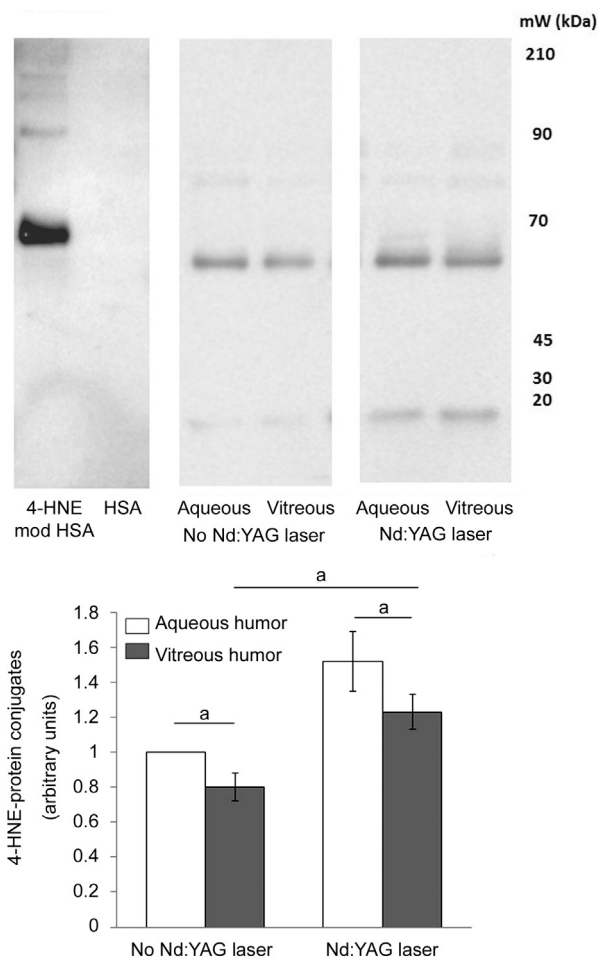


Figure 5 Western blotting analysis of 4-HNE-proteins conjugates in vitreous and aqueous humor in patients received or not the laser treatment for PCO treatment Significance of difference is signed if ^a $P < 0.01$.

protect against the harmful side effects of ROS, an increase of the concentration of TBARS, a product of membrane lipid

peroxidation, and an increase of 4-HNE-protein conjugates formation, indicating structural modifications in proteins due to lipoperoxidation, all reliable indicators for a redox imbalance. The method used in the Nd:YAG laser beam production is the “Q-switched” one that compresses energy in a single high energy nanosecond pulse instead of repeated low energy pulsations typical of “mode-locked”. The “Q-switched” technology produces the photodisruption due to chiefly pulses whose main effect is mechanical and buckling. The effect of this method is the creation of energy in the pipe let through in short impulses, and during the still interval energy is kept, accumulated and enhanced, so that each impulse has a very high performing power^[22]. Unfortunately, the excellent results in PCO treatment, which led to the immediate restoration of visual acuity in all of our treated patients, were accompanied by OS in the aqueous and vitreous compartments, due to the high and cumulative energy delivered by Q-switched Nd:YAG. Lipid containing biological structures underwent lipoperoxidation when exposed to high energy levels, a process that we observed after PCO treatment by Q-switched Nd:YAG, too. Both, the direct lipid radical production by the high energy pulses or the indirect one by oxygen radicals generated by inflammatory cells which plausibly are activated by laser-elicited tissue debris could trigger a progressive lipoperoxidation. The shown increase of proteins conjugated with the final lipoperoxidation product 4-HNE after laser treatment should not be considered just another parameter of OS, but might be of pathomechanistic importance for structural changes in the vitreous. The origin of 4-HNE are cellular membranes and lipoproteins as vulnerable targets for the peroxidation due to their high yield of polyunsaturated fatty acids which are transformed into unstable peroxides that give rise to spontaneous breakdown products such as 4-HNE. The last has been proven in different tissues to be an excellent, very sensible parameter for lipoperoxidation^[16]; 4-HNE is a stable and biologically very active molecule whose activity is not restrained to the site of its origin, as it is able to diffuse across the membrane barrier. It exerts its biologic activity firstly, by interfering with cellular signalling and gene expression profile secondly, by promoting local inflammation by chemotaxis and thirdly, by modifying posttranslationally in order to modify specific proteins of functional importance^[23-24,16]. We show here that the cell-free vitreo is targeted by this molecule, although aging was reported to increase specific protein-4-HNE conjugates in retinal proteins^[25]. Indeed 4-HNE accumulates in this relatively separated compartment giving rise to the conjugated humor proteins after laser treatment. Binding of 4-HNE to functionally relevant amino acid residues of antioxidative enzymes could explain the low activity of SOD and catalase. Indeed, catalase had been described to lose activity after HNE binding^[26]. The impairment of the antioxidative activity by

the lipoperoxidation product will make the vitreous more vulnerable to the pro-oxidative effect of the laser. Further, the lysin-rich collagen is an easy target for 4-HNE^[27] and we suggest a critical role of 4-HNE-conjugation as the 9 carbon atom long molecule might disturb the regular structure of the molecule and even introduce crosslinks between collagen fibres which may contribute to the liquidification process or similar functional changes in the vitreous^[19]. Further analysis is needed to identify the specific target proteins for 4-HNE in the vitreous to confirm their potential role in the vitreo pathologies. Finally, HNE could interfere with the expression of distinct collagen subtypes changing the expression profile of the collagen subclasses which might have consequences for the functional intactness of the vitreous as seen in the aging eye^[28]. The herein shown strong correlation of the amount of total energy delivered *via* Nd:YAG laser treatment with the level of thiobarbituric acid-reactive substance in vitreous humor in PCO patients clearly suggests a laser-dependent oxidative challenge and the laser as source for the pro-oxidant modifications. Although, the increase of the individual baseline oxidative values by laser treatment was not assessable as to ethical limitations that prohibited vitreous sample collection from PCO patients before laser treatment, the comparison of the post-treatment samples with samples from randomly enrolled control patients without any laser-treatment before vitrectomy seemed an appropriate alternative. The modest inter-individual variability of oxidative values in the non-laser treated control group may allow to consider them representative for the pre-treatment oxidative state.

Taken together these data, suggest that any change that Q-switched Nd:YAG photo disruption may cause in aqueous and vitreous compartments, resulting in a higher level of oxidative damage, might be of considerable clinical significance, particularly by accelerating the aging of the anterior and posterior segments of the eye and by worsening the intraocular pressure, the uveal, the retinal and especially macular pathologies.

ACKNOWLEDGEMENTS

Thanks are to the patients who agreed to be enrolled in the study and Heyman Belfort from Belfort English Services for editing of the paper.

Authors' contributions: Bergandi L performed samples preparation, measurements of TBARS, NO, ROS SOD and catalase. She contributed to the statistical analysis, the interpretation of the results and to write the manuscript. Skorokhod OA and La Grotta R performed Western blotting experiments. Skorokhod OA discussed the results and contributed to draft the manuscript. Franzone F was involved in patients enrolment and acquisition of clinical data. Schwarzer E supervised the experiments, participated in result discussion and interpretation and contributed to draft the manuscript.

Nuzzi R conceived the study, performed laser therapy, surgery and collected samples, contributed to the final interpretation of the data and to write the manuscript.

Foundations: Supported by Public University Funds (NUZR_autof_17_01) of University of Torino; the Italian Ministry for Research MIUR (No.2010C2LKKJ-007; No.20154JRJPP-005); the PhD and Post-doc Program of the University of Torino.

Conflicts of Interest: Bergandi L, None; Skorokhod OA, None; Franzone F, None; La Grotta R, None; Schwarzer E, None; Nuzzi R, None.

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