

RESEARCH ARTICLE

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Oxidative Stress in Cerebral Small Vessel Disease Dizziness Patients, Basally and After Polyphenol Compound Supplementation



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Abstract: Background: Leukoaraiosis (LA) is a common radiological finding in elderly, frequently associated with several clinical disorders, including unexplained dizziness. The pathogenesis of LA is multifactorial, with a dysfunction of cerebral microcirculation resulting in chronic hypoperfusion and tissue loss, with oxidative stress involved in this cascade.

Objective: The aim of this study was to analyse some oxidative stress biomarkers in a cohort of LA patients.

Method: Fifty-five consecutive patients (33 males, median age 75 years) with LA were recruited. In a subgroup of 33 patients with LA and unexplained dizziness, we have then performed an open study to evaluate if 60-day supplementation with a polyphenol compound may modify these biomarkers and influence quality of life, analysed with the Dizziness Handicap Inventory (DHI) scale.

Results: At baseline, blood oxidative stress parameters values were outside normal ranges and compared to matched healthy controls. After the two months supplementation, we observed a significant decrement of advanced oxidation protein products values and a significant improvement of DHI.

Conclusion: Oxidative stress biomarkers may be useful to detect redox imbalance in LA and to provide non-invasive tools to monitor disease status and response to therapy.

ARTICLE HISTORY

Received: March 23, 2018
Revised: May 2, 2018
Accepted: June 15, 2018

DOI:
10.2174/1566524018666180720165055

Keywords: Leukoaraiosis, cerebral small vessel disease, dizziness, oxidative stress, polyphenols, DHI.

1. INTRODUCTION

Leukoaraiosis (LA) is a common radiological finding in elderly, commonly associated with several clinical disorders such as cognitive impairment, gait disturbances, falls, and dizziness [1, 2]. LA is a purely descriptive radiological term that is related to cerebral small vessels disease (cSVD). According to STRIVE consensus [3], cSVD includes several specific radiological abnormalities: lacunes, small subcortical infarcts, white matter hyperintensities (WMH), perivascular spaces (PVS), microbleeds (MB) and brain atrophy. Typical multiple LA deep white matter abnormality (also known as WMA) appears hypodense on CT scans and hyperintense on T2-weighted MRI. The CNS load is commonly evaluated by visual rating scales, such as the Fazekas scale [4] or the Scheltens scale [5].

Beyond rare genetic forms (such as CADASIL, CARASIL, or Fabry disease), LA is usually associated

with the common vascular risk factor, especially hypertension [6-8]. The pathogenesis of LA is likely a dysfunction of cerebral microcirculation that results in chronic hypoperfusion and tissue remodelling, damage and neural loss [9]. Vessel wall change is probably the first step of chronic hypoperfusion, functional change and ischemic injury of the brain parenchyma [6, 9]. A central role of endothelial dysfunction and of oxidative stress in LA is also hypothesized and, notably, a much more wide-spread endothelial dysfunction than thought according to the neuroradiological evidences is demonstrated by pathological finding [1, 10].

Gait disturbances, dizziness, and falls are common manifestations of cSVD, especially in patients with evidence of deep white matter lesions [11]. Moreover, vertigo and dizziness are common neurological signs (about 30% in elderly) that frequently remain "unexplained" [11]. In a recent study, we have demonstrated that more than 80% of unexplained dizziness was associated with a higher cSVD burden [12].

To contrast oxidative stress, beyond endogenous antioxidants, there is also a large number of

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pharmaceutical antioxidant agents used in clinical practice [13], even though a clear correlation between antioxidant compounds consumption and reduced cardiovascular disease has not been demonstrated [14]. In this scenario, bioactive compounds such as polyphenols might mediate the benefits independently of the abundant antioxidants [15, 16]. These substances can improve endothelial function, modulate platelet aggregation [17] and inflammation [18], and improve plasma lipid profile [19]. Moreover, polyphenols can improve ROS balance through lowering ROS and reactive nitrogen species [20, 21], and stimulating the transcription of essential detoxifying enzymes (such as glutathione-S-transferase, NADPH: quinone oxidoreductase 1, haem oxygenase 1 and glutamyl-cysteine ligase [16, 22-24]. Epidemiological studies demonstrate that polyphenols might play a multifaceted protective action into the endothelial function and, therefore, reduction of sCVD progression [25-28].

Given those evidences, the aim of the present study was to monitor three markers of oxidative stress [advanced oxidation protein products (AOPP), ferric reducing antioxidant power (FRAP), and Thiols] in a cohort of patients with LA and unexplained dizziness.

We have subsequently performed an open study to evaluate if 60-day supplementation with a polyphenol compound (Vertigoval[®], Valeas, Milan, Italy) may modify these biomarkers and influence/improve quality of life in these patients.

2. PATIENTS AND METHODS

2.1. Human Subjects and Study Design

Fifty-five consecutive patients (33 males, 22 females, median age 75 years; IQR 13 yrs) were recruited at both Neurological and ENT institutes of Pisa University (demographic features presented in Table 1). The inclusion criteria were: age between 18 and 85 years; diagnosis of sCVD supported by TC scan or MRI; dizziness sensation, with no ENT or medical explanation. The exclusion criteria were: diagnosis of other otoneural, neurological or vestibular diseases accountable for dizziness; diagnosis of serious psychiatric disorders, patients taking pharmacologic supplements with antioxidant activity (e.g., vitamin E, α -lipoic acid, coenzyme Q10).

During the first visit (T0), inclusion and exclusion criteria were verified; a clinical scale for evaluating and assessing the impact of dizziness on quality of life (Dizziness handicap Inventory, DHI) was performed, and a blood sample for oxidative stress biomarker evaluation was taken. Compliance with supplementation was examined. Every patient was given a polyphenolic preparation (Vertigoval[®]) containing the recommended daily intake (RDI, 150 mg) for 60 days. Vertigoval[®] is a nutraceutical compound containing ViNitrox, Vit. B6, Citicoline, Melissa officinalis, Ginger.

Table 1. Demographic features.

Features	Number (%)
Sex (M)	33 (36%), median age 71 (11)
Sex (F)	22 (64%) median age 75 (13)
Diabetes	7 (12%)
Dyslipidemia	21 (38%)
Smoking	11 (20%)
Hypertension	30 (54%)

M = males. F = females.

After 60 days, patients were again evaluated (T1); neurological examination, DHI and blood samples for oxidative stress evaluation were repeated.

2.2. Standard Protocol Approvals, Registrations, and Patient Consents

The study was reviewed by the local Ethical Committee and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. All persons gave their informed consent prior to their inclusion in the study.

2.3. Blood Assays

For each plasma assay, blood samples, obtained in the morning at fasting, were centrifuged at 3000 rpm (1300 g), within 30 min from the drawing in order to obtain plasma which was stored at -80°C until analysis.

Oxidative stress parameters investigated in this study are advanced oxidation protein products (AOPP), ferric reducing ability of plasma (FRAP) and Plasmatic Total Thiols (-SH). Those biomarkers have been already investigated in several neurological diseases, such as mitochondrial, Parkinson and Alzheimer diseases, ALS, Huntington's disease and others [29-32].

AOPP were determined according to Witko-Sarsat *et al.* [33]. On a 96-well plate 30 μl of plasma were mixed with 170 μl of PBS, 20 μl of acetic acid, and 10 μl of potassium iodide [1,16 M]. Each evaluation was carried out in duplicate and the absorbance was read spectrophotometrically at 340 nm, after 1 minute of incubation at room temperature.

The AOPP concentration of each sample was calculated using the equation obtained from the linear regression of the standard curve acquired from a stock solution of chloramine T (10 mM) dissolved in the same buffer. The data were expressed as mmol/L of chloramine equivalents and related to plasma total protein, albumin, and immunoglobulin concentration.

Evaluation of FRAP was assessed according to Benzie *et al.* [34]. The FRAP reagent consisting of sodium-acetate buffer (0.3 M, pH 3.6), 2,4,6-tripyridyl-s-triazine (10 mM) in hydrochloric acid (40 mM), and ferric chloride (20 mM) in H₂O, in ratio 10:1:1, and pre-warmed at 37°C for 10 minutes, was mixed with 8 µl of plasma. Each evaluation was carried out in duplicate and the absorbance was read after 4 min. at 620nm. The FRAP concentration of each sample was calculated using the equation obtained from the linear regression of the standard curve acquired from a stock solution of FeSO₄*7H₂O (4mM) in HCl (0.01M). The content of plasmatic total thiols was estimated by evaluation of the sulphhydryl groups (-SH) present in the molecules, following the protocol described by Hu [35]. At the time of determination, 200 µl of plasma was added to 600 µl of a buffer consisting of TrisBase (250 mM-EDTA (20mM), pH 8.2, 40 µl of 2,2-dithiobisnitrobenzoic acid (DTNB, 10 mM in methanol), and 3.6 ml of absolute methanol. After an incubation of 15 minutes at room temperature, the samples were centrifuged at room temperature, at 3000 g for 10 minutes. The absorbance of the supernatant was assessed at 405 nm (A) and subtracted from a DTNB blank (B) and a blank consisting of the sample without DTNB (C). Total -SH groups were calculated using the following conventional formula:

$$(A-B-C) \times (4.0/0.2)/13.6 = (A-B-C) \times 1.47 \text{ mM}$$

Data obtained were compared to 49 age and sex-matched healthy controls.

2.4. Statistical Analysis

First, we performed a Shapiro-Wilk normality test with DHI scale (only in cases), AOPP, FRAP and Thiols

values (these last in both cases and controls). If there was a distribution discrepancy between the samples (*i.e.*, the same continuous variable was normal in one group and non-normal in the other), we made a log-10 transformation of the values and repeated the normality assessment. Accordingly, we used a Mann-Whitney or a Student t test to compare, between the two groups, the plasma concentrations of the aforementioned substrates at T0. Then, considering only the Vertigoval[®]-treated group, a test for related samples (Wilcoxon or Student) was performed to assess if the difference between T0 and T1 oxidative stress markers values and DHI score was statistically significant. Finally, a Mann-Whitney test was performed to investigate if oxidative stress markers values depended on other cerebrovascular risk factors. The software Statistical Package for the Social Science (SPSS, IBM[®]) 17.0, for Windows operating system, was used.

3. RESULTS

Of the enrolled patients, 33 decided to participate in the study with Vertigoval[®] supplementation. Of those 33 patients, six dropped out: four for dyspepsia, one for headache, and one for poor compliance. None of the enrolled patients suffered serious adverse events or had to change the basal therapy for new illnesses.

At baseline, blood oxidative stress parameters mean values in all 55 patients were outside normal ranges [33-35] and compared to our matched healthy controls, being AOPP the most impaired parameter (Table 2). Oxidative stress load was not influenced by age, gender, hypertension, diabetes, smoking attitude or dyslipidemia (data not shown). No relation was found between chronic ischemic burden evaluated at CT or MRI scans and oxidative stress biomarkers.

Table 2. Oxidative stress markers median values in patients and controls. Statistical significant differences in bold.

	Median Value (IQR) Patients (n=55)	Median Value (IQR) Controls (n=49)	P
AOPP nmol/ml	315,7 (151)	192,6 (18.2)	<0.001
FRAP mmol/l	0,59 (0,16)	0,82 (0,1)	<0.001
THIOLS mmol/l	0,26 (0,09)	0,50 (0,15)	<0.001

IQR = interquartile range.

Table 3. Oxidative stress markers median values in the 33 patients who underwent the polyphenol supplementation and DHI scale mean values at T0 and after 60 days supplementation with Vertigoval[®]. Statistical significant differences in bold.

N= 33	Median Value (IQR) T0	Median Value (IQR) Controls (n=49)	P	Median Value (IQR) T1	P
AOPP nmol/ml	305,7 (151)	192,6 (18.2)	<0.001	230,9 (226)	0.007
FRAP mmol/l	0,62 (0,16)	0,82 (0,1)	<0.001	0,61 (0,15)	0.10
THIOLS mmol/l	0,28 (0,09)	0,50 (0,15)	<0.001	0,29 (0,09)	0.899
DHI score	35,6 (17,1)*	-	-	24,1 (17,0)*	<0.001

*DHI (Dizziness Handicap Inventory scale) values are expressed in mean values (SD).

After two months of supplementation with Vertigoval[®], we found a significant decrease of AOPP values, whereas FRAP and Thiols did not show a significant change (Table 3). Moreover, quality of life, measured with DHI scale, showed a significant improvement after 60 days of treatment (Table 3). Finally, no correlation was found between the improvement of DHI and AOPP values.

4. DISCUSSION

The notion that oxidative stress is pathogenic is one of the most challenging hypotheses in cSVD. Here, we have observed an impaired oxidative stress balance in patients with cSVD and dizziness. Over the last few years, over hundreds papers (PubMed MeSH search terms: blood biomarker oxidative stress and stroke; cerebral vascular disease) report a strong link between cerebral vascular disease and blood circulating biomarkers. Although the exact role of oxidative stress in the pathogenesis of sCVD is still unknown, activation of the defense, repair and inflammation vascular mechanisms are considered to be involved, and the resultant increased formation of free radicals and ROS may both promote and enlarge tissue damage [36, 37]. Experimental and clinical evidences indicate that vascular oxidative stress predisposes to endothelial dysfunction through a reduced NO production and availability [36]. Common cerebrovascular risk factors (such as hypertension, diabetes mellitus, hypercholesterolemia and smoking) are associated with higher oxidative stress level [36, 38]. However, in our patients, none of them resulted in an independent risk factor for oxidative stress. Moreover, in another study, investigating AOPP values in patients with acute coronary disease, no correlation was found either [39]. Therefore, even though a contribution of common risk factors may be involved in the redox imbalance, it could be likely that those molecules might act as systemic markers of endothelial dysfunction that link together several pathological condition in the so-called "circulatory syndrome" [1]. In fact, AOPP were found higher in several vascular diseases involving kidneys, heart or brain [39-42]. In this scenario, leukoaraiosis could be seen as a manifestation of a multisystemic circulatory syndrome [1, 40].

AOPP, FRAP and -SH were significantly impaired in our patients, basally. The significant reduction of AOPP value together with the improvement of the clinical symptom (measured trough DHI score) after Vertigoval[®] supplementation supports a link between oxidative stress, cerebral vascular impairment and dizziness. The supplementation with Vertigoval[®] was not associated with an improvement of FRAP and -SH values. We do not have a definitive answer to explain that. A possibility could be that the pathways covered by those biomarkers are not linked to the ones influenced by Vertigoval[®]; another scenario could be that, in order to see modification in FRAP and thiols, a longer supplementation with Vertigoval[®] would be necessary. Moreover, despite a significant improvement after polyphenol supplementation,

oxidative stress markers were still outside normality ranges in our population. ROS and oxidative/redox balance are involved either in physiological and in pathological processes. No threshold to determine when oxidative stress levels are dangerous has been found in multiple studies [43] and a European redox consortium has been created to better comprehend "redox medicine" [43]. This consortium has concluded that different thresholds should be identified whether investigating normal or injured organs, and this recommendation might explain why, after polyphenol supplementation, oxidative stress markers were still outside normal ranges in our study. Moreover, a two months supplementation could not be enough to normalize redox status.

The clinical and biochemical improvement after the polyphenol supplementation supports the hypothesis that oxidative stress plays a role in cSVD. Despite no pathophysiological cause has been identified for dizziness in patients with cSVD, it is likely that a reduced white matter perfusion could interfere with subcortical networks involved in gait [11, 12]. Oxidative stress is associated with NO production, which is known to have a protective role in endothelium and to empower blood flow, especially in small vessel circulation [44]. The correction of oxidative stress imbalance could empower NO production and cerebral blood flow. However, an improved availability of NO after a reduction of oxidative stress load was never proven. This hypothesis deserves further investigations, for example with perfusion imaging studies, sonographic exams or by dosing NO products after supplementations with anti-oxidant therapy.

Our study presents some limitations. Firstly, it was not a placebo-controlled study, and this could have biased the clinical benefit seen after the therapy. Secondly, the control group used to compare oxidative stress markers load small (49 subjects). However, normal ranges for those markers have been previously described and, especially for AOPP values, patients were far outside those ranges. Thirdly, additional biomarkers, exploring other possible damaged products (*i.e.*, lipid peroxidation) were not explored. Finally, the low number of patients enrolled in this study may not have allowed us to find significant differences between different groups or correlations between oxidative stress load and cSVD burden.

Despite these limitations, we can assess that patients with LA and dizziness have an impaired oxidative stress balance, as seen in other vascular multisystemic condition. The supplementation with Vertigoval[®] reduces oxidative stress load in patients with a pre-existing imbalance and seems to improve dizziness symptoms.

CONCLUSION

Oxidative stress biomarkers may be useful to detect redox imbalance in LA and to provide non-invasive tools to monitor disease status and response to therapy. A double-blind placebo-controlled study is needed to confirm this preliminary data.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was reviewed and approved by the local Ethical Committee of the Neurological Institute, University of Pisa, Pisa, Italy.

HUMAN AND ANIMAL RIGHTS

All the procedures involving human subjects were performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its subsequent revisions. No animals were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

All persons gave their informed consent prior to their inclusion in the study.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

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