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Oxidative stress, nitric oxide, endothelial dysfunction and tinnitus

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Abstract

To assess whether pathogenic endothelial dysfunction is involved in acute idiopathic tinnitus we enrolled 44 patients and 25 healthy volunteers. In blood from the internal jugular vein and brachial vein we determined malonaldehyde, 4-hydroxynonenal, mieloperoxidase, glutathione peroxidase, nitric oxide, L-arginine and L-ornitine, thrombomodulin (TM) and von Willebrand factor (vWF) activity during tinnitus and asymptomatic period.

Higher plasma concentrations of oxidative markers and L-arginine, and lower nitric oxide and L-ornitine levels were observed in jugular blood of patients with tinnitus, there being a significant difference between brachial and jugular veins. TM and vWF activity were significantly higher in patients' jugular blood than in brachial blood.

Our results suggest oxidant, TM, vWF activity production are increased and nitric oxide production reduced in brain circulation reflux blood of patients with acute tinnitus. These conditions are able to cause a general cerebro-vascular endothelial dysfunction, which in turn induce a dysfunction of microcirculation in the inner ear.

Keywords: *Tinnitus, inner ear, endothelial dysfunction, thrombomodulin*

Introduction

Free radicals (FR) are a very reactive chemical species, able to damage tissues directly by modifying the intracellular spaces, cell membrane, protein synthesis and cell nucleus. They also act indirectly on the circulation and are involved in the formation of atherosclerosis, inflammation and vascular damage [1–3].

Major endothelial damage and consequent endothelial dysfunction occur in the microcirculation, in particular in the terminal tracts, as observed in the renal glomerulus, retina, ear and central nervous system [4,5].

Studies [4–7] have shown that some drugs, autoimmune diseases, ischemia reperfusion events, endotoxinemia and inflammation can determine endothelial dysfunction and damage the labyrinth sensorineural epithelium as well as the auditory peripheral and central vestibular tracts. The aim of our study was to assess whether pathogenic endothelial dysfunction is involved in idiopathic tinnitus.

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Materials and methods

We recruited 115 patients presenting recurrent tinnitus at the Department of Medical and Surgical Specialty, Padua University (Italy) and at the Department of Othorinolangology, Catania University (Italy); 25 healthy non-smoker volunteers recruited from our medical and paramedical staff.

Exclusion criteria:

- diseases of the middle ear, inner ear and retrocochlear region;
- associated labyrinthine vertigo and chronic dysmetabolic diseases;
- hypertension (blood pressure $>140/90$ mm Hg after a mean of three consecutive readings in the setting position, using a mercurial sphygmomanometer on the right arm after 5 min rest) [8];
- diabetes and other metabolic diseases;
- alcohol abuse;
- family history of diabetes;
- smoking; and
- case history of cardiovascular and acute and chronic cerebro-vascular (angina pectoris, IMA, stroke, TIA) diseases and autoimmune and systemic inflammatory diseases.

Moreover, patients on chronic or antioxidant pharmacologic treatment and radiation therapy and those with reduced creatinine clearance were not included in the investigation. Cerebral computed tomography (CT) was performed to rule out the presence of neoplastic diseases. Common carotid artery intima-media thickness was measured by ultrasonographic B-mode imaging (Apogèe 800 equipped with a 7 MHz rectilinear electronic probe) to reveal onset of atherosclerosis and vascular disease progression, and subjects presenting carotid stenosis and plaque were not included in the study cohort.

The final study cohorts was made up of:

(a) Forty-four selected non-smokers (21 males and 23 fertile females; age range 36–52 years) with crisis of idiopathic bilateral tinnitus that had onset not more that 10 days (mean 7 ± 2 days) prior to observation (group A).
(b) Twenty-five healthy non-smoker volunteers (15 males and 23 females; age range 34–50 years) who were the control group (group B).

The study was approved by the local medical committees at Padua and Catania and informed written consent was obtained from all subjects.

None of the subjects (groups A and B) presented auditory deficit and their audiogram was normal. Cholesterol and triglycerides values were within normal range in the entire study group and body mass index (BMI) ranged between 21.5 ± 2.3 kg/m^2. Blood samples were withdrawn from females during the ovulatory phase. None of the study cohort took antioxidant or herbal drugs, nor did strenuous physical activity (gymnasium) during the 5 days prior to withdrawal of blood samples. All of them adhered to a controlled alcohol free, well-balanced low cholesterol (0.3–0.4 g/day), diet (1800 kcal/day adjusted to weight) for 5 days before blood samples were taken.

In all subjects case history was recorded and impedance metric and audiometric examinations performed. Blood samples were withdrawn from the internal jugular vein and brachial vein from both groups. In 38/44 group A patients (six did not consent to the repeated control) a second blood sample was withdrawn during a symptom free period utilizing the same procedure. Posterior approach was adopted to for anesthesia and to withdraw blood samples from the jugular vein. The needle was positioned just above the intersection of the lateral edge of the sternocleidomastoid and the external jugular vein and then directed under the muscle towards the sternal manubrium.

Blood anticoagulative samples were stored immediately at 4°C and underwent refrigerated centrifugation (3000 rev/min for 15 min) to separate plasma from serum. Laboratory tests were carried out in the Department in Catania. Plasma concentrations of malonaldehyde (MDA), 4-hydroxynonenal (4-HNE) and myeloperoxidase (MPO) were used to determine oxide-reductive status as stable lipoperoxidation products *in vivo* and plasma glutathione peroxidase (GSH-Px) as oxygen radical scavenging enzyme activity. MDA using high performance liquid chromatography (HPLC JASCO; Japan) after incubation with thiobarbituric acid [9]; 4-HNE by spectrocolorimetric assay (LPO-586 method Bioxytech) according to Esterbauer and Cheeseman [10]; MPO (non-specific marker for inflammatory cells derived from neutrophil activation) by optical absorbance recorded for 4–5 min at 510 nm (after adding 1.7 nM hydrogen peroxide and 2.5 nM solution of 4-aminoantipyrine containing phenol to 100 µl of plasma). GSH-Px using spectophotometric assay (Hitachi model 200-20) [11].

Plasma concentrations of the following parameters were used to determine endothelial cell activation:

- nitrite plus nitrate (NOx) determined by photometric method after centrifugal ultra filtration (absorbance 540 nm; molecular weight cut-off 10 kD, UFC3, Millipore, Bedford MA, USA) using capillary electrophoresis (Cayman's nitrite/nitrate assay kit; Cayman Chemical Company USA) [12–14].
- L-arginine (the substrate for NO synthesis) and L-citrulline (a by-product of NO synthesis) determined by HPLC [12–14].

- von Willebrand factor antigen (vWF:Ag) assayed by enzyme-linked immunosorbent ELISA method (Boheringer Mannheim, Germany).
- Thrombomodulin (TM) (Asserachrome Thrombomodulin, Diagnostic Stago, Asnières, France).

Statistical analysis

Statistical analysis of the study data was performed using statistical software (STATS Homework. VL Bissonette, Southeastern Louisiana University, Hammond, CA, USA). Results are expressed as medians and ranges or means ± SD. Student's *t*-test (continuous variables) and/or Pearson's χ^2-test (categorical variables) were performed on the means of the data of the two groups. *p* value of less than 0.05 ($p < 0.05$) was considered statistically significant.

Results

Jugular vein sampling did not cause any complications. The results of the blood samples withdrawn from the brachial and internal jugular veins are shown in Tables I and II.

Group A (patients with idiopathic tinnitus) presented a significant increment ($p < 0.005$) of oxidative damage makers (MDA, 4-HNE and MPO) concentrations and plasma scavenger activity in jugular compared with brachial blood, accompanied by increased FR concentrations and reduced plasma glutathione peroxidase activity. They also presented an evident, significant fall ($p < 0.05$) in peripheral and jugular concentrations of NO, L-arginine and L-citrulline and an increment in L-arginine values in jugular vein reflux blood compared with peripheral blood. Moreover, group A patients had higher TM and vWF levels in jugular blood, and the difference between brachial and jugular values was significant. No significant difference between jugular and brachial blood was observed either in group A during the asymptomatic period, or in group B. Regarding the oxidant/antioxidant profile markers, no significant differences between subjects of group A, group B and group A in asymptomatic period was observed on the brachial blood while the mean values of oxidant/antioxidant profile markers was significantly higher in group A compared with controls and subjects in asymptomatic period.

Table I. Oxidants, antioxidant (GSH-Px) level in group A (pathologic), group B (control) and in patients of group A (39/44) during symptom free period. Statistical difference between brachial and jugular veins, means and SD.

	Jugular	Brachial		
		Group A		
MDA	2.61 ± 0.2	1.98 ± 0.5	µmol/dl	$p < 0.05$
4HNE	2.42 ± 0.18	1.46 ± 0.21	µmol/dl	$p < 0.05$
MPO	0.71 ± 0.03	0.32 ± 0.05	µmol/dl	$p < 0.05$
GSH-Px	2.24 ± 0.16	5.37 ± 0.4	µmol/dl	$p < 0.05$
		Group B		
MDA	2.15 ± 0.2	1.98 ± 0.16	µmol/dl	$p > 0.05$
4HNE	1.49 ± 0.2	1.44 ± 0.17	µmol/dl	$p > 0.05$
MPO	0.36 ± 0.1	0.34 ± 0.1	µmol/dl	$p > 0.05$
GSH-Px	5.65 ± 0.35	6.08 ± 0.4	µmol/dl	$p > 0.05$
		Group A (asymptomatic period)		
MDA	2.18 ± 0.1	2.01 ± 0.1	µmol/dl	$p > 0.05$
4HNE	1.38 ± 0.1	1.36 ± 0.2	µmol/dl	$p > 0.05$
MPO	0.40 ± 0.2	0.44 ± 0.1	µmol/dl	$p > 0.05$
GSH-Px	7.04 ± 0.3	6.42 ± 0.4	µmol/dl	$p > 0.05$

Table II. Nitric oxide (NO), L-arginine, L-citrulline, vWF:Ag, TM level in group A (pathologic), group B (controls) and in patients of group A (39/44) during symptom free period. Statistical significance between brachial and jugular veins, mean and SD.

	Jugular	Brachial		
		Group A		
vWF:A	116 ± 5.2	82 ± 11	IU/ml	$p < 0.05$
TM	54 ± 2.1	40.2 ± 1.4	IU/ml	$p < 0.05$
NO	48.17 ± 1.57	55 ± 1.7	µmol/dl	$p < 0.05$
L-arginine	38.05 ± 1.61	30.5 ± 0.9	µmol/dl	$p < 0.05$
L-citrulline	25.73 ± 2.26	40 ± 2.9	µmol/dl	$p < 0.05$
		Group B		
vWF:A	84 ± 3.5	81 ± 1.0	IU/ml	$p > 0.05$
TM	42.3 ± 2.0	40.1 ± 1.6	IU/ml	$p > 0.05$
NO	53.4 ± 4.7	53 ± 3.5	µmol/dl	$p > 0.05$
L-arginine	32.3 ± 2.1	31.5 ± 2.1	µmol/dl	$p > 0.05$
L-citrulline	41.3 ± 3.1	40.8 ± 2	µmol/dl	$p > 0.05$
		Group A (asymptomatic period)		
vWF:A	81 ± 3.5	82 ± 1.0	IU/ml	$p > 0.05$
TM	41.3 ± 2.0	41.4 ± 1.9	IU/ml	$p > 0.05$
NO	52.1 ± 4.2	54.3 ± 3.4	µmol/dl	$p > 0.05$
L-arginine	34.2 ± 1.4	36.2 ± 2.3	µmol/dl	$p > 0.05$
L-citrulline	40.1 ± 2.0	40.6 ± 2.1	µmol/dl	$p > 0.05$

Discussion

Oxidative stress can be considered one of the causal agents of cell and tissue damage in many diseases [1–3]. Elevated levels of FR are also related to life style (smoking, etc.) and administration of some drugs. Indubitably, the endothelium is a sensitive and elective target of FR cytotoxic action [1–3]. It mainly affects the microcirculation where it impairs the terminal tracts, such as the ear, renal glomerulus and retina [3–5].

Experimentally induced oxidative stress increased FR levels in the labyrinth, auditory and vestibular tracts [4,5] acting at the level of auditory sensorineural cells, where oxidants may interact with the cell phospholipidic membrane and determine direct lipoperoxidation lesions, and may mediate apoptosis

in auditory neurons and ciliated cells [13]. Experimental studies on the semicircular canals have shown that injections of substances able to determine oxidative stress can inhibit endolymph production and neuromediators release, and that concomitant administration of antioxidants can restore the various bioelectric activities [5].

Basal nitric oxide production can regulate cochlear blood flow [6]. Nitric oxide, whose formation is catalyzed by NO synthase (NOs) enzyme, is a signal gas and constant endothelial production of said gas regulates vascular tone [6,12–15]. Various NO isoforms play a role in triggering off other functions, such as intercellular neurotransmission, in cytotoxic mechanisms against infectious diseases, autoimmune events and in the regulation of cell apoptosis [6,12–15]. Several studies [12–15] indicate that NO acts in different key phases of atherosclerosis and inhibits platelet adhesion and aggregation, monocyte chemotactic adhesion, and LDL oxidation.

Our study results indicate there is a significant increase of plasma oxidants in brain circulation reflux blood in patients with idiopathic tinnitus and our data suggest that FR can be involved in this disease. Moreover, the investigation revealed a significant reduction in NO and L-citrulline concentrations and a rise in L-arginine, and the precursor of these substances, in the patients' jugular blood. This can be the result of endothelial dysfunction and deficient synthethase expression. Reduced NO concentrations is able to upset the antagonist-vasoconstrictor balance and modify haemodynamics, especially in the terminal tracts of the microcirculation including the labyrinth and semicircular canals. Our study also revealed changes in values of TM and vWf that are both released by activated or damaged endothelial cells. These differences were not present in healthy subjects and in group A patients during asymptomatic period.

Undeniably, endothelial dysfunction can be cause or effect of the haematochemical changes observed in our study. As the volume of blood effluent from the inner ear represents an extremely small fraction of total effluent volume of the brain, it is not clear whether oxidative stress and endothelial dysfunction originate from, and are specific to, inner ear microcirculation.

Nevertheless we believe that our results indicate the presence of endothelial dysfunction characterized by oxide-reductive balance accompanied by modified clotting (at least locally) and changes in the endothelial barrier properties in brain circulation reflux blood in patients with recent idiopathic tinnitus.

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References

[1] Davies KJA. Oxidative stress: The paradox of aerobic life. In: Rice-Evans C, et al. editor. Free radicals and oxidative stress. London: Portland Press; 1995.
[2] Halliwell B. Current status review: Free radicals, reactive oxygen species and human disease: A critical evaluation with special reference to atherosclerosis. Br J Exp Path 1989; 70:737–757.
[3] McCord J. Oxygen-derived free radicals in post-ischemic tissue injury. N Engl J Med 1985;312:159–163.
[4] Gloddek B, Lamm K, Arnold W. Pharmacological influence on inner ear endothelial cells in relation to the pathogenesis of sensorineural hearing loss. Adv Otorhinolaryngol 2002; 59:75–83.
[5] Grammas P, Liu GJ, Wood K, Floyd RA. Anoxia-reoxygenation induces hydroxyl free radical formation in brain micro vessel. Free Radic Biol Med 1993;14:553–557.
[6] Brechtlsbauer PB, Nuttel AC, Muller JM. Basal nitric oxide production in regulation of cochlear blood flow. Hear Res 1994;77(1/2):38–42.
[7] Cadoni G, Fetoni S, Agostino S, De Santis A, Manna R, Ottaviani F, et al. Autoimmunity in sudden sensorineural hearing loss: Possible role of anti endothelial cell antibodies. Acta Otorhinolaryngol 2002;548(suppl):30–33.
[8] Rose GA, Blackburn H. Cardiovascular survey methods. Geneva: World Health Organization; 1968.
[9] Yagi K. Assay for serum lipid peroxide level and its chemical significance. In: Yagi K, editor. Lipid peroxides in biology and medicine. New York: Academic Press; 1982. p 223–242.
[10] Esterbauer H, Cheeseman KH. Determination of aldehyde lipid peroxidation products: Malonaldehyde and 4-hydroxynoneal. Methods Enzymol 1990;186:407.
[11] Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization on erythrocyte glutathione peroxidase. J Lab Clin Med 1967;70:158.
[12] Ueda T, Maekawa T, Sadamitsu D, Oshita S, Ogino K, Nakamura K. The determination of nitrite and nitrate in human blood plasma by capillary zone electrophoresis. Electrophoresis 1995;16:1002–1004.
[13] Thippeswamy T, McKay JS, Morris R. Bax and caspase are inhibited by endogenous nitric oxide in dorsal root ganglion neurons *in vitro*. Eur J Neurosci 2001;14(8):1229–1236.
[14] Otha K, Shiamtzu K, Komatsumoto S, Araki N, Shibata M, Fukuuchi Y. Modification of striate arginine and citrulline metabolism by nitric oxide synthase inhibitors. Neuroreport 1994;5:766–768.
[15] Dimmler S, Zeiher AM. Nitric oxide. An endothelial cell survival factor. Cell Death Differ 1999;6(10):964–968.