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ORIGINAL ARTICLE

A Comprehensive Study of Oxidative Stress in Tinnitus Patients

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Abstract Oxidative stress is considered to be one of the molecular changes that are the underlying causes of tinnitus. The aim of this study was to investigate the dynamic thiol/disulphide homeostasis as a new oxidative stress parameter in tinnitus patients as well as to investigate the lipid hydroperoxide (LOOH), total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) parameters and compare the results with the results of the healthy control group. A prospective controlled trial was performed on tinnitus patients in Harran University hospital. A total of 70 subjects, including 35 tinnitus patients and 35 healthy individuals participated in this study. Their total thiol, native thiol levels and LOOH, TAS, TOS levels were measured in plasma of all tinnitus patients and healthy volunteers participants. TOS and OSI levels were significantly increased, and TAS levels were significantly lower in the patient groups compared with the control group ($p < 0.01$). Native thiol levels and Native thiol/total thiol ratios were significantly lower in the tinnitus group compared to the control group ($p < 0.01$). Disulphide level and disulphide/native thiol and disulphide/total thiol ratios were significantly higher in the patients ($p < 0.01$). Also, LOOH ratios were significantly higher in the tinnitus group ($p < 0.01$). The results of this study reveal that in tinnitus cases, the oxidative stress and antioxidant enzyme imbalance were more significant than in healthy control group. The nature of the relationship between oxidative stress and tinnitus should be clarified with larger studies.

Keywords Tinnitus · Thiol · Disulphide · Homeostasis · Oxidative stress

Introduction

Tinnitus is defined as the sound of the ear or head without any external acoustic stimuli [1]. Tinnitus can be classified objectively and subjectively. Objective tinnitus is sounds that can be heard using a stethoscope or an external auditory meatus, usually due to musculoskeletal and vascular pathologies. In Subjective tinnitus, sounds that are thought to be developed by the cause of abnormal neural activity without sound stimuli and can not be heard from outside [1]. The prevalence of tinnitus increases with age. It is seen in 10–17% of the general population and 33% of the elderly population [2, 3]. Although many factors, including acoustic trauma, stress, ototoxic drugs, metabolic diseases, otologic diseases, neurological diseases are accused in the etiopathogenesis, most of the cases remain idiopathic [4, 5]. Various psychiatric problems have been reported in 30% of tinnitus patients [6]. Tinnitus causes various psychiatric disorders and sleep disorders that are present in 1–2% of patients and adversely affects the social cohesion and quality of life of patients [7]. For these reasons, it is important to be able to elucidate the etiology. Reactive oxygen species (ROS) is very important in the disorders of

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neurootology may be expected to have an effect on the etiopathogenesis of tinnitus [8–11].

Oxygen molecules are indispensable molecules for aerobic organisms to survive. During metabolism, however, intermediates are formed which are known to be free radical sources and are highly reactive. Free radicals are reactive molecules that form during the energy conversion of food to oxygen. These molecules, known as ROS, have a seriously detrimental effect on cellular constituent such as lipids, proteins and deoxyribonucleic acid (DNA) found in structure and cells of the human body. The damage that free oxygen groups bring to the tissues and the most important consequence of them is lipid peroxidation. Lipid peroxidation can lead to the formation of free oxygen groups, which can ultimately lead to toxic effects. Antioxidant defense systems have been developed in aerobic organisms to control the formation of free radicals and to prevent the harmful effects of these molecules. However, in some cases, the available antioxidant defense system is missing for avoiding to the toxic effects of free radicals and a condition called oxidative stress occurs and a condition called oxidative stress occurs [8, 12–15].

Lipid hydroperoxide (LOOH) is mentioned as oxidative stress marker composed of unsaturated phospholipids, glycolipids and cholesterol [12]. Total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) are used to defined as the oxidant/antioxidant balance. TOS, TAS, and OSI are the parameters used as oxidative stress markers [12, 13]. Another important group of physiological antioxidant defense system is the group of sulfhydryl (–SH). Sulfhydryl (–SH) groups interact with electrophilic groups of reactive oxigen species. In this way it contributes to the protection of cells and tissues against oxidative injury [12–15]. Total sulfhydryl (–SH) group is the other kind of antioxidants. Thiols are organic compounds containing a hydrogen atom bonded to a carbon atom and a sulfhydryl group (-SH) made up of a sulfur atom. Thiols react with free radicals to prevent cellular and tissue damage caused by reactive oxygen products. Under oxidative stress, thiols (-sulfhydryl groups) are converted to disulfides. The resulting reversible disulfide compounds can be converted back to the thiols. This loop, called TDH, is a continuously dynamic state. TDH has a significant part in metabolic events such as oxidative stress, apoptosis, detoxification, antioxidant protection and enzymatic activities [14]. In 2014, Erel and Neselioglu developed a new and automatic method that directly measures serum thiol/disulphide levels [14]. Although TOS, TAS, OSI were studied by other groups, no study has investigated LOOH, dynamic thiol-disulphide homeostasis (TDH) levels in the serum for tinnitus.

The purpose of this paper was study the novel oxidative stress markers thiol/disulphide balance, which had not been studied previously in tinnitus patients, and to compare the values obtained with oxidative stess markers such as LOOH, TAS, TOS, OSI and healthy controls.

Materials and Methods

Subjects

This study is a prospective, controlled study in patients with tinnitus. Patient data were collected from the Harran University Ear, Nose and Throat Clinic in Sanliurfa, Turkey. The Harran University Local Ethical Committee approved this study (11.05.2017-05/19). Signed consent forms were received from all patients and the healthy control group. The procedures were made in accordance with the Helsinki declaration.

The study group comprised 35 patients (mean age 44.77 ± 7.21 years) with bilateral tinnitus attending our hospital's outpatient ear, nose, and throat clinic. The healty control group consisted of 35 (mean age 42.16 ± 8.82 years) age, sex, and body mass index (BMI) matched healthy subjects (Table 1).

All participants were carefully questioned for medical history. In all patients, routine ear, nose and throat examinations and audiologic measurements such as püre tone, speech, admittance audiometry and acoustic reflex test were performed. In addition, complete neuro-otologic evaluation and blood tests were performed. Temporal MRI was performed in the tinnitus group.

The main criteria for participation were the presence of persistent tinnitus lasting more than six months, between the ages of 27 and 56, bilateral chronic subjective cochlear tinnitus. All participants had normal otorhinolaryngologic, audiological evaluations.

Patients with pathological conditions such as neuro-otologic surgery, central-peripheral vertiginous disorders, acute-chronic otologic infective conditions and patients with used antitinnitus drug were excluded from the study. In addition, patients with chronic bad habits such as smoking and alcohol use; chronic drug use; systemic disorders such as pulmoner, cardiovascular, liver, renal, hematological, metabolic, neuropsychiatric diseases and patients with a history of malignancy were excluded from the study.

Blood Samples Collection

Peripheral venous blood samples were taken after 10–12 h fasting, and were centrifuged at 3000 $\times g$ for 10 min. The samples taken were stored at − 80°. Stored samples were used to assess the oxidative status by measuring thiol-

Table 1 The demographic data of patients with tinnitus and the control group

Variable	Tinnitus group (n:35)	Control group (n:35)	*p* value
Age (years)	44.77 ± 7.21	42.16 ± 8.82	0.174
Gender (male/female)	7/28	9/26	0.174
BMI (kg/m^2)	24.62 ± 1.04	24.28 ± 1.04	0.169

Values are presented as median(min–max) (mean ± SD)

$p < 0.05$ was considered significant for statistical analyses

BMI body mass index

disulphide hemostasis markers, TOS, TAS and OSI and lipid hydroperoxide.

Measurement of TOS

The TOS of the serum was determined by Rel Assay (Rel Assay Diagnostics kit, Mega Tip, Gaziantep, Turkey) as described by the manufacturer [15]. Briefly, ferric ion complex gets oxidized by the free oxidants and oxidation reaction was enhanced by glycerol. Then this enhanced oxisidization state reacts with xylenol to have coloration. The intensity of color indicates the amount of free radical produced during the redox reactions initiated. The results are expressed in terms of micromolar hydrogen peroxide equivalents per liter (μmol H_2O_2 equiv/L).

Measurement of the TAS

The TAS measurement was conducted with Rel Assay (Rel Assay Diagnostics kit, Mega Tip, Gaziantep, Turkey) as instructed by the vendor. Briefly, free radical reactions were initiated by Fenton reaction and monitored by absorbance of the dianisidyl radicals. Using this method, the antioxidative effect was measured by relative amount of free dianisidyl radicals [15]. The data were expressed in μmol Trolox Equiv/L.

OSI Calculation

To calculate the OSI, the resulting TAS units were converted to mmol/L, and the OSI value was calculated according to the following formula: OSI (arbitrary unit) = TOS (mmol H_2O_2 equivalent/L)/TAS (mmol Trolox equivalent/L) [15].

Measurement of the Thiol/Disulphide Hemeostasis

In present study, blood samples taken from all participants were studied using an automated novel technique defined by Erel and Neselioglu [14] and dynamic thiol-disulphide homeostasis was assessed. Native thiol content was subtracted from total thiol content and half of this difference was calculated; this value was amount of dynamic disulphide bonds. In addition, disulphide bonds/native thiol, disulphide bonds/total thiol and native thiol/total thiol ratios were calculated using these parameters. Thiol/disulphide ratios were evaluated by the method of Erel and Neselioglu [14]. Total thiol, native thiol, disulphide, disulphide/total thiol, disulphide/native thiol, and native thiol/total thiol levels were recorded to determine from collected laboratory results.

Lipid Hydroperoxide (LOOH) Measurements

An automated xylenol orange method was used and serum LOOH levels were detected [16, 17]. In this method, ferrous ions are oxidized by LOOH to ferric ions, which can be measured with xyleneol orange [16, 17]. The measured absorbance ratio is 570 nm.

Statistical Analysis

Assessment of all statistical analyses were done with the SPSS software version 19.0 (SPSS Inc., Chicago, IL, USA). Gender comparison between the both groups was performed using Pearson's Chi square test. The results of the comparison were presented as counts and percentages. Evaluation of the normal whether distribution of the variables was done with the Kolmogorov–Smirnov test. Comparison of continuous variables between the two groups was done with the independent sample *t* test. Continuous variables were presented as mean (standard deviation). Variables of continuous were recorded as mean (standard deviation). *p*-values were considered statistically significant, if *p* value is less than 0.05 ($p < 0.05$).

Results

General Characteristics

The study group consisted of 35 patients, (28 male (80%), 7 female(20%)), between the ages of 27 and 56 years (mean age, 44.77 ± 7.21 years). The control group had 35

individuals (26 male (74.28%), 9 female (25.71%) between the ages of 28 and 56 (mean age, 42.16 ± 8.82). No statistically significant difference was found between patient and control groups in terms of age, gender and body mass index (BMI) variables (Table 1). All patients were diagnosed as bilateral subjective tinnitus.

Statistical Comparisons

Median value of total thiol levels (403.29 ± 39.15 mmol/L) in tinnitus group were lower in healthy group (431.86 ± 48.64 mmol/L) ($p < 0.09$). Median value of native thiol levels (352.89 ± 48.15 mmol/L) tinnitus group were significantly lower, than native thiol level (398.61 ± 46.69 mmol/L) in healthy individuals ($p < 0.01$). Median value of dynamic disulphide bond level (25.20 ± 13.45 mmol/L) in patients group was significantly higher than healthy control (16.63 ± 6.27 mmol/L) ($p = 0.01$) (Table 2).

As presented in Table 2, mean value of disulphide/native thiol ratio of tinnitus group (7.68 ± 5.50%) was significantly higher than control group (4.23 ± 1.62%) ($p = 0.01$). Mean value of disulphide/total thiol ratio in tinnitus patients (6.33 ± 3.60%) were significantly higher than healthy control (3.86 ± 1.39%) ($p < 0.01$). The mean value of native thiol/total thiol ratio of patients with tinnitus (87.35 ± 7.21%) were significantly lower than healthy control group (92.29 ± 2.77%) ($p < 0.01$).

Serum total oxidant status (TOS) levels (14.72 ± 2.12 μmol H_2O_2 equiv/L) and oxidative stress index (OSI) level (1.47 ± 0.26 arbitrary units) were significantly higher in the tinnitus patients than the controls ($p < 0.01$). Serum total antioxidant status (TAS) levels (1.01 ± 0.11 mmol Trolox Equivalent/L) were significantly lower in the tinnitus patients ($p < 0.01$). LOOH levels (5.28 ± 0.63 μmol/L) were significantly higher in the tinnitus group ($p < 0.01$) (Table 2).

Discussion

In order to evaluate the correlation between tinnitus and oxidative stress, TAS, TOS, OSI, LOOH as well as thiol/disulphide hemostasis, a new oxidative stress marker, were evaluated. In a previous study, TAS, TOS, OSI levels were examined in patients with tinnitus [13]. However, as far as the current literature is concerned, there has been no study investigating the thiol/disulphide balance in tinnitus patients. Therefore, our study has novel aspect that we evaluated both TAS, TOS, OSI, LOOH levels and thiol/disulphide hemostasis.

Reactive oxygen species, composed of superoxid radicals, hydroxyl radicals and hydrogen peroxide, are produced during normal aerobic metabolism in the cochlea [13]. The antioxidant system, which is classified as enzymatic and nonenzymatic, forms the defense system against the damaging effects of ROS. The enzymatic system consists of glutathione reductase, glutathione peroxidase, superoxide dismutase, catalase enzymes. The nonenzymatic system consists of retinol, bilirubin, vitamin C, vitamin E, beta carotene, glutathione, uric acid [11, 18, 19].

ROS is in balanced state, as it can be synthysized or removed at the same time. This circulation is called oxidative hemostasis. As long as the oxidative balance can be sustained, the whole body structures of the organism is protected from harmful effects of the ROS. If oxidant

Table 2 Results of oxidative stress markers

Serum oxidative stress markers	Tinnitus group (mean ± SD)	Control group (mean ± SD)	*p*-value
Native thiol (mmol/L)	352.89 ± 48.15	398.61 ± 46.69	*<0.01*
Total thiol (mmol/L)	403.29 ± 39.15	431.86 ± 48.64	0.09
Disulphide (mmol/L)	25.20 ± 13.45	16.63 ± 6.27	*0.01*
Disulphide/native thiol (%)	7.68 ± 5.50	4.23 ± 1.62	*0.01*
Disulphide/total thiol (%)	6.33 ± 3.60	3.86 ± 1.39	*<0.01*
Native thiol/total thiol (%)	87.35 ± 7.21	92.29 ± 2.77	*<0.01*
Lipid hydroperoxide, LOOH, (μmol/L)	5.28 ± 0.63	4.39 ± 0.59	*<0.01*
TAS (mmol Trolox Equiv/L)	1.01 ± 0.11	1.29 ± 0.22	*<0.01*
TOS (μmol H_2O_2 equiv/L)	14.72 ± 2.12	11.04 ± 1.02	*<0.01*
OSI (arbitrary units)	1.47 ± 0.26	0.88 ± 0.15	*<0.01*

Values were expressed as mean ± standard deviation (SD). Italic values indicate statistically significant

p value is calculated by 2-tailed t-test

TAS total antioxidant status, *TOS* total oxidant status, *OSI* oxidative stress index

products are present or the antioxidant system is deficient, oxidative stress occurs [20]. In the case of oxidative stress, many systems in the organism are exposed to the harmful effects of ROS. Increased amount of ROS cause cytotoxic and neurotoxic effects and destruction of the acoustic system and labyrinthine auditory hair cells [13]. The harmful effects of oxidative stress are well known in some inner ear diseases such as Meniere's disease, impulsive induced hearing loss, drug ototoxicity, labyrinthitis [21, 22].

The lipid peroxidation produced by ROS is one of the major causes of cell and tissue damage. Lipid peroxidation can be used to evaluate the role of oxidative damage in pathophysiological disorders. Peroxidation of both saturated and unsaturated lipids leads to the formation of highly reactive and unstable hydroperoxides. The resulting lipid hydroperoxides disrupt the integrity of the cell membrane. Lipid hydroperoxide has been immunohistochemically identified in various pathological conditions such as atherosclerosis, Alzheimer's disease, Parkinson's disease, liver damage from chemical material and renal tubular damage in humans and experimental animals. These evidences reveal that lipid hydroperoxide is closely related to the etiopathogenesis of these disorders in humans and experimental animals [23–26].

Thiols react with free radicals to prevent cellular and tissue damage caused by reactive oxygen species. TDH is a dynamic state that is continuously functioning. TDH has a significant part in vital metabolic events such as oxidative stress, apoptosis, detoxification, antioxidant protection and enzymatic activities [14]. TDH is an important component of the antioxidant defense system that protects cells and tissues in our body against oxidative injuries. The TDH imbalance occurs when disulphide compounds increase or thiols decrease, which lead to the emergence of important pathologies in many systems in our body.

The etiology of tinnitus has not yet been fully explained. The majority of patients are accused of damage to the cochlea from the development of tinnitus. It has been suggested that this damage caused by various causes is caused by spontaneous stimulation in the cochlear hairy cells and in the 8th fiber [27]. It has been suggested that tinnitus may be effective in oxidative stress and free oxygen radicals in etiology. In some experimental studies, it has been suggested that ROS in the cochlea may cause sensory epithelial damage [28]. Findings from another study suggest that oxidative stress plays an crucial role in the etiopathogenesis of tinnitus [29]. It has been shown that noise induces ROS to realease local superoxide radicals (O2-), hydroxyl radicals (OH–) and hydrogen peroxide (H2O2) which may cause damage to the cochlear sensorineural epithelium [21, 22].

Tinnitus is a complicated, multifactorial process involving a large number of etiologic origins. The main cause of ear tinnitus is the deterioration of the cochlear hearing cells, whether or not the hearing cortex pathway is associated with biochemical changes, inflammation, and damage caused by ROS [30]. Normally, cochlear tissues contain vitamins and enzymes that are members of the antioxidant defense system. Certain antioxidant defense systems in certain inner ear diseases can not adequately detoxify as a result of imbalance between ROS and the excretion of these reagents, and oxidative stress develops [20]. The sensorineural epithelial tissues of the cochlea are more susceptible to deleterious effects caused by free radicals than other tissues of the body [13]. There are studies suggesting that oxidative stress may cause oxidative injuries in the acoustico-vestibuler system tissues [20].

Levine [31] suggested that a reduction in cochlear nerve entry was perceived as a disinhibition of the dorsal cochlear nucleus and hyperactivity of the auditory cortex as eardrum tinnitus. Savastano et al. [32] and Gopal et al. [33] expressed that in tinnitus cases, antioxidant treatment reduced the intensity of subjective symptoms and tinnitus, suggesting that oxidative stress may be crucial in the etiopathogenesis of tinnitus. In contrast, Polanski et al. [34] reported no benefit from using antioxidant drugs for tinnitus in tinnitus cases. Experimental studies on cochlear have shown that the control of oxidative stress can reduce the release of neuromedias and that injecting antioxidants may reduce bioelectric stimulation [29].

In this study, native thiol values were significantly lower in the patient group with tinnitus than in the healthy control group. Total thiol values were lower in the patient group with tinnitus. Disulphide values were higher in the patients group with tinnitus. Disulphide/native thiol, disulphide/total thiol and native thiol/total thiol values were lower in the patients group with tinnitus. LOOH ratios were significantly higher in the patient group with tinnitus than in the healthy control group. TOS and OSI levels were significantly higher in the patient group with tinnitus than in the healthy control group. However, TAS levels in the patient group with tinnitus were significantly lower than the healthy control group. Our results are supported by the previous study [13].

In conclusion, our study showed that patients with tinnitus are affected and exposed to potent oxidative stress. It is imperative to clarify the etiopathogenesis of tinnitus. There is a need for further study of etiology to understand the relationship between tinnitus and oxidative stress.

Compliance with Ethical Standards

Conflicts of interest Authors declare that they have no conflict of interest.

Ethical Approval The study was approved by the local ethics committee of the Harran University Faculty of Medicine (11.05.2017-05/19). All subject were informed about the research verbally and written informed consent was obtained from all.

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