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Protective effect of polyphenols on presbycusis via oxidative/nitrosative stress suppression in rats

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ABSTRACT

Age-related hearing loss (AHL) –presbycusis– is the number one neurodegenerative disorder and top communication deficit of our aged population. Experimental evidence suggests that mitochondrial dysfunction associated with reactive oxygen species (ROS) plays a central role in the aging process of cochlear cells. Dietary antioxidants, in particular polyphenols, have been found to be beneficial in protecting against the generation of ROS in various diseases associated with oxidative stress, such as cancer, neurodegenerative diseases and aging.

Objectives: This study was designed to investigate the effects of polyphenols on AHL and to determine whether oxidative stress plays a role in the pathophysiology of AHL.

Methods: Sprague-Dawley rats (n = 100) were divided into five groups according to their age (3, 6, 12, 18 and 24 months old) and treated with 100 mg/kg/day body weight of polyphenols dissolved in tap water for half of the life of the animal. Auditory steady-state responses (ASSR) threshold shifts were measured before sacrificing the rats. Then, cochleae were harvested to measure total superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities, reactive oxidative and nitrogen species levels, superoxide anions and nitrotyrosine levels.

Results: Increased levels of ROS and RNS in cochlea observed with age decreases with polyphenol treatment. In addition, the activity of SOD and GPx enzymes in older rats recovered after the administration of polyphenols.

Conclusion: The reduction in oxidative and nitrosative stress in the presence of polyphenols correlates with significant improvements in ASSR threshold shifts.

1. Introduction

Age-related hearing loss (AHL), or presbyacusis, is a complex degenerative disease that affects tens of millions of people worldwide. It is one of the most prevalent chronic conditions of the aged, afflicting approximately half of those over age 65 in the United States (Gopinath et al., 2009). AHL can cause people to withdraw from friends and become isolated and depressed (Kalayam et al., 1995a). Research into the causes of and treatment of presbyacusis is increasingly urgent, as the populations of industrialized countries grow older. For example, between 1965 and 1994, the incidence of presbyacusis in people age 50–59 increased by 150% (Adams and Marano, 1994).

AHL is thought to be the result of aging, oxidative damage, mitochondrial impairment, and environmental factors (Kokotas et al., 2007; Liu and Yan, 2007).

Oxidative injury caused by free-radical damage is perhaps the most fundamental cause of age-related pathology in the biological aging of cells. Oxidative damage may be an important intrinsic factor in the pathogenesis of presbycusis. Increased concentrations of free radicals (reactive oxygen species [ROS] and reactive nitrogen species [RNS]) are implicated as a mediator of oxidative stress and damage. It is now widely accepted that mitochondria are a major source of ROS/RNS and a major site of ROS/RNS induced oxidative damage, and that ROS/RNS production increases with age (Balaban et al. et al., 2005; Beckman and Ames, 1998; Shigenaga et al., 1994; Wallace, 2005). Numerous studies have focused on the hypothesis that age-related mitochondrial dysfunction is an underlying pathology that can cause or hasten presbyacusis. The cell normally uses a network of proteins and antioxidants to ensure that it has sufficient, but not excessive, ROS/RNS (Finkel, 2011). This network becomes less efficient with age, leading to the increased ROS/RNS levels believed to cause a variety of age-associated maladies, including hearing loss (Cui et al., 2012). Age related cochlear

Abbreviations: AHL, age-related hearing loss; ASSR, auditory steady-state responses; DHE, dihydroethidium; GPx, glutathione peroxidase; ROS, reactive oxygen species; RNS, reactive nitrosative species; SOD, superoxide dismutase.

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hair cell loss is enhanced in mice lacking the antioxidant enzyme Sod1 (McFadden et al., 1999a), while mice lacking the antioxidant enzymes Gpx1 or Sod1 show enhanced susceptibility to noise-induced hearing loss (Fortunato et al., 2004; Ohlemiller et al., 2000a). Moreover, oxidative protein damage increases with age in the cochleae of CBA mice (Staecker et al., 2001). Han and Someya (Han and Someya, 2013) found that activation of mitochondrial Sirt3 (a member of the sirtuin family) or glutathione reductase, or increased levels of mitochondrial glutathione has great potential for maintaining good hearing by mimicking the anti-aging effects of caloric restriction (CR) in cells of the human inner ear.

If ROS/RNS plays a causal role in AHL, then it is likely that enhancing antioxidant defenses through antioxidant supplementation can reduce oxidative cochlear cell damage and delay the onset of AHL. In recent years, great attention has been paid to natural dietary antioxidants notably polyphenols (Visioli and Galli, 2002) Polyphenols have an anti-inflammatory, anticarcinogenic, and antioxidant role, and are generally abundantly available in our food diets. Polyphenols are essential in our diet as they are micronutrients that our body cannot synthesize.

Polyphenols in red wine, tea and plant extracts have a large capacity for eliminating free radicals, inhibiting the synthesis of nitric oxide (NO), reducing lipid peroxidation and protecting against apoptosis in rats (Manach et al., 2004). Huang et al. (2007) have shown that the cisplatin-induced loss of rat cochlear hair cells is lower in rats treated with *Ginkgo biloba* extract than in rats not treated with the extract (Huang et al., 2007).

We have recently shown the benefits of the polyphenols, which generated a significant protection against AHL, with significantly improved ASSR (Auditory Steady State Response) and tone-burst ABR (Auditory Brainstem Response) auditory thresholds in rats receiving treatment with polyphenols mixture. A significant decrease in the audition was detected with ABR and ASSR in both non-treated groups, as the different groups became older. This deterioration was more accurately measured at acute frequencies. Significantly lower thresholds were observed in the treated rats in the 12, 18 and 24-month-old group compared with the control group (Sanz-Fernandez et al., 2016).

The aim of this study was to explore the benefits of different polyphenols in the diet on hearing impairment and its correlation with oxidative stress associated with the aging process in the rat cochlea.

2. Materials and methods

2.1. Animals

Healthy female Sprague–Dawley (SD) rats were used for this study. All of the animals were obtained from and maintained at the University Hospital of Getafe's animal care facility until use. The animals were housed in plastic cages with water and food provided “ad libitum” and maintained with a 12-h light/dark cycle. Rats with skin lesions, splenomegaly, macroscopically visible tumors or inner ear infections were excluded.

2.2. Polyphenols administration

A mixture of polyphenols with tannic acid, resveratrol, quercetin, rutin, gallic acid and morin (Sigma Aldrich, St. Louis, MO, USA) was administered to the treated group. Each of the six polyphenols was present in equal proportion in the mixture, which was added to the drinking water. Since the water was changed every day, we estimate a 100 mg/kg body weight of daily intake for each animal.

The dose of polyphenols used was shown to be non-toxic. Possible side effects of treatment with polyphenols were monitored throughout the study; no difference was observed when comparing control rats and treated rats.

Five groups were created based on the age of the rats, in months: 3, 6, 12, 18 and 24 months old. There were 20 SD rats per group, with a total of 100 animals. All of the measurements were obtained for both the right and left ears, yielding a total of 200 ears that were examined.

Two additional groups were created based on the treatment received by the animals. Each age group (3, 6, 12, 18 and 24 months old) had 10 subjects in the control group were assigned to no treatment in the drinking water and 10 subjects in the treatment group were given a vehicle mixture of polyphenols in the drinking water for half of the life of the animal before euthanization.

Mortality and excluded rats for the longer period of treatment (9 and 12 months) accounted for 5% of the total. No mortality or excluded rats were noted in the shorter period of polyphenol treatment (2, 3 and 6 months).

2.3. Extraction of protein homogenates of cochleae

Left cochleae were used to different analyses like ELISA kits and western blot. Thus, protein extracts of whole cochlea were obtained by using mammalian protein extraction reagent, with the addition of Complete mini protease inhibitor cocktail (Boehringer Mannheim GmbH, Mannheim, Germany), according to the manufacturer's instructions. The homogenates were treated for 3 min with a homogenizer ultra-Turrax T8 (IKA, Staufen, Germany) followed by 1 min on ice. This treatment was repeated twice. The lysate was cleared by centrifugation at 16.100*g* for 10 min at 4 °C to pellet the cell debris, and the supernatant was used or immediately stored at −80 °C. The protein concentration was determined by the BCA protein assay (Thermo Scientific. Rockford, IL, USA), is according to the manufacturer's instructions.

2.4. Total superoxide dismutase (SOD) activity measurements

Total superoxide dismutase (SOD) activity was measured in whole left cochlear homogenate using a SOD assay kit (Cayman Chemical Company, Ann Arbor, MI, USA), a colorimetric method. This kit measures total SOD activity (Cu/Zn-, Mn- and Fe-SOD) and utilizes a tetrazolium salt for the detection of the dismutation of superoxide radicals generated by xanthine oxidase to hypoxanthine in the tissue homogenates. SOD activity was standardized using the cytochrome-c and xanthine oxidase coupled assay. One unit of activity is defined as the activity of enzyme required to inhibit the production of formazan by 50%. Color intensity was read using a microplate GENios Plus (TECAN Trading AG, Männedorf, Switzerland).

2.5. Glutathione peroxidase (GPx) activity measurements

The GPx Assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione, produced upon reduction of hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in the absorbance at 340 nm. Under conditions in which the GPx activity is rate limiting, the rate of decrease in the A340 is directly proportional to the GPx activity in the sample.

GPx activity was measured in whole left rat cochlear homogenate. Color intensity was read using a microplate GENios Plus (TECAN Trading AG, Männedorf, Switzerland) set to 340 nm.

2.6. Reactive oxidative and nitrogen species detection

The OxiSelect™ In Vitro ROS/RNS Assay Kit (Cell Biolabs, Inc. San Diego, CA. USA) is used to measure the total free radical present in left rat cochlear homogenized. The assay employs a proprietary quenched fluorogenic probe, dichlorodihydrofluorescein DiOxyQ (DCFH-DiOxyQ), which is a specific ROS/RNS probe. Fluorescence intensity is proportional to the total ROS/RNS levels within the sample. The DCFH-DiOxyQ probe can react with hydrogen peroxide (H2O2), peroxyl radical (ROO·), nitric oxide (NO), and peroxynitrite anion (ONOO-), which are