

Prevention of Peroxide-Induced Biochemical Damage to the Neural Retina by Caffeine: A Preliminary Report

Hegde Kavita R* and Brown Destiny D

Department of Natural Sciences, Coppin State University, Science and Technology Center-Suite 200, Baltimore, MD 21216, USA

Abstract

Oxidative stress is one of the significant factors in the pathogenesis of several retinal diseases, viz. age-related macular degeneration, diabetic retinopathy, etc. Available treatments are not fully effective in attenuating tissue damage and the associated vision loss. Hence development of newer therapeutic compounds is highly desirable. We have previously demonstrated the effectiveness of metabolic and nutritional antioxidants such as pyruvate and caffeine in preventing oxidative damage to the lens. However, so far, studies investigating the protective effect of caffeine on the neural retina exposed to direct oxidative stress are lacking. The primary goal of this study was therefore to examine the efficacy of caffeine in preventing biochemical damage to the neural retina exposed to oxyradicals, in terms of maintaining the concentration of glutathione (GSH), a major endogenous antioxidant reserve. In vitro short-term tissue culture studies were conducted using freshly isolated neural retinas exposed to H₂O₂ in a medium with/without caffeine supplementation. Bovine neural retinas were incubated in medium 199 for 4 hours. Oxidative stress was induced by incubating the tissue with 9 mM H₂O₂. Caffeine group was incubated with 9 mM H₂O₂+ caffeine (5mM). Controls were incubated without H₂O₂ and caffeine. Tissue damage was assessed by measuring GSH content following incubation. Incubation of neural retina with H₂O₂ decreased GSH level to ~40% of the controls. Caffeine, however, maintained it at ~95% of the controls, indicating its effectiveness in preventing retinal oxidative stress. This novel effect of caffeine in the neural retina has been shown for the first time. The results are highly encouraging with regard to pursuing further studies investigating its other possible mechanisms of action, and its potential neuroprotective effect.

Keywords: Oxidative stress; Caffeine; Antioxidant; Glutathione; Neural Retina

Introduction

Utilization of oxygen to generate ATP (Adenosine Triphosphate) is associated with simultaneous production of partially reduced species of oxygen, especially during cellular respiration in the mitochondria, even under physiological conditions. These oxygen species, commonly known as Reactive Oxygen Species (ROS), Reactive Oxygen Intermediates (ROI) or oxyradicals, are the singlet oxygen (¹O₂), superoxide (O₂^{•-}), Hydrogen Peroxide (H₂O₂), and Hydroxyl radical (OH[•]). The hydroxyl radical is known to be the most reactive, while H₂O₂, although not a radical, is nonetheless highly reactive and potentially damaging to biological cells by generating the hydroxyl radical via Fenton's reaction (H₂O₂+Fe²⁺ → Fe³⁺+OH⁻+OH[•]) [1,2]. The cellular components susceptible to damage by ROS are the enzymatic and non-enzymatic proteins, lipids (including membrane lipids), nucleic acids as well as carbohydrates. Oxidation of proteins can lead to modification of their conformation and eventual loss of ability to perform their normal function, such as maintaining cell structure if cytoskeletal proteins are affected. Similar oxidative modification of enzymatic proteins can lead to their inactivation and consequent metabolic aberrations adversely affecting ATP generation and hence many biological processes. Oxidation of membrane lipids and proteins can alter membrane permeability and cause disturbances in ionic balance inside and outside the cells. All the above changes have the potential to cause cell death [3,4]. Fortunately, the small amounts of ROS generated physiologically are scavenged by endogenous cellular enzymatic and non-enzymatic defense mechanisms. Enzymatic defenses consist of superoxide dismutase which dismutates O₂^{•-} to the less reactive H₂O₂, catalase which catalyzes the conversion of H₂O₂ to H₂O, and glutathione peroxidase which also scavenges H₂O₂. Additional antioxidant enzymes such as thioredoxin reductase, glutathione reductase, etc. also contribute to the neutralization of ROS. The major non-enzymatic defense molecule is the tripeptide glutathione (GSH, λ-glutamylcysteinylglycine), which is present in high concentration in

many tissues, especially in the skin and the eye which are constantly exposed to solar radiation [5,6]. Exposure to UV rays is known to induce a cascade of reactions initiated by endogenous photosensitizers resulting in cyclic and excessive ROS generation [5]. High concentration of GSH in these tissues is nature's own way of protecting them from damage. The above defense systems are considerably effective in preventing oxidative damage to the cells under physiological conditions. However, when ROS generation increases, as with aging, as well as in certain diseases viz. diabetes, or with excessive exposure to ultraviolet (UV) radiation, these defenses become overwhelmed, leaving un-scavenged radicals available to react with cell components [7,8]. In addition, the activities of defense enzymes are known to decrease with aging. Hence the use of exogenous ROS-scavenging compounds can be potentially beneficial in protecting the tissues from such damage and help in preventing age-associated cell damage as well as retarding disease progression [9-13].

ROS-induced damage is one of the known factors involved in the pathogenesis of many ocular diseases, such as cataract, diabetic retinopathy (DR), age-related macular degeneration (AMD), glaucoma etc., causing significant debility due to vision impairment and even blindness [5,14-16]. Most of these conditions are multifactorial, hence effective treatments to prevent/retard their progression are limited. It is desirable to identify newer and more effective pharmacological

*Corresponding author: Kavita Hegde R, Associate Professor, Department of Natural Sciences, Coppin State University, Science and Technology Center-Suite 200, Baltimore, MD 21216, USA, Tel: +410-951-4137; Fax: 410-951-2632; E-mail: khegde@coppin.edu

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compounds that could be effective in preventing oxidative damage to the retina, and can become potentially useful for therapeutic purpose. Caffeine, or 1,3,7 trimethyl xanthine, is an efficient free radical scavenger previously shown to be effective in preventing oxidative damage to the lens and in preventing cataract formation [17]. It is therefore hypothesized that it will be effective in preventing oxidative stress to the neural retina as well, the neural component therein being primarily dependent on oxidative metabolism for its ATP needs. The hypothesis was verified by inducing oxidative stress in neural retina by incubating it with H_2O_2 with and without caffeine supplementation. The extent of biochemical damage was assessed by measuring the level of the major antioxidant glutathione (GSH) in the tissue.

Materials and Methods

Freshly enucleated bovine eyes were obtained from the local abattoir (Ruppersberger, Baltimore). Care was taken to keep the eyes on ice until they were brought to the laboratory. Neural retinas were then dissected out and divided into 3 incubation groups. Control group was incubated in medium 199 (11043-023, Gibco, Grand Island, NY) without any additions. Experimental group was incubated in medium 199 containing 9 mM H_2O_2 (H325, Fisher, Fair Lawn, NJ) as the oxidant, whereas caffeine group was incubated in medium 199 containing 9 mM H_2O_2 and 5 mM caffeine (C0750, Sigma, St. Louis, MO). Incubations were conducted in a humidified incubator maintained at 37°C and 5% CO_2 . Duration of incubation was 4 hours.

The retinas were then removed from the medium and processed for biochemical studies following their homogenization in ice-cold dH_2O . The homogenate was centrifuged at 34000 rpm and the supernatant used for determination of protein and glutathione content.

Protein determination

Water-soluble protein concentration in the supernatant was determined by Bradford's assay using Coomassie blue dye-based reagent (B6916, Sigma, St. Louis, MO). An aliquot of the supernatant was reacted with the dye and the resulting color read spectrophotometrically at 595 nm. Bovine serum albumin standards of known concentration were run simultaneously. The concentration of water-soluble protein in the sample was then calculated by comparing the absorbance of the standards with absorbance of the tissue samples.

Statistical analysis to assess the significance of the data was done using Student's t test and determining the p value.

Glutathione assay

The supernatant obtained as described above was deproteinized with the addition of 100% trichloroacetic acid (TCA) to a final concentration of 5%. The sample was then centrifuged to remove the protein precipitate, and the protein-free supernatant was used for GSH assay.

GSH was determined spectrophotometrically using Ellman's reaction as done previously [4,11]. The assay is based on the reaction of -SH with Ellman's reagent containing 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to produce a yellow-colored product thionitrobenzoate which absorbs at 412 nm. The reagent was prepared by dissolving 4 mg DTNB in 10 ml of 1% trisodium citrate solution. An aliquot of the acidified supernatant prepared as above was mixed with 0.6M Na_2HPO_4 to a neutral pH. Ellman's reagent was then added and the yellow color produced was measured spectrophotometrically at 412 nm. Standards with known concentrations of GSH were run simultaneously.

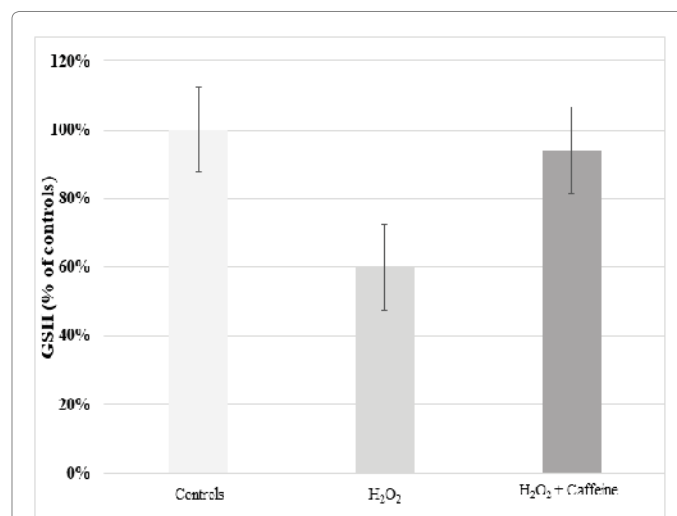


Figure 1: Effect of H_2O_2 (9mM) on retinal glutathione (GSH) levels in the absence and presence of caffeine (5mM); Results are expressed as mean \pm S.D. of $n \geq 10$ in each group. Results of H_2O_2 and H_2O_2 + caffeine groups are expressed as % of controls (No H_2O_2 and caffeine). $p < 0.001$ between controls and H_2O_2 , as well as between H_2O_2 and H_2O_2 + caffeine groups.

Results

The primary purpose of this study was to determine whether caffeine can prevent oxidative damage to the neural retina. Since glutathione is a major antioxidant in the tissue, concentration of this tripeptide was measured in the incubated retinas exposed to ROS in the absence and presence of caffeine.

The level of GSH was calculated as nanomoles/mg protein. Due to the difference in the protein concentration among the individual retinas and the inherent physiological and biochemical variations in the tissue obtained from the abattoir, the final GSH results are expressed relative to the controls, the controls considered as 100%.

As shown in Figure 1, the level of GSH decreased substantially to ~60% of the controls in the tissues incubated with 9mM H_2O_2 . However, such decrease was prevented to a significant extent when the incubation medium contained 5 mM caffeine in addition to 9 mM H_2O_2 . As evident, GSH in the caffeine group was maintained close to the controls. $p < 0.001$ between controls and H_2O_2 , as well as between H_2O_2 and H_2O_2 + caffeine groups.

Caffeine was thus found to exert a significant protective effect against peroxide-induced depletion of GSH. Such an effect of caffeine in the neural retina has been demonstrated for the first time.

Discussion

Ocular diseases such as DR & AMD are known to be multifactorial in origin, oxidative insult being one of the major inducers of tissue damage. Both these diseases are associated with the onset of early (often pre-symptomatic) indications of retinal changes. For example, preclinical signs of DR include decrease in contrast sensitivity with associated electrophysiological abnormalities as determined by electroretinography and visually evoked potentials. These aberrations suggest damage to the neural retina very early in the disease even before the onset of ophthalmoscopically visible microvascular changes characteristic of clinical DR [18-22]. Early AMD is characterized by the appearance of drusen between the retinal pigment epithelium

(RPE) and Bruch's membrane visible ophthalmoscopically in the macula and also often in the peripheral retina. This is called the dry AMD, which can eventually progress to formation of excessive drusen, degeneration of the overlying RPE, degeneration of the photoreceptors which depend on the RPE for their maintenance, and consequent damage to the neural retina, choroidal neovascularization with leaky blood vessels (wet AMD) and scarring of the retina. The associated vision loss is permanent and is usually progressive. The Age-related Eye Disease Study (AREDS) concluded that an antioxidant formulation consisting of ascorbate, beta-carotene, vitamin E, copper, zinc, lutein and zeaxanthin may have some role in retarding progression of dry AMD (atrophic AMD) [23]. However, even early intervention with this treatment has limited benefit which is seen only in a small percentage of patients. This could be attributable to the possible tendency of some of these antioxidants to become pro-oxidant, especially in the presence of trace metals, and also due to the involvement of other factors such as genetic predisposition and smoking in the pathogenesis of this disease. Hence there is a need for the development of newer antioxidants which will not have a pro-oxidant effect under physiological conditions and are effective in preventing oxidative stress to the retina. Such therapies would perhaps be most beneficial if initiated at the pre-clinical stages.

Caffeine has been shown to be an effective scavenger of reactive oxygen, especially OH^\cdot . The rate constants of its reaction with OH^\cdot and $^1\text{O}_2$, as determined by electron spin resonance and pulse radiolysis are ~ 5.9 to $6.9 \times 10^9 \text{ M}^{-1} \text{ S}^{-1}$ and $2.9 \times 10^7 \text{ M}^{-1} \text{ S}^{-1}$, respectively. It also scavenges $\text{O}_2^{\cdot-}$, albeit less efficiently, with a rate constant of $7.5 \times 10 \text{ M}^{-1} \text{ S}^{-1}$ [24,25]. We have previously demonstrated its effectiveness in protecting the lens against UV-damage *in vitro*. It was also effective in preventing cataract formation *in vivo* in the selenite cataract model, as well in galactose-induced cataract in rats [17,26].

Results in the present study demonstrating the effectiveness of caffeine in preventing the decrease in GSH level in the neural retina exposed to peroxide are highly encouraging. Such an effect of caffeine in the neural retina has been shown for the first time. Further studies are in progress to investigate its other possible effects on the neural retina, such as its effect on neural retinal metabolism.

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