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Prevention of Oxidative Stress-induced Metabolic Aberrations in the Neural Retina by Caffeine

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Abstract: Oxyradical-induced damage to the retina has been implicated as one of the contributing factors in the pathogenesis of vision-impairing diseases such as diabetic retinopathy (DR) and age-related macular degeneration (AMD). It is hypothesized that caffeine, a nutraceutical antioxidant, will be effective in preventing metabolic aberrations in the neural retina exposed to oxygen radicals. This hypothesis is based on our previous studies demonstrating its effectiveness in preventing oxidative damage to the lens, and in protecting the neural retina against UV-A- and peroxide-induced biochemical damage. Bovine neural retinas were incubated in medium 199 at 37°C for 6 hours. Xanthine (XA)-xanthine oxidase (XO) were used to generate reactive oxygen species (ROS). Incubations were conducted in 3 groups- control, experimental (with XA+XO), and caffeine group (XA + XO+ 5mM caffeine). Retinas were then processed for determining protein, lactate and pyruvate concentrations. Lactate concentration in the controls was 2.62±0.43mM/mg protein, decreasing to 1.04±0.3 mM/mg protein in the presence of XA+XO. Its level in the caffeine group was significantly higher, 2.44±0.65 mM/mg protein, close to the controls. Pyruvate concentration in the controls was 0.16±0.05mM/mg protein, which declined significantly with XA+XO to 0.066±0.02 mM/mg protein. Such decrease was substantially prevented in the caffeine group, wherein its concentration was 0.156±0.03mM/mg protein. Caffeine was thus found to be highly effective in preventing metabolic aberrations, due to its ability to scavenge oxyradicals and thereby possibly prevent inactivation of key enzymes. Such effect of caffeine in maintaining metabolism of the neural retina exposed to ROS has been shown for the first time.

Keywords: Caffeine, Retinal Metabolism, Lactate, Oxidative Stress

1. Introduction

Excessive generation of reactive oxygen species (ROS) is known to be a significant factor in the pathogenesis of tissue damage found in many ocular diseases such as cataracts, age-related macular degeneration, diabetic retinopathy, glaucoma, etc. [1-4]. These free radicals, viz. superoxide, hydrogen peroxide and the hydroxyl radical, readily react with cellular macromolecules such as proteins, lipids and nucleic acids, inducing a variety of structural and functional aberrations in the cell. Oxidation of proteins can induce permanent modifications of their conformation causing aberrations in cellular architecture in case of structural proteins, and disruption in metabolic and other cellular activities in case of enzymatic proteins. Oxidative damage to cellular lipids, especially the membrane lipids, is known to alter membrane permeability with serious consequences on ionic composition of the cytosol, swelling of the cell, and eventual cell death. Membrane proteins can get similarly affected with disruption of ion channels and transporters, with consequent ion imbalance and cell death. Oxidative modification of nucleic acid bases are known to induce aberrations in DNA replication as well as transcription, with eventual synthesis of abnormal proteins leading to cellular dysfunction [5].

It is known that ROS are produced even physiologically, albeit in minor quantities. It has been shown that~1-3% of the respired oxygen gets converted to its partially reduced entities that leak out from the mitochondria [5, 6]. These are prevented from inducing cell damage due to presence of enzymatic free radical scavengers such as superoxide dismutase, catalase, glutathione peroxidase, etc. and the primary non-enzymatic antioxidant glutathione. However,

when such radicals are produced in excessive amounts, the above antioxidant mechanisms become overwhelmed, leaving the radicals un-scavenged and free to react with cellular molecules. ROS are known to be generated in increasing amounts as a function of age, as well as in certain diseases such as diabetes. Furthermore, exposure to solar radiation, particularly the ultraviolet wavelengths, combined with high oxygen consumption, and high content of polyunsaturated fatty acid in photoreceptors, increases vulnerability of the retina to oxidative stress [7, 8]. Hence supplementation with exogenous antioxidants has been considered to be a possible way to neutralize the excessive ROS and prevent oxidative stress. In this regard, several compounds viz. ascorbate, vitamin E, pyruvate, have been demonstrated to be effective antioxidants, preventing oxidative damage to the ocular lens in vitro as well as in vivo, with potential protective effect on other ocular tissues as well [8-11].

Retinal neurons depend primarily on oxidative metabolism to fulfil their ATP requirements. Müller cell, the major macroglial cell of the retina, is known to be particularly important for optimal function and metabolism of the neurons. Studies have shown that Müller cells primarily metabolize glucose to produce lactate which is shuttled extracellularly and taken up by photoreceptors and other retinal neurons wherein it is oxidized to pyruvate-a source of acetyl CoA for Krebs cycle, with eventual oxidative phosphorylation producing ATP [12].

Müller cells actively take up extracellular glutamate and λ-amino butyric acid (GABA) released from photoreceptors and during neuronal excitation, thereby protecting the neurons from neurotransmitter excitotoxicity. Glutamine synthetase, expressed almost exclusively in the glial cells, converts glutamate to glutamine which is then released for uptake by photoreceptors and neurons as a precursor for re-synthesis of glutamate [13]. Glutamate in Müller cells is also used for synthesis of the antioxidant glutathione (GSH) [14]. During oxidative stress, this glutathione is released by the Müller cells and is taken up by neurons where it reduces H_2O_2 to water, the reaction being catalyzed by the selenium-containing enzyme glutathione peroxidase.

We have recently shown that caffeine, a compound present in widely-consumed beverages, is highly effective in preventing biochemical damage to the neural retina exposed to H_2O_2. The decrease in GSH content of the neuroretina incubated with H_2O_2 was significantly prevented by caffeine supplementation, maintaining the level of this important antioxidant close to control levels [15].

In view of the above findings, we considered it interesting to determine whether it has a beneficial effect on retinal metabolism as well. The primary objective of the current study, therefore, was to investigate the effect of oxidative stress on the metabolic status of the neuroretina, specifically of glycolysis, reflected by the production of lactate and pyruvate, and to examine the effect of caffeine therein.

2. Materials and Methods

2.1. Incubation Conditions

Freshly enucleated bovine eyes were obtained from a local abattoir. Eyes were kept on ice until they were brought to the lab, total time not exceeding 10 minutes. Neural retina was then dissected out and incubated in medium 199 (Gibco 11043-023, Grand Island, NY) in a humidified incubator gassed with 5% CO_2 and maintained at 37°C. ROS were generated by the addition of xanthine (XA) and xanthine oxidase (XO) to the medium.

2.2. Incubation Groups

Retinas in the control group were incubated in medium 199 containing 0.5mM sodium xanthine (Sigma X3627, St. Louis, MO), whereas retinas in the Experimental group were incubated in the above medium with the addition of 10µl XO (Sigma X2252, St. Louis, MO). Medium in the caffeine group contained 5mM caffeine (Sigma C0750, St. Louis, MO) in addition to XA & XO. XO was added to the experimental and caffeine groups after 10 minutes of pre-incubation of the tissue in their respective medium. A control group with the addition of caffeine to a concentration of 5mM was also included to determine whether caffeine has any effect on retina metabolism without the generation of free radicals by XA+XO in the medium. The duration of incubation was 6 hours, after which the tissues were processed for biochemical assays. The retinas were homogenized in their own medium with a mechanical homogenizer. The homogenate was centrifuged at 4°C for 10 minutes at 34,000 rpm. The supernatant was used for measuring the contents of protein, pyruvate and lactate.

2.3. Protein Assay

The amount of water-soluble protein in the supernatant was measured by Bradford's assay based on protein-binding by Coomassie Blue dye-based reagent (Sigma B6916, St. Louis, MO) and determining the spectrophotometric absorbance of the dye-bound protein at 595nm. The amount of protein in the sample was calculated in reference to bovine serum albumin standards run simultaneously.

2.4. Lactate Assay

Lactate was determined enzymatically using the lactate dehydrogenase (LDH)-catalyzed oxidation of lactate to pyruvate with simultaneous reduction of its cofactor NAD^+ to NADH [11, 16]. Briefly, the assay was done as follows: 10µl aliquots of the supernatant, obtained as described above, were added to 500µl of a reagent mixture containing NAD (0.09 mM) and lactate dehydrogenase (0.1U/ µl) (Sigma 10127876001, St. Louis MO) in glycine (1M)-hydrazine (0.4M) pH 9.5 buffer, in quartz cuvettes. A reagent blank was run simultaneously using the unincubated medium. Reaction was carried out at 37°C and absorbance recorded for approximately 15 minutes to ensure the completion of the reaction. The increase in absorbance/optical density (OD) at

340nm due to formation of NADH during the reaction, which stoichiometrically reflects the amount of lactate oxidized to pyruvate, was recorded. The change in absorbance and the extinction coefficient of NADH at λ_{340nm} ($6.22\times10^{3}M^{-1}$ cm^{-1}) were then used to calculate the concentration of lactate in the sample using Beer Lambert law.

2.5. Pyruvate Assay

Pyruvate concentration was also determined enzymatically by LDH and monitoring spectrophotometrically the decrease in absorbance at 340 nm due to the NADH-dependent reduction of pyruvate to lactate by LDH [11]. The assay was done at 37°C using quartz cuvettes. A reagent mixture consisting of 0.09 mM NADH and LDH (0.1Units/ µl) in 0.05M phosphate buffer, pH 7.4 was prepared. The initial absorbance (OD_1) was noted. An aliquot of the supernatant was then added to the reaction mixture and OD_2 was read after 15 min. A reagent blank was run in parallel using un-incubated medium. Pyruvate concentration, represented by the difference in absorption (OD_1–OD_2), was calculated with reference to the extinction coefficient of NADH using Beer Lambert equation.

2.6. Statistical Analysis

Significance of the data was assessed using student's t test and determining the *p* value between control and experimental groups, and between experimental and caffeine groups, for both lactate as well as pyruvate concentrations; n≥10 in each group.

3. Results

3.1. Lactate Levels

Exposure of the neural retina to an oxyradical-generating medium resulted in a substantial inhibition of glycolysis, reflected in the lactate and pyruvate levels. As shown in figure 1, the concentration of lactate in the control group was 2.62 ± 0.44 mM/mg protein. The experimental group incubated in medium containing XA+XO exhibited a significant decrease in lactate levels to 1.04 ± 0.3 mM/mg protein. Supplementation of the medium with 5mM caffeine was highly effective in preventing this decrease, the lactate content in this group being 2.44 ± 0.66 mM/mg protein.

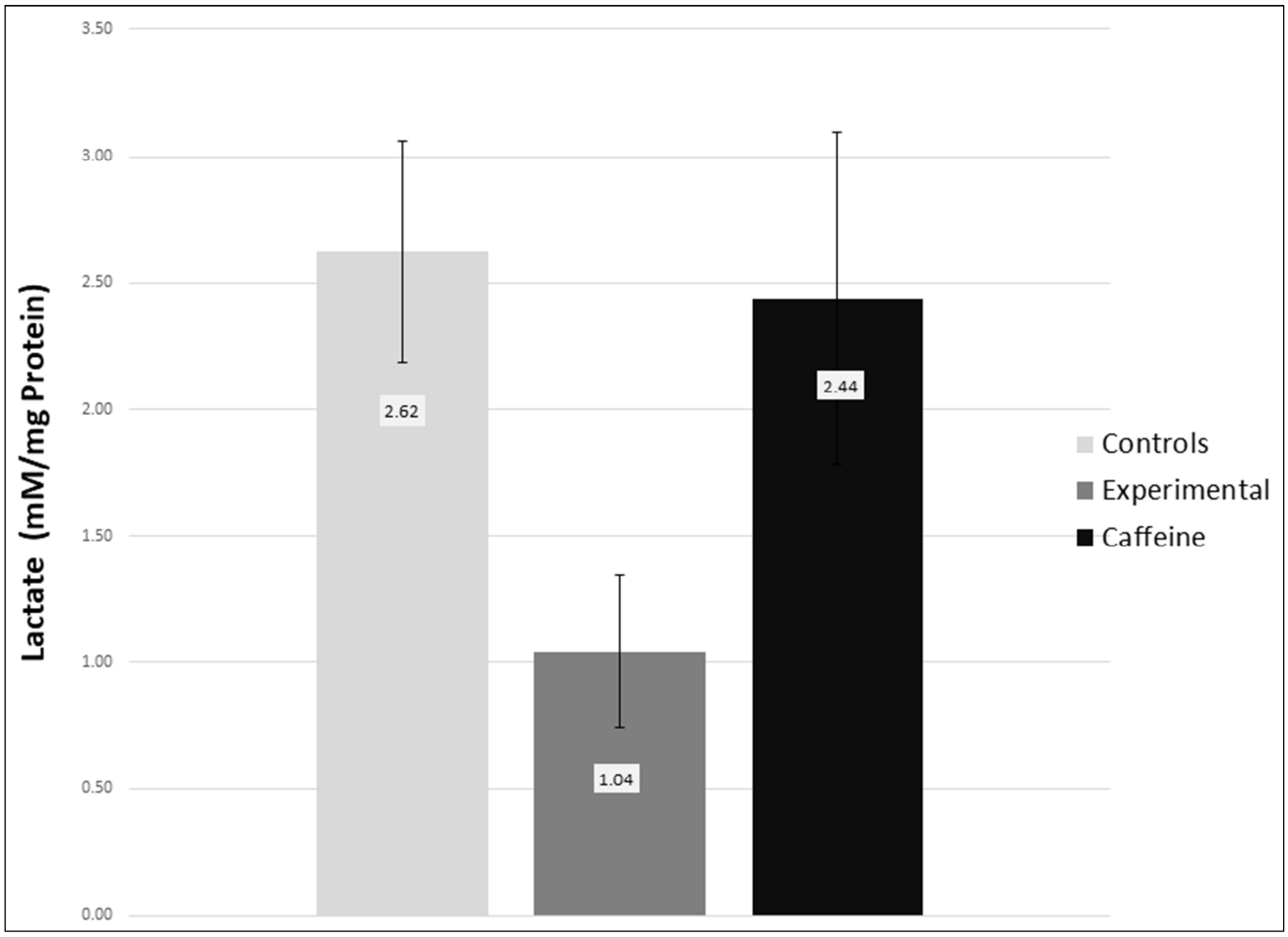

Figure 1. *Lactate Concentration (mM/mg Protein).*

Figure 1: Lactate concentration of the neural retina exposed to ROS in the absence and presence of caffeine (5mM). Control group was incubated without ROS. A highly significant decrease in lactate induced by ROS was also completely prevented by caffeine. N≥10 in each group; p<0.001 between control and experimental groups, as well as between experimental and caffeine groups.

3.2. Pyruvate Levels

Pyruvate levels demonstrated a similar trend, as shown in figure 2. Its concentration in the control group was 0.16 ± 0.06 mM/mg protein. Incubation with XA+XO induced a substantial decrease to 0.066 ± 0.02mM/mg protein. Addition of caffeine to the medium maintained the pyruvate concentration closer to the controls, the level in the caffeine group being 0.156 ± 0.035 mM/mg protein.

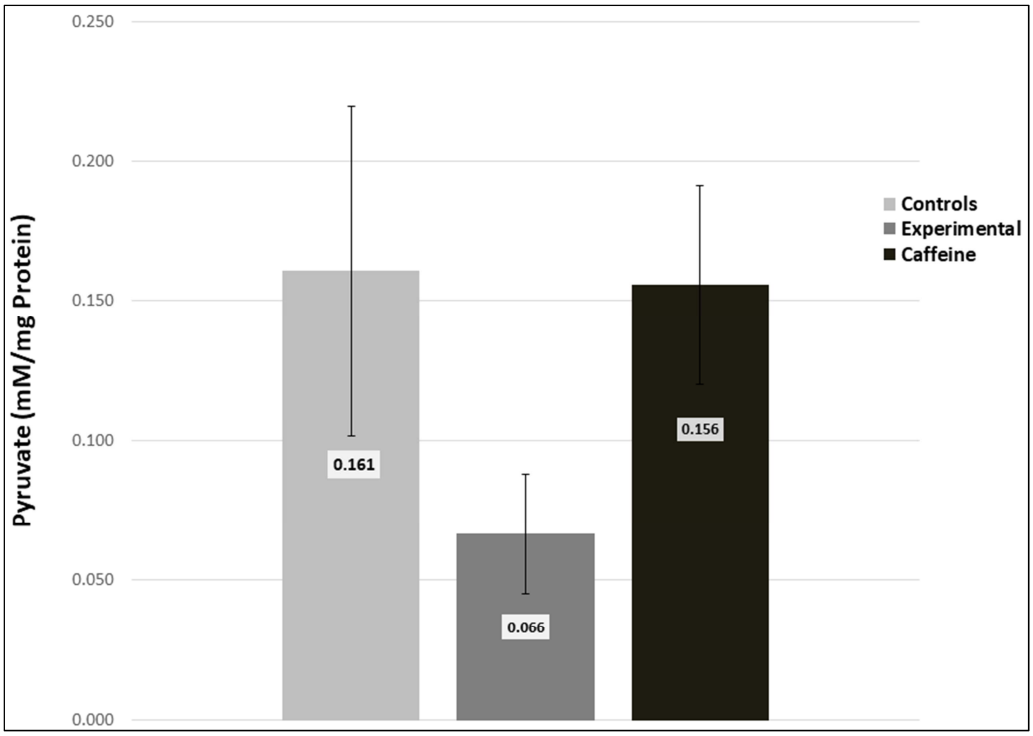

Figure 2. *Pyruvate Concentration (mM/mg Protein).*

Figure 2: Pyruvate concentration of the neural retina incubated with ROS ± 5mM caffeine. ROS induced a substantial decline in pyruvate levels, which was significantly prevented by caffeine supplementation. N≥10 in each group; p<0.001 between control and experimental groups, and between experimental and caffeine groups.

Lactate and pyruvate contents of the neural retinas incubated in the control medium (medium 199 + XA, without XO) with addition of 5mM caffeine, was similar to their contents in the control group incubated without caffeine. *p* value was <0.001 between control and experimental groups, and between experimental and caffeine groups, for both lactate as well as pyruvate concentrations.

4. Discussion

We have recently shown that caffeine protects the neural retina against peroxide-induced depletion of glutathione, a major antioxidant [15]. This effect of caffeine is due to its ability to scavenge various reactive oxygen species, especially the OH•, with high rate constants. The present study further demonstrates the beneficial effect of caffeine in the neural retina, in this case with regard to retinal metabolism. As described in "Results", caffeine was significantly effective in maintaining the levels of the end-products of glycolysis-lactate and pyruvate in neural retina incubated in a medium ROS. The level of lactate in the experimental group incubated with ROS decreased to~40% of the controls. However, retinas incubated with ROS + caffeine maintained the lactate level significantly higher, at~93% of the control value. The concentration of pyruvate in retinas incubated with ROS dropped to 41% of the controls, while it was maintained at~97% of the controls in the ROS + caffeine group.

The results clearly indicate that the ROS-generating medium induces significant metabolic aberrations in the retina which are prevented by caffeine, supporting our previously reported results demonstrating the protective effect of caffeine against ultraviolet-A radiation induced damage to retinal morphology [17], as well as our recently published report on maintenance of GSH content [15].

Since the major source of pyruvate and lactate in the cell is glycolysis, it is possible that ROS may modulate the activity of glycolytic enzymes, through possible oxidative inactivation of–SH at the active site of certain key glycolytic enzymes, especially the NAD^+-dependent glyceraldehyde-3-phopshate dehydrogenase (GAPD) [18]. Pyruvate kinase, which catalyzes the conversion of phosphoenolpyruvate to pyruvate, is also vulnerable to oxidative deactivation [19]. Whether caffeine does indeed prevent enzymatic inactivation, remains to be seen and is a subject of further investigations.

It is known that the retina consumes higher amount of ATP

compared to other tissues, and demonstrates the Warburg effect, wherein glucose is preferentially converted to lactate, instead of CO_2 and H_2O through oxidative phosphorylation. The Warburg effect enables rapid and significant amount of ATP production required to drive various biosynthetic and membrane transport processes. Breakdown of glucose to pyruvate, which is then reduced to lactate by NAD+-dependent LDH, enables regeneration of NAD^+ required for the GAPD reaction, thereby allowing glycolytic pathway to continue. As mentioned above, lactate generated through glycolysis in Müller cells is actively transported extracellularly, to be taken up by the neurons and RPE for oxidative metabolism. Such metabolic coupling and inter-dependence between glial cells and the photoreceptors-neurons suggests that glial cell dysfunction can directly impact the photoreceptor and neuron signaling functions [20]. Overall, our recent data clearly demonstrate the protective effect of caffeine in maintaining glutathione concentrations as well as in supporting retinal metabolism when the tissue was exposed to ROS.

5. Conclusions

As hypothesized, caffeine was found to be effective in maintaining retinal metabolism when the tissue was exposed to ROS. Current results, along with those previously published with regard to maintenance of level of the endogenous antioxidant glutathione in the ROS-exposed neural retina, are indicative of the efficacy of caffeine in maintaining the important biochemical and metabolic parameters of this tissue. The beneficial effect is attributable to its ROS-scavenging properties. Other possible mechanisms of action are currently being investigated.

Author Contributions

KRH has conceptualized the experiments and prepared the manuscript with significant contributions from the co-author. KD has conducted most experiments and has provided useful feedback during manuscript preparation.

Conflict of Interest Statement

Authors have no conflict of interest to declare.

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