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The Antioxidant Cocktail, Effective Microorganism X (EM-X), Protects Retinal Neurons in Rats Against *N*-methyl-D-aspartate Excitotoxicity *In Vivo*

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Injection of the glutamate agonist *N*-methyl-D-aspartate (NMDA) into the vitreous body of rats resulted in severe degeneration of neurons in the retina, with a loss of 81% of ganglion cells and 43% of non-ganglion cells. The cocktail EM-X is a novel antioxidant drink derived from ferment of unpolished rice, papaya and sea-weeds with effective microorganisms (EM-X). In animals treated with an intraperitoneal injection of EM-X, the loss of ganglion cells was reduced to 55% and that of non-ganglion cells to 34% when compared to untreated NMDA-injected retinas. Cell degeneration resulting from NMDA excitotoxicity, is thought to be mediated via oxidative stress mechanisms. The neuroprotective effect of the EM-X in this system is therefore likely to be due, at least in part, to its flavonoids, saponins, vitamin E and ascorbic content.

Keywords: EM-X; *N*-methyl-D-aspartate; Retinal ganglion cells; Alzheimer's disease; Flavonoids; Antioxidants

INTRODUCTION

The nervous system is particularly vulnerable to reactive oxygen species (ROS) and nitrogen species (RNS) primarily as a consequence of high metabolic rate and deficient antioxidant defenses. Mechanisms involving oxidative stress and deregulation of glutamate metabolism in the pathology of a variety of chronic neuroinflammatory conditions including Alzheimer's disease, Down syndrome, multiple sclerosis, hypoxia reperfusion, trauma, epilepsy, tardive dyskinesia, etc are widely discussed in the literature.[1–8] The *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor has many important roles in normal brain function, including synaptic transmission, learning and memory and neuronal development.[9] The dysfunctions in glutamate metabolism and the associated injury to neurons in the CNS may be caused by the over-stimulation of receptors for excitatory amino acids including glutamate and aspartate.[1,2,5–10] Although increased free radical production results from the over-stimulation of the NMDA glutamate receptor subtype,[11–15] the molecular mechanisms associated with this release and antioxidant protection as well as how they are related to the neuropharmacology of glutamatergic synaptic transmission still remain unclear.

The mechanism of antioxidant action *in vivo* may involve direct inhibition of the generation of ROS, or the scavenging of free radicals. It is important to identify the role of ROS and the factors that may regulate and/or combat ROS mediated tissue damage.[16–18] In order to establish neuroprotective properties of antioxidants, it is prudent to measure "markers" of oxidative damage in the central nervous system and examine how they are affected

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by changes in diet or by antioxidant supplements. One such marker is neuronal loss itself. It has been shown that the injection of aggregated amyloid peptides (Aβ) into the vitreal body of a rat results in severe degeneration of neurons in the retina, which can be prevented by co-treatment with intravitreal injection of the antioxidant vitamin E.[19] Thisz supports the concept that oxidative stress *in vivo* plays a vital role in causing degeneration of neurons in the CNS. The mechanisms of neurotoxicity associated with Aβ deposition and the role of oxidative stress in the pathogenesis of Alzheimer's disease is widely discussed in the literature.[3–5,20] Excitotoxicity can be triggered by high levels of glutamate, which stimulate the entry of calcium into the cytoplasm of cells at concentrations that can activate oxidative enzymes such as phospholipase A_2 and xanthine oxidase, deplete cells of cysteine and glutathione, cause mitochondrial release of free radicals and cell death characterized by a mixture of apoptosis and necrosis.[1,2,21,22]

The mammalian retina is an integral part of the central nervous system. It is peripherally located and therefore highly accessible for experimental manipulation. The retina has biochemically and structurally well defined glial and neuronal populations and the closed nature of the system means that injected compounds remain in the area of interest for much longer than they might in other areas of the CNS.[19,23,24] The degeneration of retinal neurons is accompanied by dysfunction of the glial cells and this has been shown in rodents receiving intravitreal injection of Aβ,[19] and in the retinas of patients with Alzheimer's disease.[25,26] Thus the retinal model makes it possible to study the interaction between glial cells and neurons *in vivo*.

The novel antioxidant drink EM-X is derived from ferment of unpolished rice, papaya and sea-weeds with effective microorganisms (EM) selected from actobacillaceae, saccharomycetes, funguses, actinomyces and photosynthetic bacteria.[27] EM-X contains over 40 minerals, α-tocopherol, lycopene, ubiquinone, ascorbic acid, saponin and flavonoids, such as quercetin, quercetin-3-*O*-glucopyranoside and quercetin-3-*O*-rhamnopyranoside (Fig. 1). EM-X is widely available in South East Asia as a beverage and is widely accepted in clinical practice in the region. EM-X increases the serum levels of superoxide dismutase, modulates immunological functions in animals, decreases malondialdehyde (MDA) levels in D-galactose-induced aging in mice and prolongs survival time of mice at high temperatures as well as under hypoxic conditions.[27] High concentrations of glutamate are known to kill neurons in the retina.[2,28] We investigated the effect of chronic administration of EM-X in protecting against the loss of neurons in the retina caused by the glutamate agonist NMDA.

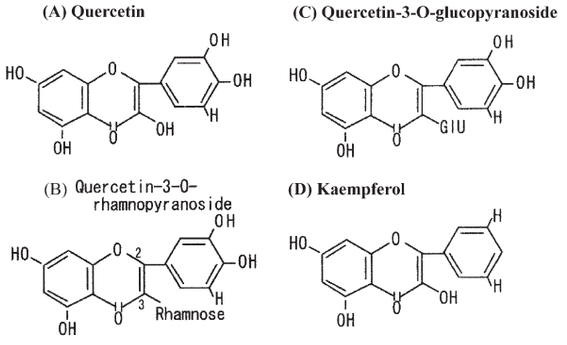

FIGURE 1 Phenolic structures in EM-X.

MATERIALS AND METHODS

Unless otherwise stated, all of the biochemical reagents used in this study were purchased from Sigma–Aldrich (Poole, UK). EM-X was obtained from EM Research Organization, Okinawa, Japan.

Animal and Treatments

Young adult female Sprague–Dawley rats were used in all experiments. The animals were supplied by Harlan, England. Animal procedures used were in accordance with regulations of the Home Office, UK. The animals were divided into three groups. The first group consisted of six normal rats that received no treatment. Another nine animals were anesthetized with Hypnorm (0.2 mg of fentanyl citrate and 0.54 mg fluanisone/100 g body weight) and Hypnovel (0.27 mg midazolam/100 g body weight) before they received unilateral intravitreal injection of 5 μl of 4 mM NMDA to the vitreous body of the left eyes, with the un-injected right eyes serving as controls. The injection of NMDA was performed using a Hamilton syringe equipped with a 10μl needle. The site of the injection is at the upper temporal corneo–scleral junction. As the rats were albino with little or no pigment behind the retinal tissue, it was possible to introduce and guide the tip of the needle to the center of the vitreous body under visual guidance with a Zeiss operation microscope. After perfusion, the retinas were dissected under the microscope. All cases included in this study showed no sign of detachment or physical distortion as retinal detachment itself can result in the loss of the retinal cells. Six of the animals received additional intraperitoneal injection of 0.2 ml of EM-X, or phosphate buffer saline (PBS) as vehicle control 24 h and half an hour before injection of NMDA. Further intraperitoneal injection of EM-X, or PBS was performed at 1, 24, 48 and 72 h time points, and three injections per week for another three weeks. At the end of the sixth week

after injection of NMDA, all animals were anaesthetized deeply again and perfused with physiological saline followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). The eyeballs were collected in the same fixative and post-fixed for another half an hour before the retinas were dissected out in PBS. For each retina, four radial cuts were made before the retinas were flatly mounted onto gelatin-coated slides, and air-dried slowly in a moist chamber for 2–3 days. The retinal wholemounts were then stained for cresyl violet and cover slipped.

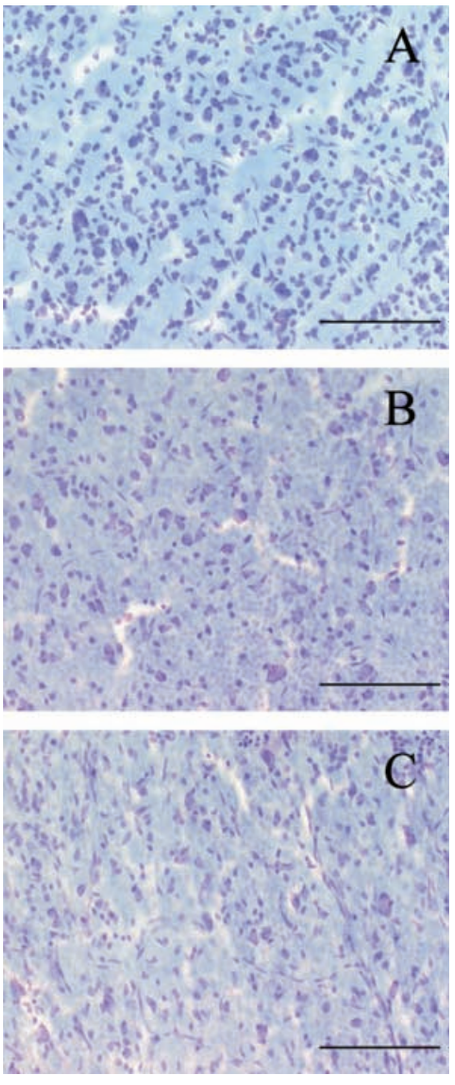

FIGURE 2 Photomicrographs showing cells in the retinal ganglion cell layer in cresyl viloet-stained retinal wholemounts from animals that received unilateral intravitreal injection of NMDA solution to the left eyes and intraperitoneal injection of the antioxidant EM-X (A,B) or PBS (C, i.e. NMDA alone). A and B are the right (A) and left (B) retinas from a rat treated with EM-X, and C is the left retina from a rat treated with PBS. Note a significant loss of neurons in the retinal ganglion cell layer in B and C, and that the retina is less healthy in C as compared with B. Scale bar: 100 μm.

Analysis was performed under a Wild microscope equipped with a drawing tube. The number of retinal neurons in the retinal ganglion cell layer was counted and cell sizes measured under the microscope at a magnification of 300 × and in an area of 150 × 150 μm in the central, intermediate and peripheral parts of the four retinal quadrants. The neurons counted were divided into two groups: those cells with somata smaller than 6 μm and those with somata equal to or larger than 6 μm in diameter. The great majority of neurons larger than 6 μm are retinal ganglion cells, which are the projection neurons in the retina and neurons with smaller somata are primarily nonganglion cells or displaced amacrine cells.[25,26,29] The numbers of cells with somata smaller or larger than 6 μm were counted in a total of 12 fields of individual retinas, and compared.

Statistical Analysis

The data are expressed as mean ± SEM. Differences between values were compared by a one-way ANOVA.

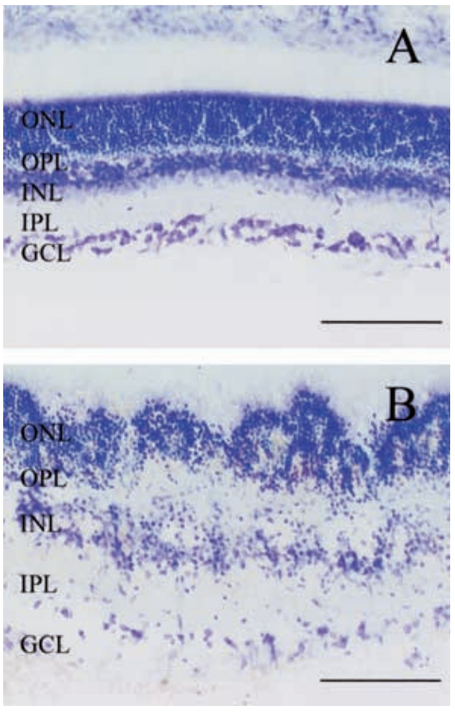

FIGURE 3 Photomicrographs showing the left and right retinas from a rat that received intravitreal injection of NMDA to the left eye. Note that the NMDA-injected left retina (B) is highly degenerated as compared with the uninjected right retina (A). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Scale: 100 μm.

RESULTS

The rat retinas injected with NMDA were morphologically different from the un-injected control and normal retinas, as indicated by an apparent reduction in the density and sizes of neurons as well as an alteration in the vascular patterns in wholemounted preparations (Fig. 2). The retinal tissue, as revealed in sections stained for cresyl violet was distorted and degenerative in histological sections and in some cases was necrotic in appearance (Fig. 3).

In animals that received intravitreal injection of NMDA and who were treated with PBS, there was a 58% reduction in entire population of cells. The cell loss for the ganglion cell population was 81% and for the non-ganglion cells 43% (Figs. 3 and 4). In contrast, there was a loss of 15% of ganglion cells and 8% of non-ganglion cells in the un-injected retinas (Fig. 4). In animals treated with EM-X, a loss of 55% of ganglion cells and 34% of non-ganglion cells was seen in the NMDA injected retinas. The un-injected control eyes from these animals showed a loss of 10 and 5% in these populations. On average, the loss of the entire retinal neuron population was reduced to 42% as a consequence of EM-X treatment.

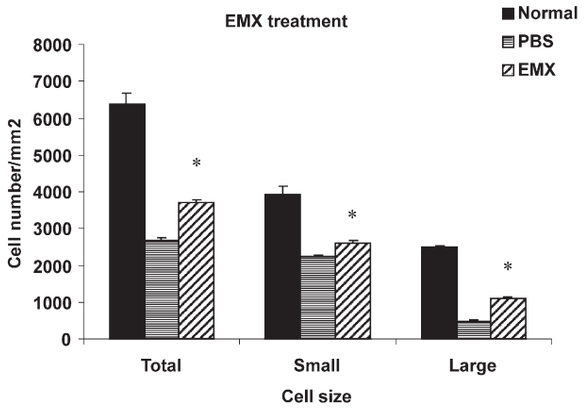

FIGURE 4 The neurons counted were divided into two groups with somata smaller than 6 μm, or equal to or larger than 6 μm in diameter. The great majority of neurons larger than 6 μm are retinal ganglion cells which are the projection neurons in the retina and are glutamatergic. Neurons with smaller somata are primarily non-ganglion cells or displaced amarcine cells. The effect of NMDA treatment is shown by the horizontal bar and the protection by EM-X shown in the diagonal bar (*$p < 0.001$ as compared to PBS).

DISCUSSION

The retinal–vitreal system is a useful experimental model for studying neurotoxicity and the underlying mechanisms. This is important for the development of potentially useful therapeutic strategies based on dietary antioxidants for the treatment and management of neurodegenerative diseases characterized by overt inflammation.

Although a variety of neuronal and glial markers for studying nerve cells in the retina has been described in the literature, e.g. Ref. [30–35], the labeling methods are not able to label the entire population of cells concerned. Retinal wholemounts stained for creysl violet, on the other hand, not only reveal the entire population of cells in the ganglion cell layer,[25,26,36,37] but also allow accurate assessment of retinal neurons in the ganglion cell layer on the basis of cell density and sizes, which in turn is essential for resolving the issue of whether there is actual loss and/or atrophy of nerve cells over time. For this reason, cell count and size measurement were performed in retinal wholemounts 6 weeks after injection of NMDA. In normal retinas, the total average cell density is 6394 cells/mm^2 a value comparable with previous reports.[29,36] Of these, 61% are non-ganglion cells and 38.8% are considered as ganglion cells on the basis of their somal diameter. Neurons with small somata are known as displaced amacrine cells.[29]

NMDA is excitotoxic to neurons in the central nervous system. The results confirm the neurotoxic effect of NMDA on retinal neurons in the ganglion cell layer and provide evidence of an *in vivo* effect of NMDA in causing significant degeneration and loss of both ganglion and displaced amacrine cells which are known to receive excitatory input or express NMDA receptors and are therefore susceptible to excitotoxicity.[39,40–44] The cytotoxic effect of NMDA on retinal neurons and glial cells was confirmed by the observation in previous studies that there is a severe loss of immunoreactivity to amyloid precursor protein and glial fibrillary acidic protein in ganglion cells and astrocytes, respectively.[37]

We show that the antioxidant beverage EM-X can prevent the degeneration and loss of both ganglion and non-ganglion cell populations in the retina caused by NMDA. The observed reduction of small cell populations with somal diameter (less than 6 μm) in addition to a reduction of the larger ganglion cells 6 weeks after intravitreal injection of NMDA indicates that there is actual loss of the neuronal ganglion cells. This is in line with previous observations.[40,42] The loss of non-ganglion cells or displaced amacrine cells was lower suggesting that these cells are less vulnerable to glutamate excitotoxicty, presumably due to a lack or relatively low density of the NMDA receptor expressed by these cells. The exact molecular entities responsible for the antioxidant action is not clear (Fig. 1) but the flavonoid and other antioxidant molecules, ascorbic acid, saponins, vitamin E may have contributed. EM-X also modulated the slight reduction/degeneration in cell density of the total neuronal population in the un-injected retina, suggesting that this non-specific and presumably,

systemic effect of unilateral injection of neurotoxic chemical compounds can be modulated by antioxidants.

Retinal glial cells play an important role in maintaining the normal function and survival of retinal neurons, and there is evidence in culture studies that dysfunction of these cells could be a precipitating factor of chronic neuronal degeneration after intravitreal injection of a number of neurotoxic chemical compounds.[23,34,38,45] Glutamate in the extracellular space is taken up mainly via specific high affinity plasma membrane transporters among which the GLT-1 found in neurons and astrocytes are regulated by Aβ and ROS.[46,47] The NMDA receptor subtype is widespread among retinal cell types[4,48] and thus represents the primary receptor involved in glutamate excitotoxicity. Glial dysfunction may also be causative for chronic neuronal degeneration after intravitreal injection of NMDA in this study. Aβ-peptides can induce both the microglial activation and TNF-α release,[45] which are causative factors in neuronal injury. The stimulation of the NMDA glutamate sub-receptor leads to activation of several oxidative enzymes including xanthine oxidase and phospholipase-A_2[1,49,50] whose actions would increase oxidative burden *in vivo*. Severe neuronal loss is common to many neurodegenerative diseases characterized by neuroinflammation, which may be precipitated by necrosis or apoptosis. Whilst both mechanisms are likely, their contribution to disease progression remains unclear.[50,51] Nevertheless, NMDA can induce both necrosis and apoptosis in neuronal cells depending on concentrations.[22,52–55] Understanding the molecular mechanisms of neurotoxin associated free radical damage to neurons and glial cells is critical for the development of neuroprotective strategies based on antioxidants. Extracts of Gingko biloba leaves are consumed as dietary supplements to counteract chronic and age related neurologic disorders and it has been shown by Watanabe *et al.*[56] to possess neuromodulatory effects. The anti-inflammatory drug ebselen has continued to receive attention as a neuromodulator and this may be in part, due to its actions as a modulator of NMDA receptor redox modulatory sites.[12] However, the ability of ebselen to block the quinolic acid induced production of thiobarbituric acid reactive species[14] intrinsically point to ebselen acting on post receptor events. Calzada *et al.*[57] have shown that the calcium channel blocker dextromethorphan attenuates the glutamate mediated excitotoxicity secondary to laser induced cell death in the retinal ganglion cell layer. Indeed, the difficulties that need to be overcome in assessing *in vivo* protection of plant extracts include the delineation of the molecular entity and concentrations of the bioactive compounds responsible for the observed effects. Whilst the regular strength EM-X used in this study was neuroprotective, it is anticipated that use of optimized concentrations of the bioactive flavonoids, saponins, vitamin E and ascorbic acid in EM-X could enable greater understanding of the molecular mechanisms of neuroprotection *in vivo*. It is particularly intriguing that ginseng total saponins have been reported to modulate NMDA receptor-mediated signals,[58] by inhibiting the influx of Ca^{2+} in cultured and isolated hippocampal cells. However, that EM-X protect neurons from NMDA excitotoxicity in the retinal–vitreal *in vivo* model described in this paper calls for the application of this model in targeted research to delineate the associated molecular mechanisms of antioxidant protection and establishment of how this relates to other receptor–agonist–antagonist relationships and the neuropharmacology of glutamatergic synaptic transmission.

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