Université de Montréal

CYANOCOBALAMIN IS A SUPEROXIDE SCAVENGER AND NEUROPROTECTANT IN NEURONAL CELLS

LA CYANOCOBALAMINE PEUT CAPTER L'ANION SUPEROXYDE ET AGIR COMME NEUROPROTECTEUR DANS LES CELLULES NEURONALES

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ABSTRACT

Damage to the optic nerve (optic neuropathy) can result in permanent loss of vision or blindness through retinal ganglion cell (RGC) death. Our prior work identified a burst of superoxide anion as a critical molecular event in RGCs prior to injury-induced cell death. Recently, Suarez-Moreira *et al* (JACS 131:15078, 2009) demonstrated that vitamin B_{12} scavenges superoxide as effectively as superoxide dismutase. Vitamin B_{12} deficiency can lead to optic neuropathy through unknown mechanisms. We investigated the relationship between superoxide scavenging by cyanocobalamin, the most abundant vitamin B_{12} vitamer, and its neuroprotective properties in neuronal cells.

Cyanocobalamin at concentrations of 10 μM and 100 μM reduced the rate of superoxide generation by 34% and 79% in cell free assays, respectively. In menadione-treated RGC-5 cells, cyanocobalamin concentrations above 10 nM scavenged superoxide anion similar to those treated with pegylated-SOD. Cyanocobalamin at concentrations of 100 μM and 1 mM reduced RGC-5 cell death from menadione by 20% and 32%, respectively. In rats with unilateral optic nerve transection, a single intravitreal dose of 667 μM cyanocobalamin significantly reduced the number of RGCs with superoxide. This dose also increased RGC survival rate compared to rats injected with saline control.

These data suggest that vitamin B_{12} may be an important neuroprotectant, which could cause death of RGCs when depleted in nutritional deficiency. Vitamin B_{12} could also potentially be used as a therapy to slow progression of RGC death in patients with optic neuropathies characterized by overproduction of superoxide.

Key Words: Vitamin B_{12}, superoxide anion, optic neuropathy, neuroprotection

RÉSUMÉ

Les dommages au nerf optique (neuropathie optique) peuvent entraîner la perte permanente de la vision ou la cécité causée par la mort des cellules ganglionnaires de la rétine (CGR). Nous avons identifié qu'une surproduction de l'anion superoxyde constitue un événement moléculaire critique précédant la mort cellulaire induite par des lésions. Récemment, Suarez-Moreira *et al* (JACS 131:15078, 2009) ont démontré que la vitamine B_{12} peut capter l'anion superoxyde aussi efficacement que l'enzyme superoxyde dismutase. La carence en vitamine B_{12} peut conduire à une neuropathie optique causée par des mécanismes inconnus. Nous avons étudié la relation entre la captation de superoxyde par la cyanocobalamine (forme de vitamine B_{12} la plus abondante) et ses propriétés neuroprotectrices dans les cellules neuronales.

La cyanocobalamine aux concentrations de 10 μM et 100 μM a réduit le taux de production de superoxyde respectivement par 34% et 79% dans les essais sans-cellule. Dans les cellules RGC-5 traités avec la ménadione, les concentrations de cyanocobalamine supérieures à 10 nM ont diminué l'anion superoxyde à des valeurs similaires à celles traitées par PEG-SOD. La cyanocobalamine aux concentrations de 100 μM et 1 μM a réduit la mort des cellules RGC-5 exposées à la ménadione par 20% et 32%, respectivement. Chez les rats avec section du nerf optique unilatérale, une dose intravitréenne de 667 μM de cyanocobalamine a réduit le nombre de CGRs exposées au superoxyde. Cette dose a également augmenté le taux de survie des CGRs comparativement aux rats injectés avec la solution témoin. Ces données suggèrent que la vitamine B_{12}

peut être un neuroprotecteur important, et sa carence nutritionnelle pourrait causer la mort de CGRs. La vitamine B_{12} pourrait aussi potentiellement être utilisée comme une thérapie pour ralentir la progression de la mort CGR chez les patients avec les neuropathies optiques caractérisés par une surproduction de superoxyde.

Mots Clés : La vitamine B_{12}, l'anion superoxyde, neuropathie optique, la neuroprotection

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LIST OF ABBREVIATIONS

4-Di-10-Asp	4-(4-(didecylamino)styryl)-N-methylpyridinium iodide
AdoCbl	5'-deoxydenosylcobalamin
ATP	Adenosine-5'-triphosphate
CNCbl	Cyanocobalamin
CSLO	Confocal scanning laser ophthalmoscopy
DMEM	Dulbecco's modified Eagle's medium
EGF	Epidermal growth factor
Et	Ethidium
HEt	Hydroethidine or Dihydroethidium
LHON	Leber's hereditary optic neuropathy
MeCbl	Methylcobalamin
MELAS	Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes
mtDNA	Mitochondrial DNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NARP	Neuropathy, ataxia, and retinitis pigmentosa
OHCbl	Hydroxocobalamin
OH-Et	2-Hydroxyethidium
PBS	Phosphate buffered saline
PEG-SOD	Polyethlylene glycol conjugated superoxide dismutase
PI	Propidium iodide
RFU	Relative fluorescence units
RGC	Retinal ganglion cell
RPE	Retinal pigment epithelium
SOD	Superoxide dismutase
TCA	Tricarboxylic acid
THF	Tetrahydrofolate
TNF-α	Tumour necrosis factor alpha

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I. INTRODUCTION

Vision is arguably the most important of the five senses, and the sensory modality that is most easily taken for granted. There is hardly a task we do in our daily lives that does not require sight. The visual system is composed of the eye, the retina, the optic nerve, and the brain. Light passes through the anterior chamber and lens of the eye, and focuses on the retina. The retina converts the light stimuli into electrical signals, which are then sent through the optic nerve to processing centers in the brain for vision (the lateral geniculate nucleus and then the visual cortex), pupillary responses (pretectal nuclei), sleep-wake cycling (suprachiasmatic nucleus of the hypothalamus), and orientation in space (superior colliculus). While this sounds deceptively simple, there is actually a tremendous amount of complex cellular interactions in the retina and the brain that allow us to perceive our visually complex environment. Abnormalities, whether genetic, molecular, cellular, or anatomical anywhere in the visual system will often cause disruptions in vision. While all the structures of the visual system are critical for normal vision, the focus of this thesis will be on the retina and the optic nerve.

1.1 The Retina and Optic Nerve

The retina is the translucent sensory layer of the eye responsible for transmitting visual stimuli to the brain. The retina itself is also organized into layers: starting from the outer retina are the photoreceptor (rods and cones) phototransduction elements, the outer nuclear layer containing the photoreceptor nuclei, the outer plexiform (synaptic) layer, the inner nuclear layer, the inner

plexiform layer, the ganglion cell layer, and the nerve fibre layer. When light is focused onto the retina, it must first reach the photoreceptors (rods and cones), located in the outermost layer of the retina, right next to the retinal pigment epithelium (RPE) (Kandel et al., 2000). The photoreceptors transform this light stimulus into an electrical signal that is then transmitted to bipolar, horizontal, and amacrine cells for refinement before finally reaching the retinal ganglion cells (RGCs). Since the light-sensitive photoreceptor cells are in the outermost layer, the axons of RGCs are unmyelinated in most species so that they remain translucent for light to pass through.

The RGC axons converge to form the optic nerve. In vertebrate retinas, a 'blind spot' arises from where the RGC axons exit the retina due to the lack of retinal cells in the optic disc area (Kandel et al., 2000). The optic nerve is responsible for transmitting action potentials from the RGCs to the brain. Up until the lamina cribrosa, the axons of the optic nerve are unmyelinated, requiring an abundance of mitochondria to propel the action potential and restore membrane potential (Waxman, 1978, Carelli et al., 2004). The axons of the optic nerve become myelinated after passing through the lamina cribrosa and capable of saltatory conduction, requiring less mitochondria. Before reaching their targets in the brain, the optic nerve of each eye converge to form the optic chiasm, approximately 50 mm behind the eye in humans. In primates, RGC axons from the nasal half of the retina cross-over at the optic chiasm to join the contralateral retina's temporal RGC axons in the optic tract, before continuing towards the lateral geniculate nucleus (Kandel et al., 2000). RGC axons from the temporal half

of the retina do not cross-over at the optic chiasm. In rodents, most of the axons from one eye cross over to the opposite optic tract.

1.2 Optic Neuropathies

When the optic nerve is afflicted by injury or disease, it signals the RGC soma to activate a programmed cell death pathway (apoptosis). Much like the brain, the neurons of the retina are not able to regenerate, resulting in irreversible vision loss, or an optic neuropathy. Optic neuropathies are the leading cause of blindness worldwide, with glaucoma being the most prevalent (Quigley, 1996). In the United States alone, an estimated 2.2 million people are currently affected by glaucoma (Quigley and Broman, 2006, Dirani et al., 2011), the prevalence of which is expected to increase with the aging population. Optic neuropathies can arise from several etiologies. Some examples include abnormalities of the lamina cribrosa (Quigley and Addicks, 1981), ischemia (Selles-Navarro et al., 1996), inflammation (Trip et al., 2005), or trauma (Steinsapir, 1999).

The lamina cribrosa is a multilayered network of collagen fibres located behind the optic disc, through which the optic nerve exits the eye. Since the optic nerve passes through the lamina cribrosa on its journey to the brain, abnormalities in structure or composition can lead to injury to the optic nerve. Individuals with glaucoma appear to have compression of lamina cribrosa sheets and eventual backward bowing of the entire structure. This is believed to compress the optic nerve from the superior and inferior poles and damage RGC axons (Quigley and Addicks, 1981, Quigley et al., 1983). Ischemia occurs when there is limited or a

lack of blood supply to tissues, causing oxygen and nutrient deprivation. In cases of ischemic optic neuropathy, the RGC soma die from lack of oxygen and other required trophic factors the blood supply provides. Optic neuropathies can also arise from inflammation of the optic nerve as a result of infection or autoimmune diseases. Trauma causes RGC death by direct traumatic damage or severing of RGC axons

Though often overlooked, deficiencies of critical vitamins and nutrients can also lead to vision problems (Whatham et al., 2008). A well known example of this is vitamin A deficiency causing visual impairment during dim lighting (Whatham et al., 2008). The cause of visual impairment in this case is more overt, since vitamin A plays a central role in phototransduction. Rhodopsin, the visual pigment in rod cells, is composed of a transmembrane opsin protein and retinaldehyde (vitamin A aldehyde). In the dark, retinaldehyde fits well with opsin as the 11-*cis* isomer. Upon absorption of a photon, there is rotation around the 11-*cis* double bond, resulting in the all-*trans* retinaldehyde isomer that no longer fits well with opsin. Opsin then changes shape and activates a G-protein coupled cascade for hyperpolarization of the cell, eventually resulting in transmission of visual information through the retina. The only light dependent reaction for vision occurs at the outer segments of the photoreceptors with the help of vitamin A (Kandel et al., 2000). Deficiency in vitamin A directly affects the formation of rhodopsin and phototransduction, thus resulting in vision problems.

Deficiency in other vitamins affects the eye in less obvious mechanisms. In the case of vitamin E deficiency, the accumulation of lipofuscin granules

(remnants of lipid and fatty acid oxidation) in the RPE seems to disrupt the outer segments of the rods and cones of the retina, eventually killing them (Robison et al., 1979, 1980). The spinal cord, cerebellum, and muscles (skeletal and cardiac) are also adversely affected (Kumar, 2010). In the retina, vitamin E is most concentrated in the outer segments of the photoreceptors and is believed to protect the cell membranes from free radical damage (Katz et al., 1978, Keys et al., 1997, Brigelius-Flohe and Traber, 1999). Though not directly involved in the visual process like vitamin A, vitamin E is critical for maintaining photoreceptor health and integrity – loss of the rods and cones leaves the retina without any light-sensitive cells, leading to blindness. Finally, vitamin B_{12} deficiency is known to lead to optic neuropathy with centrocaecal scotoma (Cohen, 1936, Larner, 2004), amongst its hematological, neurological, and neuropsychological manifestations (Oh and Brown, 2003). The mechanism for this neuropathy is not well understood.

1.3 Vitamin B_{12}

Vitamin B_{12} is a water-soluble vitamin, composed mainly of a planar corrin ring co-ordinating a central cobalt ion (Co^{+}, Co^{2+}, or Co^{3+}) and a nucleotide substituent (5,6-dimethylbenzimidazole, also co-ordinated to the cobalt ion) (Hodgkin et al., 1956, Hodgkin, 1958). This basic structure is often referred to as a cobalamin (Figure 1); different derivatives of vitamin B_{12} are possible through variations of ligand attachments to the cobalt ion. For example, cyanocobalamin (CNCbl) has a cyanide ligand, while hydroxocobalamin (OHCbl), methylcobalamin (MeCbl), and 5'-deoxydenosylcobalamin (AdoCbl) have

hydroxyl, methyl, and deoxyadenosyl ligands, respectively. The cobalt ion in CNCbl and OHCbl are typically trivalent (Co^{3+}) and must be reduced to divalent (Co^{2+}) or monvalent (Co^{+}) forms for conversion to MeCbl and AdoCbl in cells. While certain bacteria are capable of synthesizing their own vitamin B_{12}, humans must acquire it through diet, making it an essential micronutrient.

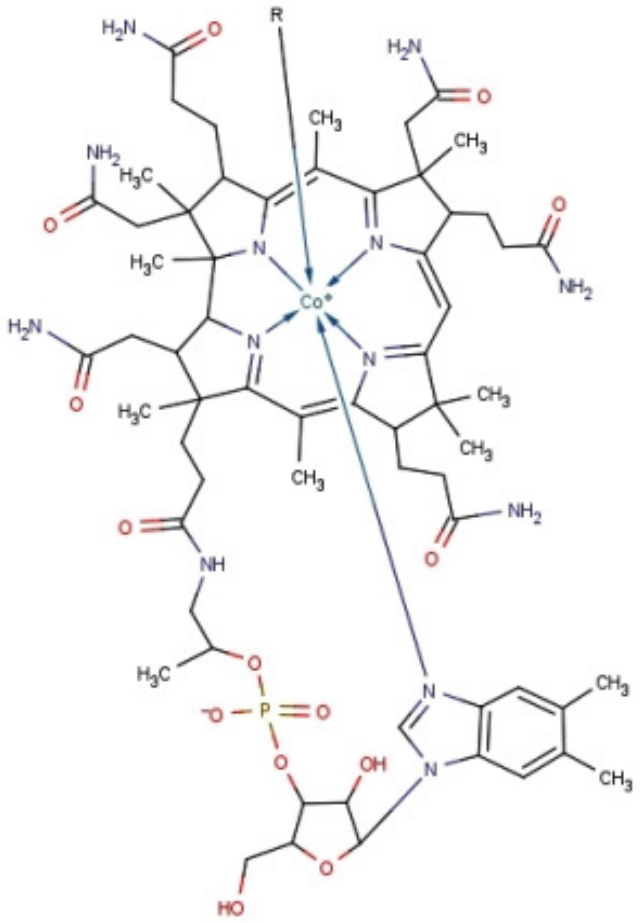

Figure 1. Chemical structure of vitamin B_{12} (cobalamin). R can be methyl, deoxydenosyl, hydroxyl, or cyanide.

Vitamin B_{12} is typically acquired through the diet from animal products (e.g. meat, eggs, fish, shellfish, and milk) (Herbert, 1988, Watanabe, 2007, Whatham et al., 2008). Certain edible algae (i.e. seaweed) also contain vitamin B_{12}, but are inactive in mammals (Watanabe, 2007). Stomach pepsin and hydrochloric acid play an important role in separating vitamin B_{12} from food, allowing it to be bound by hepatocorrin for protection against degradation in the stomach's acidic environment. Once in the duodenum, hepatocorrin is digested by pancreatic proteases and vitamin B_{12} is bound by intrinsic factor (Andres et al., 2004, Kumar, 2010). The intrinsic factor-bound vitamin B_{12} then travels through the small intestine and is eventually absorbed by the distal ileum upon attachment to receptors located on the ileal mucosal cells (Andres et al., 2004, Watanabe, 2007). Once in the mucosal cells, vitamin B_{12} is bound and transported into cells by the transcobalamin proteins and circulated systemically through the hepatic portal system (Andres et al., 2004, Watanabe, 2007).

Cyanocobalamin (CNCbl), the form of vitamin B_{12} most commonly used in supplements, needs to be converted to methylcobalamin (MeCbl) or 5'-deoxydenosylcobalamin (AdoCbl) in the body for cell use (Oh and Brown, 2003, Andres et al., 2004). MeCbl acts as a cofactor for methionine synthase in the cytoplasm, while AdoCbl is a cofactor for L-methylmalonyl Co-A mutase in the mitochondria (Figure 2). These enzymes are critical for amino acid synthesis and the citric acid cycle; deficiency in vitamin B_{12} results in enzymatic dysfunction and disruptions in cellular metabolism (Andres et al., 2004).

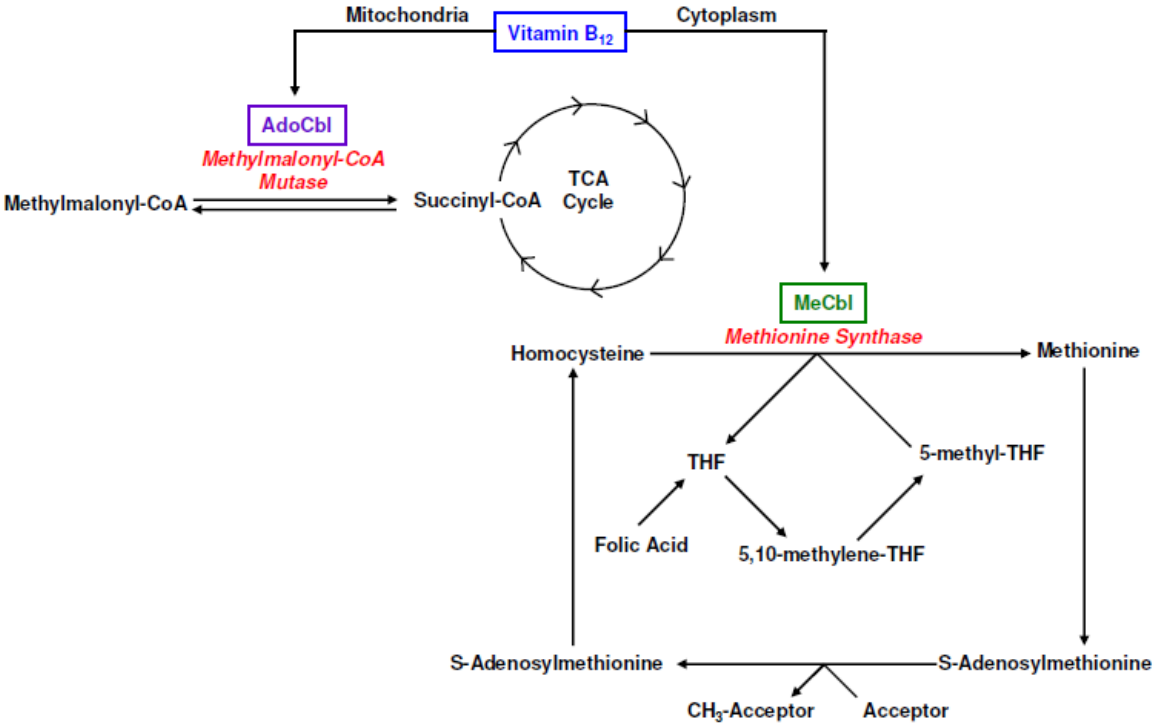

Figure 2. Metabolic co-factor roles of vitamin B_{12}. 5'-deoxydenosylcobalamin (purple) is a cofactor for methylmalonyl-CoA mutase, which transforms methylmalonyl-CoA to succinyl-CoA. Succinyl-CoA is required for the tricarboxylic acid (TCA) cycle, an important metabolic step in the generation of energy. Methylcobalamin (green) is a cofactor for methionine synthase, which is involved in the synthesis of methionine from homocysteine and 5-methyltetrahydrofolate (5-methyl-THF). TCA, tricarboxylic acid; AdoCbl, 5'-deoxydenosylcobalamin; MeCbl, methylcobalamin; THF, tetrahydrofolate.

1.4 Vitamin B_{12} Deficiency

The daily recommended allowance of vitamin B_{12} intake for adults is 2.4 μg (Stabler and Allen, 2004). Vitamin B_{12} deficiency is often diagnosed based on blood serum levels of vitamin B_{12} below 150 pmol/L (200 pg/mL) in addition to hematologic, neurologic, or psychiatric symptoms (Oh and Brown, 2003). An estimated 20% of general population in developed countries is believed to be vitamin B_{12} deficient (Carmel, 2000, Dali-Youcef and Andres, 2009). In the elderly population (adults aged 65 and above), the prevalence of vitamin B_{12} deficiency is around 12% for those in the community (Lindenbaum et al., 1994), which increases to 30-40% for sick or institutionalized individuals (van Asselt et al., 2000).

Though often difficult to determine, vitamin B_{12} deficiency can arise from several different causes. In the elderly, the most common causes of vitamin B_{12} deficiency include achlorhydria (low or absent stomach acid production), and atrophic gastritis (bacterial or autoimmune inflammation of gastric mucosa, resulting in impaired release of stomach acid, pepsin, and intrinsic factor; Carmel, 1997, Hurwitz et al., 1997). Not surprisingly, prolonged proton-pump inhibitor use (Marcuard et al., 1994) and gastric surgery (Schilling et al., 1984, Sumner et al., 1996) can also cause vitamin B_{12} deficiency . Other gastrointestinal causes for vitamin B_{12} malabsorption and deficiency include *Heliobacter pylori* infection (often associated with mucosal atrophy and achlorhydria), tropical sprue, diseases of the ileum, and parasitic infestation of tapeworms (e.g. *Diphyllobothrium latum*, which results in competition for vitamin B_{12} ; Kumar, 2010).

Any sort of hereditary genetic defect in any of the proteins involved in vitamin B_{12} absorption and transport (e.g. intrinsic factor or transcobalamins) will also cause malabsorption deficiency (Rosenblatt and Cooper, 1990). Strict vegetarians (i.e. vegans who do not consume any animal products) can also develop vitamin B_{12} deficiency over the course of several years. For the most part, indications of deficiency arising from malabsorption manifest in about 2-5 years since the liver stores 50% of available cobalamin while the rest goes towards other tissues. However, vitamin B_{12} deficiency as a result of nitrous oxide abuse (for its euphoriant properties) manifests more quickly. Nitrous oxide is used as an inhaled anaesthetic, commonly known as 'laughing gas'. In cells, nitrous oxide renders MeCbl inactive by permanently oxidizing the central cobalt ion (Deacon et al., 1978, Schilling, 1986, Marie et al., 2000). Symptoms of deficiency appear much quicker in this case due to direct inactivation of vitamin B_{12}.

The most common clinical symptoms (Table 1) of vitamin B_{12} deficiency include: haematological abnormalities such as megaloblastic anaemia (large dysfunctional red blood cells) and neurological disturbances such as degeneration or sclerosis of the spinal cord, peripheral nerves, or the optic nerve (Andres et al., 2004). While there appears to be a higher incidence of vitamin B_{12} deficiency in patients with dementia, Alzheimer's disease, Parkinson's disease, and depression, the causal relationship is not entirely clear (Carmel, 2000). The mechanism for the adverse effects of vitamin B_{12} deficiency on the nervous system, most notably the optic nerve, has yet to be elucidated.

Table 1. Clinical Symptoms of Vitamin B_{12} Deficiency

Frequency	Hematologic	Neurologic	Other
Frequent	▪ Megaloblastic anaemia ▪ Hypersegmantation of neutrophils	▪ Spinal cord sclerosis ▪ Peripheral neuropathy ▪ Myleoneuropathy ▪ Optic neuropathy	▪ Fatigue ▪ Loss of appetite ▪ Atrophic glossitis
Rare or Under Study	▪ Thrombocytopenia ▪ Neutropenia ▪ Pancytopenia ▪ Haemolytic anaemia	▪ Encephalopathy ▪ Dementia ▪ Cognitive changes ▪ Stroke	▪ Mucocutaenous ulcers ▪ Myocardial infarction ▪ Hypofertility

1.5 Superoxide Anion

Superoxide anion is the free-radical product of the single-electron reduction of molecular oxygen (O_2). Biologically, superoxide anion is generated in the mitochondria as a by-product of oxidative phosphorylation and is capable of wreaking havoc in cells by altering important cellular macromolecules (e.g. proteins and lipids) when produced in excess (Buetler et al., 2004). Our white blood cells deploy superoxide anion to combat invading pathogens; phagocytes in particular catalyze the production of large amounts of superoxide anion with the help of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Badwey and Karnovsky, 1980). Under normal physiological circumstances, the enzyme superoxide dismutase (SOD) catalyzes the conversion of superoxide anion into oxygen and hydrogen peroxide – which is further broken down to water by catalases – to protect mitochondria and cells from constant oxidative damage. Humans have three different SOD enzymes in different cellular compartments, i.e. SOD-1 (cytoplasm), SOD-2 (mitochondria), and SOD-3 (extracellular matrix) (Tainer et al., 1983, Hart et al., 1999).

Our previous work has shown that a burst of superoxide anion is a critical signal for apoptosis in axotomized neurons (Nguyen et al., 2003, Lieven et al., 2006, Kanamori et al., 2010a). We also found that post-axotomy cell death can be inhibited, both *in vitro* and *in vivo*, through exogenous delivery of pegylated-SOD (PEG-SOD) (Schlieve et al., 2006, Kanamori et al., 2010a). The neuroprotection conferred to axotomized neurons by PEG-SOD is presumed to occur through interference of superoxide signaling for apoptosis. However, the impracticality of attempting to deliver exogenous SOD as a therapy has necessitated the need investigate small molecules which also have superoxide scavenging abilities. We recently showed that the metallocorroles can scavenge superoxide anion and prevent neuronal cell death, much like SOD (Kanamori et al., 2010b). The metallocorroles are small tetrapyrrolic macrocycle molecules that hold a metallic ion in the porphyrin-like inner core (Figure 3) (Gross and Galili, 1999, Kanamori et al., 2010b). Upon closer inspection of the structural features of the metallocorroles and vitamin B_{12}, it is apparent that they both have a corrin ring and metal ion as their core chemical structure.

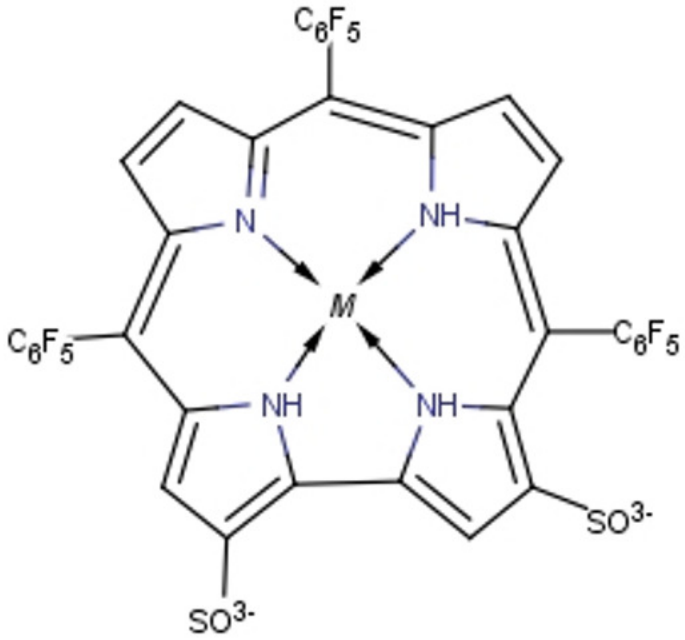

Figure 3. Chemical structure of the metallocorroles. M can be iron (Fe^{3+}), manganese (Mn^{3+}), or gallium (Ga^{3+}).

II. HYPOTHESIS AND OBJECTIVES

The mechanism for vitamin B_{12} deficiency-induced optic neuropathy is not well understood. Vitamin B_{12} has recently been found to react specifically with superoxide anion at rates approaching that of SOD (7×10^8 $M^{-1}s^{-1}$, comparable to the SOD-catalyzed rate of 2×10^9 $M^{-1}s^{-1}$) (Suarez-Moreira et al., 2009). Additionally, vitamin B_{12} has been found to act as an intracellular antioxidant in cell culture (Birch et al., 2009) while conferring increased viability to human aortic endothelial cells subjected to superoxide-induced damage, suggesting that vitamin B_{12} may play an additional role beyond its known cofactor functions (Suarez-Moreira et al., 2009, Suarez-Moreira et al., 2011).

Since the metallocorroles and vitamin B_{12} share a core structure, and both have been found to effectively scavenge superoxide anion, it is possible that the core corrin ring structure (and metal ion) plays a role in scavenging. Given that 1) superoxide anion is a key apoptotic signal in our axotomy model for optic neuropathy (Kanamori et al., 2010a) and 2) vitamin B_{12} appears to be capable of superoxide anion scavenging, it is possible that vitamin B_{12} has a role in cellular redox maintenance in addition to its traditional co-factor roles. To the best of our knowledge, the effect of vitamin B_{12} deficiency on neurons have yet to be explored, despite the clinical presentation of neurological symptoms in patients that are vitamin B_{12} deficient. **We hypothesize that vitamin B_{12} is a neuronal SOD mimetic, which during deficiency results in an increased level of superoxide anion in the cell as SOD becomes overwhelmed.** This high level of superoxide then becomes a misplaced signal that activates the cell death pathway. Besides

causing pathologies through enzymatic dysfunction when deficient, it is possible that vitamin B_{12}'s novel function as an intracellular antioxidant can provide some insight into the unknown mechanism of vitamin B_{12} deficient-optic neuropathy. The objectives of this project are to determine whether CNCbl, the form of vitamin B_{12} most commonly used in supplements, can scavenge superoxide anion in neuronal cells and be neuroprotective *in vitro* and *in vivo*. This study aims to elucidate a potential mechanism for vitamin B_{12} deficient-optic neuropathy and potentially bring insight to other neurologic presentations of vitamin B_{12} deficiency.

III. JOURNAL ARTICLE MANUSCRIPT

Cyanocobalamin is a superoxide scavenger and neuroprotectant in neuronal cells

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Keywords: Cyanocobalamin (CNCbl); superoxide anion; neuroprotection; confocal scanning laser ophthalmoscopy (CSLO)

Abstract

Damage to the optic nerve (optic neuropathy) can result in permanent loss of vision or blindness through retinal ganglion cell (RGC) death. Our prior work identified a burst of superoxide anion as a critical molecular event in RGCs prior to injury-induced cell death. Recently, Suarez-Moreira et al (JACS 131:15078, 2009) demonstrated that vitamin B_{12} scavenges superoxide as effectively as superoxide dismutase. Given that vitamin B_{12} deficiency can lead to optic neuropathy through unknown mechanisms, we investigated the relationship between superoxide scavenging by cyanocobalamin, the most abundant vitamin B_{12} vitamer, and its neuroprotective properties in neuronal cells.

Superoxide anion reacts with hydroethidine to produce a fluorescent product, 2-hydroxyethidium. Superoxide scavenging by cyanocobalamin in a cell-free system was measured with a fluorescent microplate reader. Superoxide scavenging in RGC-5 neuronal cells was assessed *in vitro* by fluorescent microscopy. Neuroprotection against menadione was evaluated by calcein-AM/propidium iodide assay to identify living and dead cells. An optic nerve transection model in Long-Evans rats was used to study superoxide scavenging and neuroprotection *in vivo,* with visualization of superoxide within retrograde-labelled RGCs by confocal scanning laser ophthalmoscopy.

Cyanocobalamin at concentrations of 10 μM and 100 μM reduced the rate of superoxide generation by 34% and 79% in cell free assays, respectively. In menadione-treated RGC-5 cells, cyanocobalamin concentrations above 10 nM scavenged superoxide anion similar to those treated with pegylated-SOD. Cyanocobalamin at concentrations of 100 μM and 1 mM reduced RGC-5 cell death from menadione by 20% and 32%, respectively. In rats with unilateral optic nerve transection, a single intravitreal dose of 667 μM cyanocobalamin significantly reduced the number of 2-hydroxyethidium–positive RGCs. This dose also increased RGC survival rate compared to rats injected with saline control.

These data suggest that vitamin B_{12} may be an endogenous neuroprotectant, which when depleted in nutritional deficiency, could cause death of retinal ganglion cells by a superoxide-dependent mechanism. Vitamin B_{12} could also potentially also be used as a therapy to slow progression of RGC death in patients with optic neuropathies characterized by overproduction of superoxide.

Introduction

The optic nerve is responsible for relaying visual stimuli from the retina to the brain and is composed of retinal ganglion cell (RGC) axons. When the optic nerve is damaged from injury or disease (i.e. an optic neuropathy), the RGC soma activates a programmed cell death pathway and undergoes apoptosis, resulting in irreversible visual loss. Optic neuropathies are the leading cause of blindness worldwide, with glaucoma being the most prevalent of the optic neuropathies (Quigley, 1996). In the United States alone, an estimated 2.2 million people are currently affected by glaucoma (Quigley and Broman, 2006, Dirani et al., 2011), the prevalence of which is expected to increase as the population ages.

Optic neuropathies arise from multiple etiologies, including intraocular pressure too high for the optic disc, ischemia (Selles-Navarro et al., 1996), tumors (Danesh-Meyer et al., 2008), infection (Trip et al., 2005), or trauma (Steinsapir, 1999). Although often overlooked, deficiencies of critical vitamins and nutrients can also lead to vision problems (Whatham et al., 2008). Vitamin B_{12} deficiency in particular is known to lead to bilateral optic neuropathy, characterized by centrocecal scotomas and the slow development of optic atrophy (Cohen, 1936, Larner, 2004), in addition to more common hematological, neurological, and neuropsychological manifestations (Oh and Brown, 2003).

Vitamin B_{12} is an essential micronutrient acquired through the diet in animal products such as meat and eggs (Herbert, 1988, Watanabe, 2007, Whatham et al., 2008). Digestion of food by stomach pepsin and hydrochloric acid release vitamin B_{12} and allow it to be bound by intrinsic factor in the duodenum (Andres et al., 2004, Kumar, 2010). The intrinsic factor-bound vitamin B_{12} then travels through the small intestine and is eventually absorbed by the distal ileum upon attachment to receptors located on the ileal muscoal cells (Andres et al., 2004, Watanabe, 2007). Once in the mucosal cells, vitamin B_{12} is bound and transported into cells by transcobalamins and circulated systemically through the hepatic portal system (Andres et al., 2004, Watanabe, 2007). Cyanocobalamin, the form of vitamin B_{12} most commonly used in supplements, is converted to methylcobalamin (MeCbl) and 5'-deoxydenosylcobalamin (AdoCbl) (Oh and Brown, 2003, Andres et al., 2004). MeCbl acts as a cofactor for methionine synthase in the cytoplasm, while AdoCbl is a cofactor for L-methylmalonyl Co-A mutase in mitochondria (Figure 1A). These enzymes are critical for amino acid synthesis and the citric acid cycle, with vitamin B_{12} deficiency resulting in enzymatic dysfunction and disrupted cellular metabolism (Andres et al., 2004).

In contrast, the mechanism for vitamin B_{12} deficiency-induced optic neuropathy is not well understood. A possible mechanism for vitamin B_{12}

deficiency-induced optic neuropathy involves the recent finding that vitamin B_{12} can act as an intracellular antioxidant (Birch et al., 2009). This suggests that vitamin B_{12} may play an additional role beyond its known cofactor functions (Figure 1B). Vitamin B_{12} reacts with superoxide anion at rates approaching that of superoxide dismutase, and protects human aortic endothelial cells from superoxide-induced damage (Suarez-Moreira et al., 2009, Suarez-Moreira et al., 2011). Superoxide anion is produced as a byproduct of electron transport chain leakage, and normally is scavenged by superoxide dismutase (SOD), thereby protecting mitochondria from constant oxidative damage. However, there is another role for superoxide in the nervous system, namely as a signal for apoptosis in axotomized neurons (Nguyen et al., 2003, Lieven et al., 2006, Kanamori et al., 2010a). Cell death after axotomy can be inhibited through exogenous delivery of pegylated superoxide dismutase-1 (PEG-SOD) (Schlieve et al., 2006, Kanamori et al., 2010a), presumably by inhibiting superoxide signaling of apoptosis.

The difficulties associated with delivering exogenous SOD therapeutically prompted the development of small molecules that scavenge superoxide. We recently showed that metallocorroles which scavenge superoxide anion (Eckshtain et al., 2009) can prevent neuronal cell death (Kanamori et al., 2010b). Metallocorroles are small tetrapyrrolic macrocycles that contain a metallic ion in the porphyrin-like inner core (Figure 1C) (Gross and Galili,

1999, Kanamori et al., 2010b). Metallocorroles and vitamin B_{12} both have a corrin ring as their core chemical structure (Figure 1C). The ability of these molecules to scavenge superoxide anion is likely related to this core corrin ring structure.

Given that superoxide anion is a signal for signaling apoptosis after RGC axotomy (Kanamori et al., 2010a) and that vitamin B_{12} shares a core structure with superoxide-scavenging and neuroprotective metallocorroles, we hypothesized that vitamin B_{12} is an endogenous SOD mimetic which when deficient would lead to RGC death. The mechanism of RGC death in vitamin B_{12} deficiency would result from elevated levels of physiological superoxide, which would aberrantly signal a pathological cell death pathway meant for axonal injury. To explore this hypothesis, we studied the role of vitamin B_{12} as a superoxide scavenger and neuroprotectant *in vitro* and *in vivo*.

Materials and Methods

Animals

Female Long Evans rats weighing 225-250g were from Charles River, Canada. Rats were housed in the Maisonneuve-Rosemont Hospital Research Centre Animal Facility in a temperature-controlled environment with a 12-hour light/dark cycle. Food and water were provided *ad libitum*. Animals were anesthetized by intraperitoneal injection of ketamine (50 mg/kg) and xylazine (100 mg/kg) prior to any surgery or imaging procedure. All animal experiments were carried out in accordance with the guidelines of the Maisonneuve-Rosemont Hospital Research Centre Animal Care Committee and the Canadian Council on Animal Care.

Materials

RGC-5 cells were a generous gift from Neeraj Agarwal, PhD. Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum were from Wisent Inc. (St. Bruno, QC). Staurosporine was from Alexis Biochemicals (San Diego, CA). Hydroethidine (dihydroethidium; HEt) was from Fluka BioChemika (Buchs, Switzerland). Penicillin-streptomycin, polyethlylene glycol conjugated to superoxide dismutase from bovine erythrocytes (PEG-SOD), menadione, xanthine, and xanthine oxidase were from Sigma-Aldrich (St. Louis, MO). Phosphate buffered saline (PBS) was from Lonza, Inc. (Walkersville, MD). Salmon sperm DNA, calcein AM, and propidium

iodide were from Invitrogen (Carlsbad, CA). 4-Di-10-Asp, [4-(4-(didecylamino)styryl)-N-methylpyridinium iodide] was from Anaspec (Fremont, CA). Cyanocobalamin was from U.S Biological (Swampscott, MA).

Cell-Free Assays

Xanthine (100 µM) was mixed with HEt (100 µM), 1 mg/mL of salmon sperm DNA, and 100 pM – 100 µM of cyanocobalamin. This master mix was incubated in quadruplicate in a black 96-well plate (Nunc, Rochester, NY), and xanthine oxidase (5 mU/mL) was added last to initiate superoxide-generation. Plates were read in a CytoFluor 4000 (Perseptive Biosystems, Framingham, MA) fluorescent plate reader every ten minutes for an hour at 360±40 nm excitation and 580±50 nm emission.

Cell Culture

RGC-5 cells were cultured in DMEM containing 1 g/L glucose, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were split every 48 to 72 hours when cells were approximately 60-85% confluent, re-plated at a 1:20 dilution in a 25 cm^2 flask in 5 mL of cell culture media and incubated at 37℃ in humidified 5% CO_2. Cells were seeded into 6-well culture plates (Sarstedt, Nümbrecht, Germany) for all *in vitro* experiments. For studies using differentiated RGC-5 cells, cells were incubated in media containing 316 nM staurosporine for 3 hours to allow cells to differentiate before further treatments. This

concentration resulted in minimal cell death when early passages (7-15) were used. All experiments were performed in triplicate.

Measurement of intracellular superoxide *in vitro*

Differentiated RGC-5 cells were treated with media containing 15 μM menadione to generate superoxide and treated with various scavengers for 24 hours. Cells were then stained with HEt (50 μM) for 30 minutes before rinsing with PBS for quantitative epifluorescence microscopy (excitation 395 ± 5.5 nm, 500 nm dichroic, emission 560 ± 20 nm; Zeiss Axio Observer A1). Microscope fields were randomly sampled and all photos were taken at the same exposure settings. ImageJ (National Institutes of Health, Bethesda, MD) was used for image analysis of the HEt fluorescence signal from cells, as previously described (Kanamori et al., 2010b).

Fluorescent live-dead assays

No. 1 glass coverslips (VWR, Radnor, PA) were sterilized by flaming in absolute ethanol prior to coating with poly-L-lysine (Sigma-Aldrich, St. Louis, MO). RGC-5 cells were seeded onto the glass coverslips in 6-well tissue culture plates (Sarstedt, Nümbrecht, Germany). After differentiation, cells were treated with various agents for 24 hours. Cells were then stained with calcein-AM (1 μM) and propidium iodide (PI; 5 μM) in PBS for 20 minutes at 37℃ in humidified 5% CO_2. Coverslips were rinsed in PBS and fixed in 4% paraformaldehyde for 30 minutes before rinsing 3 times (15 minutes each) with PBS. Coverslips were mounted on glass slides using

PermaFluor (Thermo Scientific, Walthan, MA) for epifluorescence microphotography (Zeiss Axio A1). Four random microscope fields were captured for each coverslip for analysis. An observer masked to treatment groups manually counted the number of live (calcein-positive) and dead (PI-positive) cells in each microphotograph.

Retrograde labelling of RGCs

RGCs were retrogradely labeled by stereotactic injection of 4-Di-10-Asp into both superior colliculi, as previously described (Kanamori et al., 2010a). Briefly, anesthetized rats were placed in a stereotactic apparatus and the cortex surface was exposed by drilling the parietal bone to facilitate dye injection. A total volume of 2.8 µL of 100 mM 4-Di-10-Asp was injected bilaterally at 6 mm caudal to bregma and 1.2 mm lateral to the midline to a depth of 4.5 mm from the skull surface. Animals were maintained for 4-7 days after 4-Di-10-Asp injection into the superior colliculi to ensure dye transport to RGC somas.

Optic nerve transection

After RGC labeling, intrameningeal optic nerve transection on the right eye was performed as previously described (Kanamori et al., 2010a). The left eye served as an untransected control. All animals were examined by indirect ophthalmoscopy after optic nerve transection to ensure the integrity of the retinal blood supply. Rats with abnormal retinal vessels or retinal edema were not used further.

Intravitreal injections

Intravitreal injections of HEt and CNCbl were performed as previously described (Kanamori et al., 2010b). Briefly, injections were made immediately posterior to the superotemporal limbus at a 45° angle using a 32-gauge needle attached to a 10 µL Hamilton syringe (Hamilton, Reno, NV). Figures 4A and 4D show the different injection time points relative to transection and imaging for different experiments.

***In vivo* confocal laser scanning ophthalmoscopy**

A Heidelberg Retinal Angiogram 2 (Heidelberg Engineering, Germany) confocal scanning laser ophthalmoscope (CSLO) was used for *in vivo* imaging of the rat retina, as previously described (Kanamori et al., 2010a). Briefly, rats were anesthetized and their pupils dilated with phenylephrine and atropine. The 488 nm laser was used for the excitation of OH-Et positive cells and 4-Di-10-Asp retrograde-labeled RGC somas. To ensure the plane of focus was in the ganglion cell layer, the CSLO polarization filter (red-free mode) was used to focus onto the nerve fiber layer and then adjusted posteriorly. Retinal images were captured using a 30° field of view lens with real-time averaging of at least 50 images. The same four retinal quadrants (superior, inferior, nasal, and temporal) immediately adjacent to the optic nerve were imaged over time in each rat. OH-Et positive cells or 4-Di-10-Asp-labeled cells within specific retinal sections of each image outlined by major retinal blood vessels were counted by an observer

masked to the treatment group, for quantification of superoxide production or neuroprotection, respectively.

Statistics

Statistical analysis was done using Prism v.5.0 (Graphpad Software Inc., San Diego, CA). Unpaired *t*-tests were used to compare means from two groups, with $\alpha = 0.05$. For comparisons between multiple groups, significance was calculated using one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison post-hoc test at $\alpha = 0.05$ against the negative control group. Data are presented as mean ± SEM unless otherwise indicated.

Results

Cyanocobalamin scavenges superoxide in a cell-free system

We measured the relative fluorescence units (RFU) of the reaction between superoxide and HEt to determine the degree of superoxide scavenging by CNCbl compared to SOD. HEt is the non-fluorescent reduced form of ethidium (Zhao et al., 2003). Upon reacting with superoxide, HEt oxidizes to 2-hydroxyethidium (OH-Et) and binds to DNA to yield a fluorescent signal (excitation 395 nm, emission 567 nm; Figure 2A and 2B). The reaction approaches maximal fluorescence at 30 minutes (Figure 2C). In order to compare scavenging with CNCbl (100 pM – 100 μM) or bovine erythrocyte SOD (0.36 U/mL), we calculated the slope from the first 30 minutes of each reaction and compared the rate (RFU/minute) of superoxide production in the presence or absence of scavenger. Numerically larger slopes indicated more superoxide reacting with HEt to yield fluorescent OH-Et, i.e. less superoxide scavenging, while smaller slopes indicated greater scavenging. The slope with the positive control SOD was significantly less than the no-scavenger control (0.29 ± 0.018 vs. 1.65 ± 0.049 RFU/min; $p < 0.0001$), and indicated scavenging of 82% of available superoxide. The slopes with CNCbl (10 μM or 100 μM) were also significantly less than the no-scavenger control (1.09 ± 0.030 and 0.34 ± 0.0058 RFU/min; $p < 0.0001$ for both comparisons), and indicated

scavenging of 34% and 79% of available superoxide, respectively (Figure 2D).

Cyanocobalamin scavenges superoxide *in vitro* in neuronal cells

Differentiated RGC-5 cells were treated with menadione to generate intracellular superoxide anion via mitochondrial redox cycling (Nguyen et al., 2003). HEt can passively diffuse across the plasma membrane of live cells to react with intracellular superoxide, followed by binding to nuclear DNA and thereby yielding a fluorescent signal in the nucleus. Quantification of the nuclear fluorescence therefore is an indirect measure of superoxide levels (Figure 3A). Compared to cells exposed to menadione in the absence of a scavenger, cells treated with CNCbl concentrations from 1 nM to 1 mM had significantly decreased levels of superoxide (Figure 3B). Superoxide levels of menadione-treated RGC-5 cells decreased in the presence of CNCbl, with 20% scavenging at 1 nM and 42% scavenging at 1 μM. Higher concentrations of CNCbl did not demonstrate greater superoxide scavenging.

Cyanocobalamin increases cell survival *in vitro* in neuronal cells

Differentiated RGC-5 cells were treated with menadione (15 μM) to generate superoxide in the presence or absence of CNCbl (1 pM to 1 mM) or PEG-SOD (30 U/mL) as a positive control, then stained with calcein-AM and PI to assess cell survival (Figure 3C). Cells exposed to menadione in the absence of a CNCbl or PEG-SOD had a mean 21.2 ± 8.8% survival (Figure 3D). RGC-5 survival increased significantly in the presence of as

little as 10 pM CNCbl (56.5 ± 3.58%; $p < 0.001$ compared to control), and was 87.7 ± 3.44% ($p < 0.0001$) with 10 μM CNCbl. Higher concentrations of CNCbl resulted in RGC-5 cell survival comparable to cells treated with PEG-SOD.

Cyanocobalamin scavenges superoxide in retinal ganglion cells *in vivo* after optic nerve transection

In order to determine whether vitamin B_{12} was a superoxide scavenger *in vivo*, we took advantage of our ability to use confocal scanning laser ophthalmoscopy to longitudinally image superoxide in axotomized rat RGCs (Kanamori et al., 2010a, Kanamori et al., 2010b). HEt was injected into the vitreous 3 days after optic nerve transection in Long Evans rats in the presence or absence of CNCbl, using four injection paradigms with different concentrations and timing of administration (Figure 4A). Injecting a high CNCbl dose (667 μM) sooner after transection decreased the number of superoxide-positive cells (44.2 ± 6.1 vs. 77.3 ± 7.4 in the no CNCbl control; 39% decrease; $p = 0.003$). A lower CNCbl dose (333 μM) injected later after transection non-significantly decreased the number of superoxide-positive cells (71.4 ± 6.2; 7.6% decrease; $p = 0.51$). The greatest decrease (34.7 ± 3.8; 55% decrease; $p < 0.0001$) in superoxide-positive cells after axotomy occurred when an initial 667 μM CNCbl dose was followed by a delayed 333 μM CNCbl dose (Figure 4B, 4C).

Cyanocobalamin increases cell survival of retinal ganglion cells *in vivo* after optic nerve transection

RGCc were retrograde labeled by injection of 4-Di-10-Asp into the superior colliculi 4-7 days prior to optic nerve transection (Figure 4D). Rats received an intravitreal injection of 667 μM CNCbl or vehicle control one day after transection and were longitudinally imaged by CSLO to monitor RGC survival (Figure 4E). At 5 days post-transection, rats that received an intravitreal injection of 667 μM CNCbl had 87.0 ± 2.62% RGC survival compared to the vehicle control (69.0 ± 3.66 %; $p = 0.0008$). At 7 days post-transection, CNCbl-treated retinas had a 79.7 ± 2.47% RGC survival compared to the vehicle control animals (53.1 ± 2.30%; $p < 0.0001$;). Finally, at 14 days post-transection, CNCbl-treated retinas had 59.7 ± 3.28% RGC survivial compared to those that received vehicle control (29.3 ± 2.32; $p < 0.0001$, Figure 4F)

Discussion

These data demonstrate that (1) CNCbl scavenges superoxide anion generated in a cell-free reaction between xanthine and xanthine oxidase; (2) CNCbl scavenges intracellular superoxide induced by menadione-induced redox cycling, and protects against cell death *in vitro*; and (3) CNCbl scavenges superoxide and is neuroprotective *in vivo* in rat RGCs after optic nerve transection. To the best of our knowledge, this is the first demonstration that CNCbl scavenges superoxide and is neuroprotective in neuronal cells *in vitro* and *in vivo*.

Previous reaction kinetic studies have shown that vitamin B_{12} reacts with superoxide in a second order reaction to generate H_2O_2 at a rate of 7×10^8 $M^{-1}s^{-1}$, which is comparable to the SOD-catalyzed rate of 2×10^9 $M^{-1}s^{-1}$ (Suarez-Moreira et al., 2009). The same group also demonstrated *in vitro* superoxide scavenging by CNCbl in an endothelial cell line. Clinically, patients with genetic vitamin B_{12} metabolism disorders have significantly greater levels of markers for oxidative stress in their urine (Mc Guire et al., 2009), possibly due to the lack of superoxide scavenging by vitamin B_{12}. Together with the results from our study demonstrating that vitamin B_{12} scavenges superoxide and is neuroprotective in neuronal cells, these findings suggest that vitamin B_{12} contributes to the maintenance of cellular

redox status, in addition to its several known functions as a co-factor in metabolic processes.

Such findings provide a pathophysiological explanation for an uncommon but potentially devastating disease, the optic neuropathy caused by vitamin B_{12} deficiency. This deficiency has well-known neurological manifestations, with peripheral neuropathy, subacute combined (posterior and lateral columns) degeneration of the spinal cord, cognitive changes, and optic neuropathy being the most prominent. The damage from vitamin B_{12} deficiency within the retina is specific to RGCs, and spares photoreceptors and other retinal neurons on clinical and pathological grounds (Chester et al., 1980). Therefore, a unifying explanation for the pathophysiology of vitamin B_{12} deficiency in the eye necessarily has to include why it is restricted to the RGC. It cannot be mitochondrial stress or insufficient production of ATP, because genetic mitochondrial diseases such as mitochondrial DNA (mtDNA) deletions, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS), and neuropathy, ataxia and retinitis pigmentosa (NARP) affect high energy-consuming tissues (e.g. skeletal muscle and photoreceptors), but not RGCs.

Based on the results of the present study, we instead propose that the specificity of vitamin B_{12} deficiency for RGCs is because (1) this molecule is a superoxide scavenger within RGCs, and (2) RGCs are particularly

sensitive to superoxide-induced cell death. We know from our previous studies (Lieven et al., 2006, Kanamori et al., 2010a) that RGCs use superoxide as an intracellular signaling molecule to transduce apoptosis after axonal injury. Drugs that scavenge superoxide decrease RGC death (Kanamori et al., 2010a, Kanamori et al., 2010b, Catrinescu et al., 2012), while induction of elevated superoxide by knockdown of superoxide dismutase isoforms induces neuronal or RGC death (Scott et al., 2010, Yuki et al., 2011). Given our results in the present study demonstrating that vitamin B_{12} is a neuronal superoxide scavenger *in vitro* and *in vivo*, coupled with previous studies demonstrating that elevated superoxide is an axotomy signal and induces RGC death, then it follows that vitamin B_{12} deficiency would lead to dysregulated superoxide levels and consequent RGC death, i.e. optic neuropathy.

RGCs, like all cells, contain superoxide dismutase-1 (SOD-1) in the cytoplasm and SOD-2 in the mitochondria. These endogenous superoxide scavengers would have to be overwhelmed in vitamin B_{12} deficiency, which would occur in situations where there was high levels of mitochondrial oxygen consumption and consequent superoxide leak from the mitochondrial electron transport chain (Kussmaul and Hirst, 2006). RGCs have high numbers of mitochondria in the unmyelinated portion of their axons, particularly at the optic nerve head anterior to the myelin transition zone (Waxman, 1978, Carelli et al., 2004). More mitochondria are need in

unmyelinated than myelinated axons because the lower membrane resistance and higher capacitance necessitates larger current flux for axonal conduction. Therefore, the prelaminar and laminar portion of the optic nerve head is a putative hot spot for superoxide-mediated damage resulting from vitamin B_{12} deficiency.

Support for a superoxide-dependent mechanism comes from a seemingly unrelated optic neuropathy, Leber hereditary optic neuropathy (LHON), which shares with vitamin B_{12} deficiency neuropathy an uncommon clinical phenotype, a cecocentral scotoma visual field defect (Newman et al., 2006). This maternally inherited optic neuropathy results from one of three mutations in mtDNA coding for subunits of complex I of the mitochondrial electron transport chain, and causes relatively specific damage to RGCs, with minimal effects elsewhere in the retina, CNS, or body (Kerrison and Newman, 1997). The LHON mutations do not significantly decrease ATP production in studies of fibroblast cybrids (Baracca et al., 2005). However, they greatly increase superoxide levels, and it is more likely that the superoxide induction is responsible for the the RGC-specific death in LHON (Levin, 2007).

There are several potential weaknesses in our study. We used RGC-5 cells for our *in vitro* studies, which are neuron-like but not actually RGCs (Van Bergen et al., 2009, Wood et al., 2010, Nieto et al., 2011). However,

the *in vivo* studies were with RGCs, thus making this less of a concern. We used an optic nerve transection model in rats to investigate whether CNCbl can scavenge superoxide and increase cell survival *in vivo,* with the added advantage of monitoring how transected RGCs respond to intravitreal injections of CNCbl in the same animal over time. Optic nerve transection is an acute injury, and models diseases like traumatic optic neuropathy, which is far less common than other optic nerve disease such as glaucoma. Finally, our focus on the optic nerve is not necessarily extrapolable to the spinal cord, peripheral nerves, and the cerebral cortex, which are also affected by vitamin B_{12} deficiency.

In summary, CNCbl scavenges superoxide anion and is neuroprotective for RGCs after axonal injury *in vivo*. These results extend previous studies (Birch et al., 2009, Suarez-Moreira et al., 2009, Suarez-Moreira et al., 2011) demonstrating a novel role for vitamin B_{12} in maintaining cellular redox status, and support the hypothesis that vitamin B_{12} deficiency optic neuropathy results from a misplaced signal for cell death due to unconstrained superoxide generation. These results also have implications for the ocular health of populations at risk of vitamin B_{12} deficiency, most notably the elderly, vegans, and third world populations. Further study examining the prevalence of vitamin B_{12} deficiency in patients with optic neuropathies could prove informative for implementing preventative therapy for high risk populations.

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Figure Legends

Figure 1. Association of vitamin B_{12} deficiency and optic neuropathy. (A) Vitamin B_{12} derivatives methylcobalamin (MeCbl) and 5'deoxyadenosylcobalamin (AdoCbl) are cofactors involved in methionine synthesis and propionate metabolism respectively. AdoCbl is required for the conversion of methylmalonyl-CoA to succinyl-CoA, an intermediate in the tricarboxylic acid (TCA) cycle. (B) Cobalamin has been shown to scavenge superoxide anion. Scavenging equation re-written from Suarez-Moreira et al. 2009, *JACS 141*(42): 15078 . (C) Chemical structures of cobalamin (left) and metallocorrole (right). R can be methyl (methylcobalamin), hydroxyl (hydroxocobalamin), deoxyadenosyl (5'deoxyadenosylcobalamin), or cyanide (cyanocobalamin). M can be iron (Fe^{3+}), manganese (Mn^{3+}), or gallium (Ga^{3+}). Both structures contain a corrin ring (red circle), which could underlie the superoxide scavenging properties of these compounds.

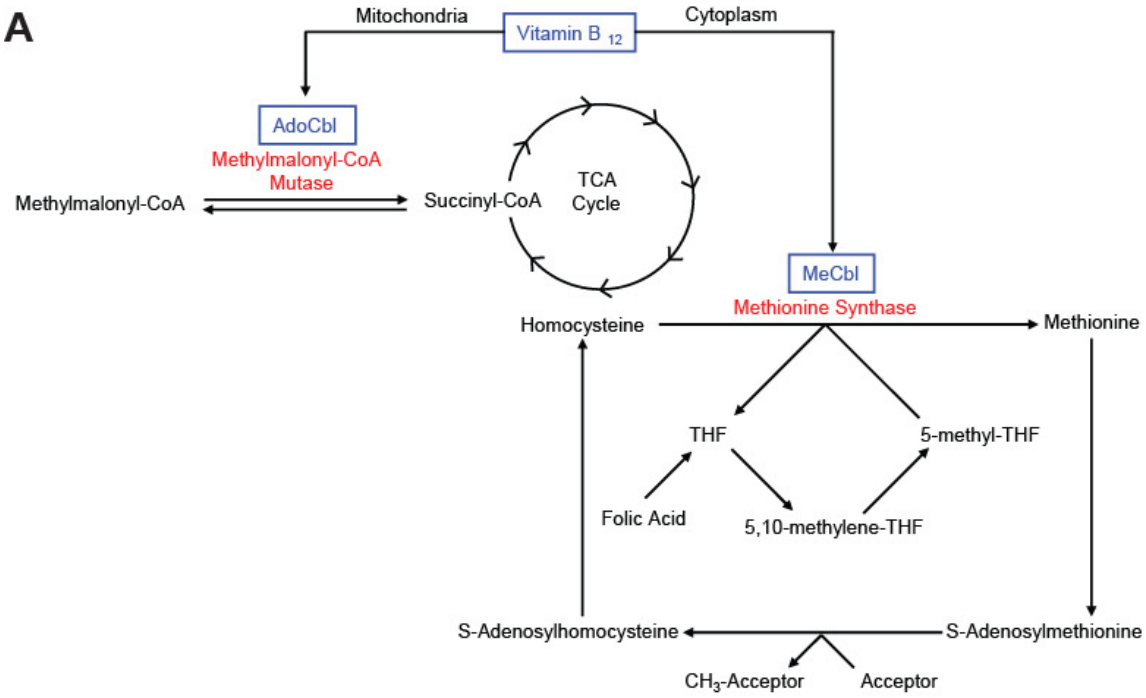
Figure 2. Vitamin B_{12} scavenges superoxide anion in a cell-free system. (A) Chemical structure of hydroethidine (HEt), ethidium (Et), and 2-hydroxyethidium (OH-Et). Et is the fluorescent product of HEt's single electron reaction while OH-Et is the production of HEt's reaction with superoxide anion. (B) Excitation and emission spectra of Et (purple) and OH-Et (green) at 510 nm and 396 nm excitation, redrawn from Kanamori et

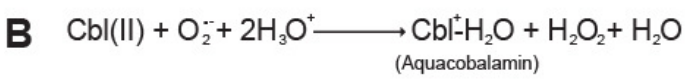
al. 2010, *Brain 133*(9): 2612-2625. (C) Curve graph of OH-Et generation over time. (D) Bar graph of slopes from Figure 2C. A greater slope indicates less superoxide scavenging.

Figure 3. Vitamin B_{12} scavenges superoxide and is neuroprotective *in vitro* in menadione-treated RGC-5 cells. Differentiated RGC-5 cells were treated with 15 uM menadione for 24 hours in the presence or absence of cyanocobalamin. (A) Representative microscope photos of HEt-stained RGC-5 cells. Fluorescence is localized in the nucleus (B) Bar graph summarizing OH-Et levels of RGC-5 cells. CNCbl concentrations greater than 1 μM do not demonstrate greater superoxide scavenging. (C) Representative microscope photos of RGC-5 cells stained with calcein (green) and PI (red). (D) Bar graph summarizing the percentage of RGC-5 cell survival.

Figure 4. Vitamin B_{12} scavenges superoxide and is neuroprotective in vivo in rats with unilateral optic nerve transection. (A) Flowchart of experimental paradigms. Day 0 (D0) marks the day of transection (ONT). HEt (100 μM) was injected intravitreally 3 days post-transection. CSLO imaging was performed 4 days post-transection, based on previous studies demonstrating that transected RGCs yield maximal OH-Et staining at that time (Kanamori et al., 2010a). (B) Representative CSLO images of OH-Et-positive cells. Retinas treated with CNCbl appear to have less OH-Et

positive cells compared to those treated with vehicle control. (C) Bar graph summerizing number of OH-Et positive cells with respect to CNCbl treatment. (D) Flowchart of experimental paradigm for determining *in vivo* neuroprotection. RGCs were retrogradely labelled (-D5) with 4-Di-10-Asp prior to transection (D0). Rats received an intravitreal injection of 667 μM CNCbl (D1) and imaged on 5, 7, and 14 days post-transection. (E) Representative CSLO images of labelled RGCs treated with CNCbl or vehicle control prior to transction and at 5 and 7 days post-transection. (F) Bar graph summerizing percentage of RGC survival at 5, 7, and 14 days post-transection when treated with CNCbl (blue) or vehicle control (yellow).





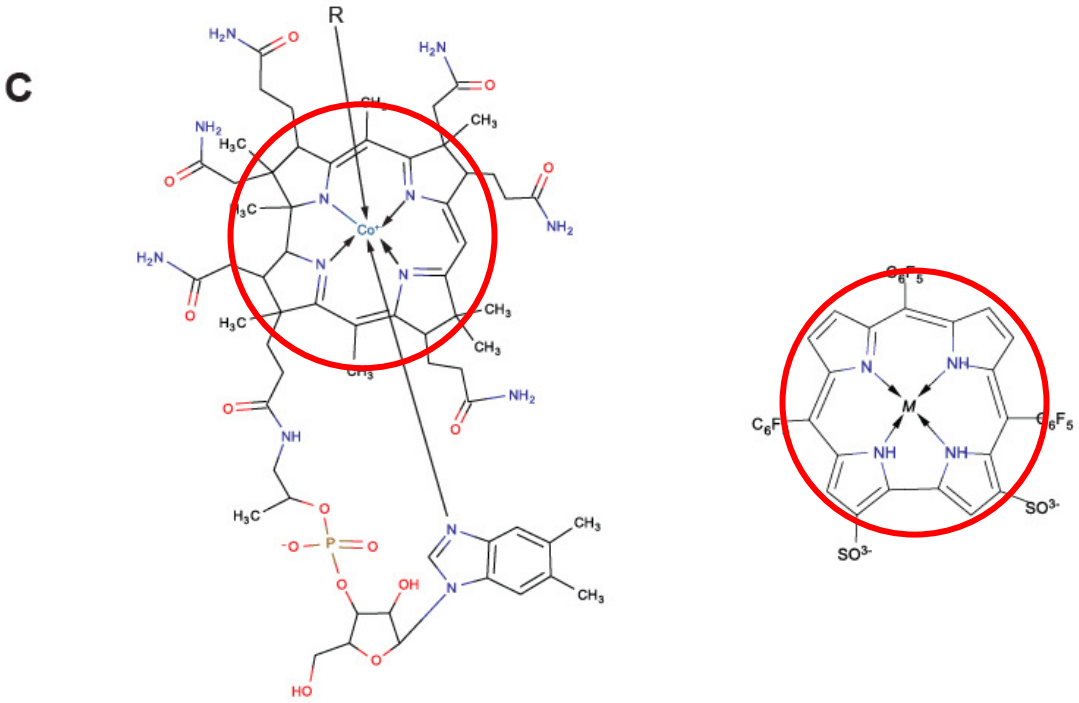


Figure 1. Association of vitamin B_{12} deficiency and optic neuropathy.

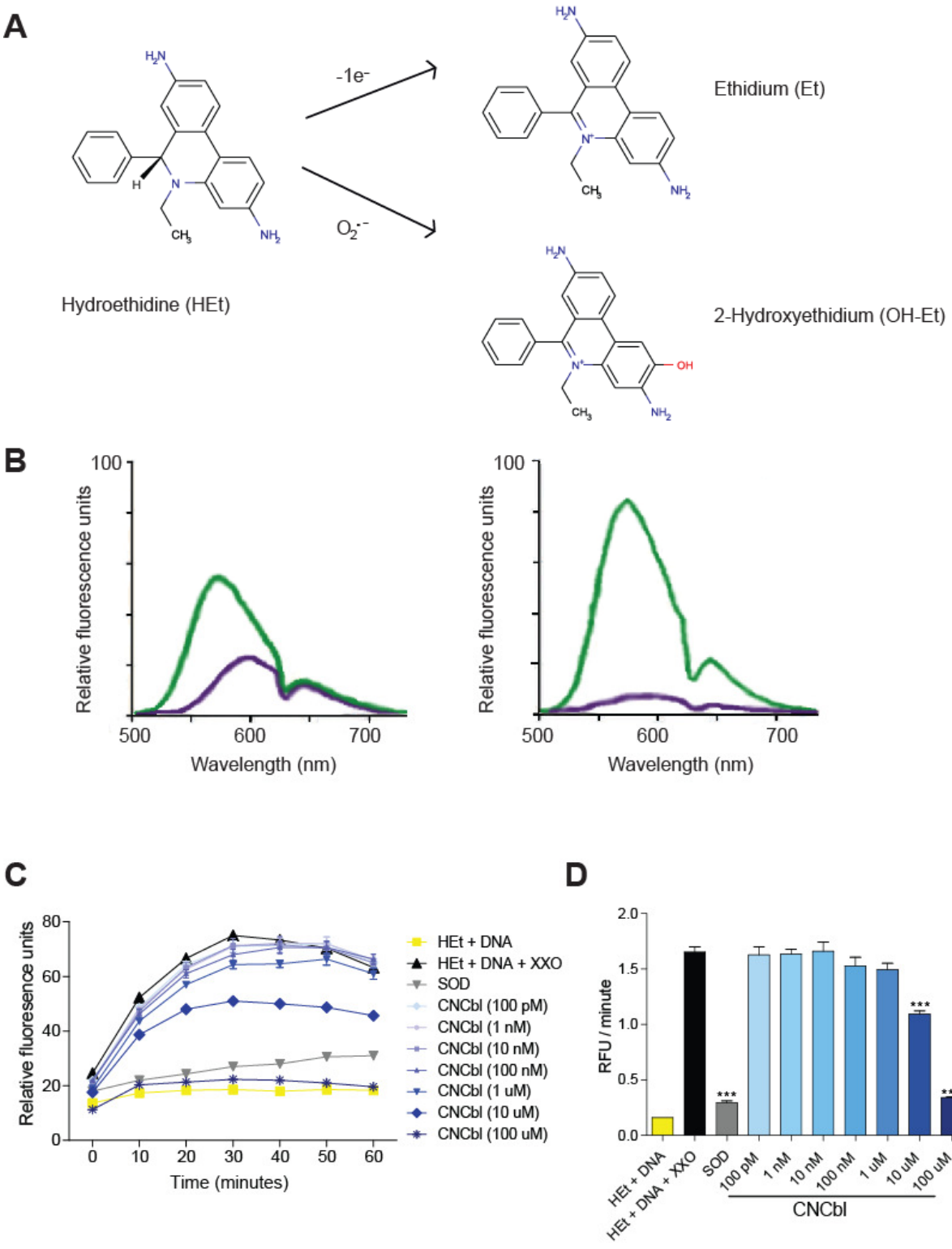


Figure 2. Vitamin B_{12} scavenges superoxide anion in a cell-free system.

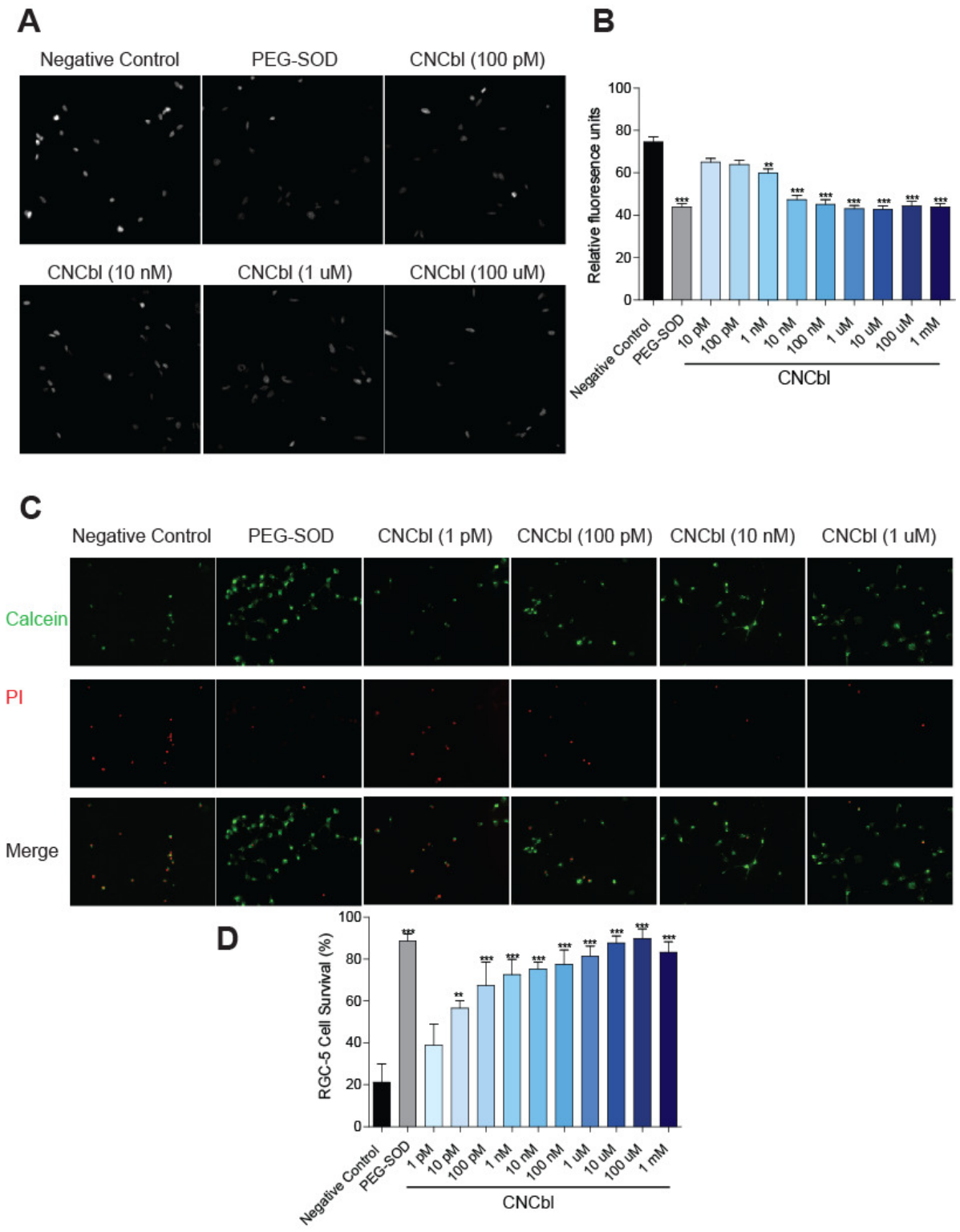


Figure 3. Vitamin B_{12} scavenges superoxide and is neuroprotective *in vitro* in menadione-treated RGC-5 cells.

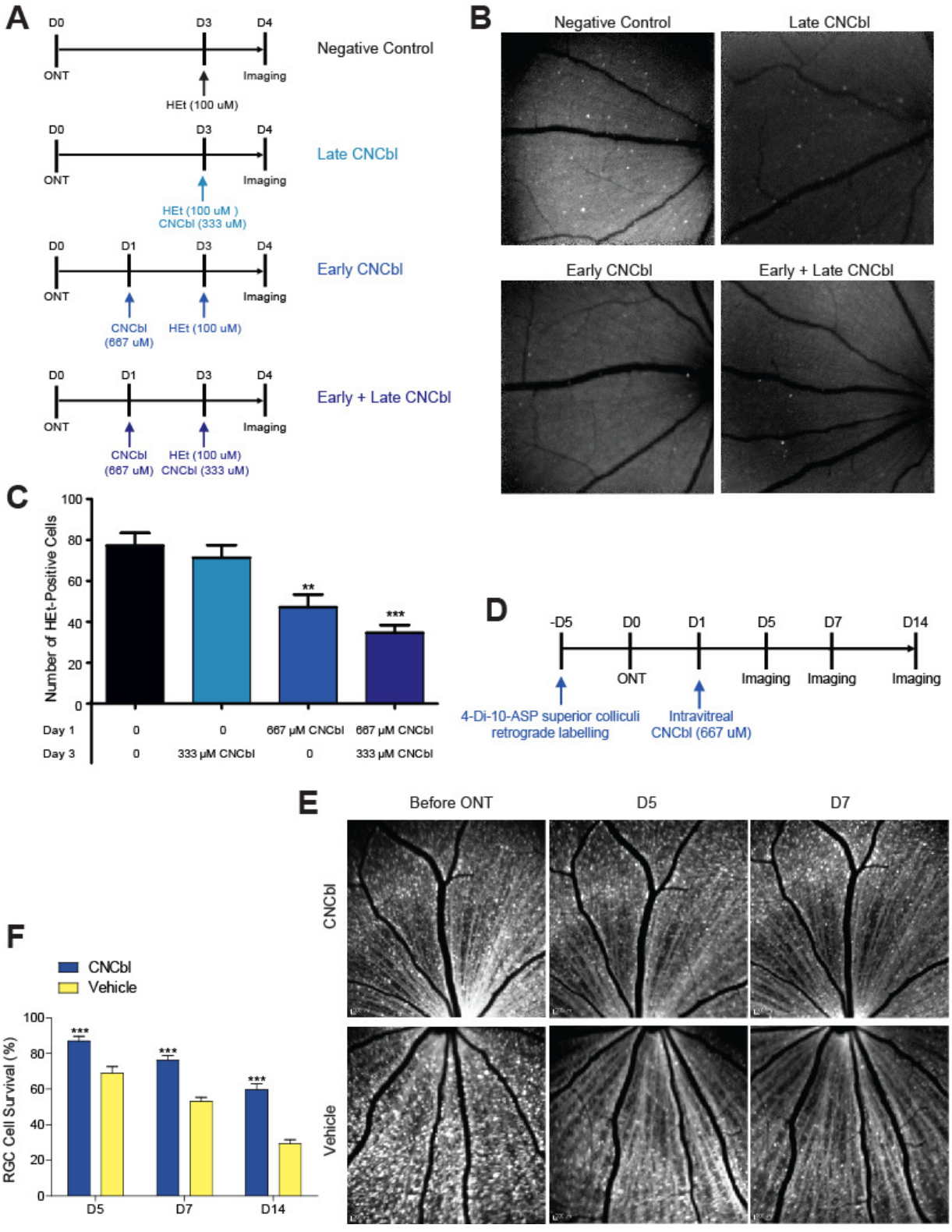


Figure 4. Vitamin B_{12} scavenges superoxide and is neuroprotective in vivo in rats with unilateral optic nerve transection

IV. DISCUSSION

4.1 Summary of Experimental Results

The results demonstrate that (i) CNCbl can scavenge superoxide anion generated in a cell free reaction between xanthine and xanthine oxidase; (ii) CNCbl is effective at scavenging superoxide produced by menadione-induced redox cycling and is also neuroprotective *in vitro* in RGC-5 neuronal cells exposed to excess superoxide; (iii) CNCbl is effective at scavenging superoxide and is neuroprotective *in vivo* in rat RGCs when CNCbl is injected into eyes that have undergone optic nerve transection.

4.2 Justification of Methods

Dihydroethidine (HEt) has two oxidation products (Figure 1): ethidium (Et), the single electron oxidation product of HEt, and 2-hydroxyethidium (OH-Et), the oxidation product of superoxide anion and HEt (Robinson et al., 2006). The emission spectra of OH-Et and Et overlap when excited at 560 nm (Robinson et al., 2006). However, OH-Et has a distinct excitation spectrum around 396 nm. Thus, to minimize excitation of both OH-Et and Et, we excited OH-Et more specifically using shorter wavelength light, with an excitation wavelength of 360 ± 40 nm for the cell-free experiments, 395 ± 5.5 nm for the *in vitro* superoxide anion experiments (epifluorescence microscopy of live cells), and 488 nm for the *in vivo* experiments (confocal laser scanning ophthalmoscopy; CSLO). While Et can also be partially excited at 488 nm, we have previously confirmed through

epifluorescent microscopy (395 ± 5.5 nm excitation) that the fluorescence detected *in vivo* by the CSLO is OH-Et (Kanamori et al., 2010a).

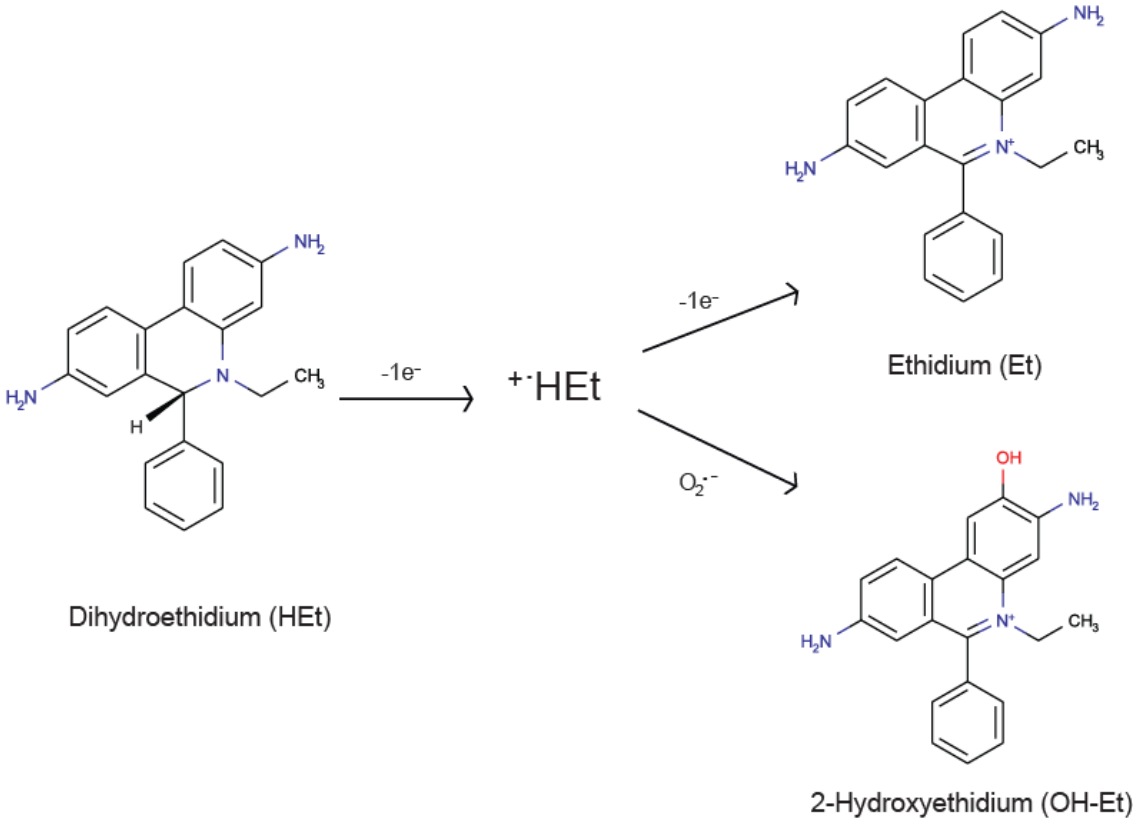


Figure 1. Chemical structures of dihydroethidium (HEt) oxidation products. Oxidation occurs in a two step fashion, involving formation of a HEt radical prior to further oxidation. Ethidium (Et) is the single electron oxidation product, while 2-hydroxyethidium (OH-Et) is the superoxide anion oxidation product. Redrawn from Kanamori, A, *et al.* (2010). Brain 133(9): 2612-2625.

The cell type and origin of the RGC-5 cells used in our *in vitro* studies are controversial (Van Bergen et al., 2009, Wood et al., 2010, Nieto et al., 2011). When differentiated with 316 nM staurosporine for 3-4 hours, RGC-5 cells stop dividing, extend neurites, and express ion channels, all of which are characteristic of neuronal cells (Frassetto et al., 2006). RGC-5 cells and cells of the mouse cone photoreceptor line 661W differentiate almost identically (Thompson et al, *unpublished data)*, consistent with the RGC-5 cell line being of retinal neuronal origin. Given that the goal of our *in vitro* model was to demonstrate whether CNCbl could scavenge superoxide and be neuroprotective for retinal neuronal cells, then the use of RGC-5 cells for *in vitro* studies was acceptable for preliminary experiments to determine the effects of vitamin B_{12} on neurons before proceeding with *in vivo* experiments.

Our optic nerve transection model in rats allows us to investigate whether CNCbl can scavenge superoxide and increase cell survival *in vivo,* with the added advantage of monitoring how transected RGCs respond to intravitreal injections of CNCbl in the same animal over time. We recognize that the axotomized RGC axons in this model are more representative of traumatic optic neuropathies than more slowly progressing neuropathies like glaucoma. However, all optic neuropathies, regardless of etiology, result in the eventual death of RGCs. Therefore using an acute injury model is appropriate, given that the goal of the study was to investigate the superoxide scavenging and neuroprotective properties of CNCbl. Studies in an experimental glaucoma model would be useful for confirming the present results.

The results from this study also apply primarily to the optic nerve and cannot necessarily be generalized to the spinal cord, peripheral nerves, or the brain, which are also affected by vitamin B_{12} deficiency. The results presented in this thesis are from laboratory experiments in a cell line and an animal model, and cannot be simply extrapolated to human patients. Further research on the effects of CNCbl in models of spinal cord, peripheral nerve, and cortical degeneration would provide greater insight into vitamin B_{12}'s role in different neurons.

4.3 General Discussion

Previous reaction kinetic studies have shown that vitamin B_{12} reacts with superoxide as a second order reaction to generate H_2O_2 at a rate of 7×10^8 $M^{-1}s^{-1}$, which is comparable to the SOD-catalyzed rate of 2×10^9 $M^{-1}s^{-1}$ (Suarez-Moreira et al., 2009). This group also demonstrated *in vitro* superoxide scavenging by CNCbl in a human aortic endothelial cell line. Clinically, patients with genetic vitamin B_{12} metabolism disorders have been found to have significantly greater levels of markers for oxidative stress in their urine (Mc Guire et al., 2009), likely due to the lack of critical superoxide scavenging by vitamin B_{12}. Furthermore, patients with chronic inflammatory diseases (Crocket, 1957, Flynn et al., 1994) and viral-induced inflammation (Wheatley, 2006) (both associated with oxidative stress) appear to benefit from vitamin B_{12} supplementation. Together with the findings from this study, vitamin B_{12} appears to play a role in the maintenance of cellular redox in addition to its known functions as a metabolic co-factor.

The mechanism of superoxide apoptotic signaling in vitamin B_{12} deficiency-optic neuropathy is not well understood. However, there appears to be a relationship between vitamin B_{12}, superoxide anion, and tumor necrosis factor (TNF-α). TNF-α is a cytokine mostly known for its role in the innate immune response and inflammation. Upon binding to either of its two receptors (TNFR1 or TNFR2), TNF-α can activate pathways involved in triggering an inflammatory response (activation of NF-κB pathway), cell proliferation or death in response to environmental stress (JNK pathway), or apoptosis through activation of caspases (Boldin et al., 1996, Hsu et al., 1996). Vitamin B_{12} has been found to mediate a balance between TNF-α and epidermal growth factor (EGF) in neurons through an unknown mechanism (Scalabrino, 2001, Miller, 2002). Clinically, patients with vitamin B_{12} deficiency have high levels of TNF-α in their cerebrospinal fluid compared to non-deficient control patients (Scalabrino et al., 2004). High levels of TNF-α in the aqueous humour of glaucoma patients have been reported (Balaiya et al., 2011). TNF-α and TNFR1 also appear to be upregulated in human glaucomatous optic nerve heads in parallel with the degree of optic nerve degeneration (Yuan and Neufeld, 2000). Experimentally, it was recently found that intravitreal injections of TNF-α lead to RGC cell death and optic nerve degeneration (Nakazawa et al., 2006, Lebrun-Julien et al., 2010).

It is possible that vitamin B_{12} mediates TNF-α through a superoxide-dependent pathway (Figure 2). Overexpression of SOD is known to inhibit apoptosis and render cells impervious to the cytotoxic effects of TNF-α (Afonso et al., 2006). One potential mechanism involves superoxide signaling TNF-α for

apoptosis when vitamin B_{12} is deficient, based on the recent findings that superoxide is an important molecule for TNF-α induction in microglia cells (Yoshino et al., 2011). Whether TNF-α is released predominately from retinal glia or neuronal cells in vitamin B_{12} deficiency is worth exploring, and further research would elucidate the cellular and molecular relationship between vitamin B_{12}, superoxide anion, and TNF-α.

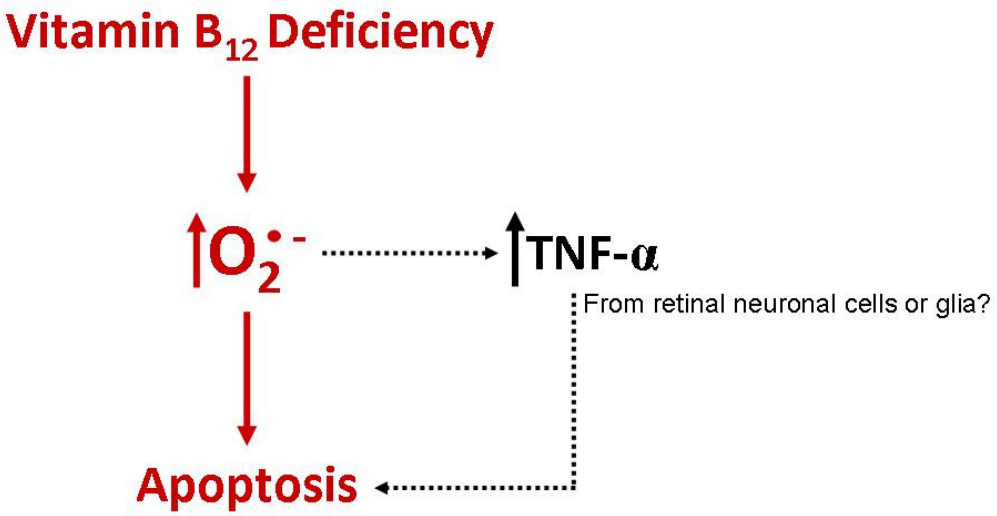


Figure 2. Potential relationship between vitamin B_{12}, superoxide anion, and tumour necrosis factor alpha (TNF-α). It is possible that increased superoxide anion production due to vitamin B_{12} deficiency signals TNF-α, which will then signal for apoptosis. It is unknown whether TNF-α is coming from the retinal neuronal cells or retinal glia.

V. CONCLUSION

Our data show that CNCbl, the form of vitamin B_{12} most commonly use in supplements, can scavenge superoxide anion and be neuroprotective for RGCs. These results corroborate previous studies demonstrating a novel role for vitamin B_{12} in maintaining cellular redox status, and support the hypothesis that optic neuropathies in patients with vitamin B_{12} deficiency may result from a misplaced signal for cell death from excess superoxide generation. Our results have important implications for the ocular health of populations at risk of vitamin B_{12} deficiency, most notably the elderly, vegans, and third world populations. Further study examining the prevalence of vitamin B_{12} deficiency in patients with optic neuropathies could prove informative for implementing preventative therapy for high risk populations.

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