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Evidence that the marine-derived multi-mineral Aquamin has anti-inflammatory effects on cortical glial-enriched cultures.

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4 **Evidence that the marine-derived multi-mineral Aquamin has anti-inflammatory**
5 **effects on cortical glial-enriched cultures.**
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30 **Short Title:** Anti-inflammatory properties of Aquamin in cortical glia
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35 **Key words:** Inflammation; Aquamin, glia, TNF α , IL-1 β , brain
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Abstract

It is well established that neuroinflammation contributes to brain aging, and that cortical cells are particularly vulnerable. Lipopolysaccharide stimulates the release of the pro-inflammatory cytokines, tumor necrosis factor-alpha and interleukin-1beta from glial cells which consequently induces an impairment in neuronal cell function. The food supplement Aquamin is a natural, multi-mineral derived from the red algae *Lithothamnion corallioides*, rich in calcium, magnesium and 72 other trace minerals. The aim of this study was to evaluate the anti-inflammatory potential of Aquamin in lipopolysaccharide-stimulated, glial-enriched primary cultures of rat cortex. We report that Aquamin prevented lipopolysaccharide-induced secretion of tumor necrosis factor-alpha and interleukin-1beta from cortical glia. These data suggest that nutritional supplements such as Aquamin may play an important role in impeding the detrimental effects of excessive inflammation in the brain.

Introduction

Minerals such as magnesium copper, zinc, manganese and selenium are now recognized as important regulators of inflammation and growing evidence suggests that mineral-rich seaweed extracts may play an important role in the regulation of inflammation (Granert *et al.*, 1999; Jung *et al.*, 2007). The food supplement, Aquamin is a natural seaweed-derived multi-mineral from the red algae *Lithothamnion corallioides* which is rich in calcium, magnesium and trace amounts of other minerals (Table 1). It has recently been shown to provide relief from the symptoms of osteoarthritis (Frestedt *et*

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4 *al.*, 2009) and to be of benefit in digestive and bone health (Aslam *et al.*, 2010a; Aslam
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7 *et al.*, 2010b).

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11 It is well established that inflammation contributes to cortical neuronal dysfunction in
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13 age-related neurological diseases such as Alzheimer's disease, amyotrophic lateral
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15 sclerosis, multiple sclerosis and Parkinson's disease (Minghetti, 2005). Microglia are
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17 considered the resident immune cells of the brain and along with astrocytes, constitute
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19 the major glial cell types. Under conditions such as neurodegenerative disease, injury,
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21 exposure to environmental toxins, infection or age, microglia become activated to
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23 release large numbers of mediators such as cytokines and free radicals. Microglial-
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25 secreted pro-inflammatory cytokines such as interleukin-1 beta (IL-1 β) and tumor
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27 necrosis factor alpha (TNF α) can influence neurons and their ability to process
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29 information and can ultimately contribute to neuronal cell death. Astrocytes provide
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31 structural and metabolic support to neurons and also play a role in brain inflammation
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33 through antigen presentation and cytokine secretion. It has previously been reported that
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35 exposure to the endotoxin lipopolysaccharide (LPS) induces release of IL-1 β and TNF α
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37 from cortical glia to contribute to neuronal degeneration (Long-Smith *et al.*, 2010). The
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39 present study was designed to investigate the anti-inflammatory potential of Aquamin in
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41 LPS-stimulated, glial-enriched rat cortical cultures to determine if nutritional
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43 supplements, such as Aquamin, may play a role in impeding the detrimental effects of
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45 excessive inflammation in the brain.
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56 **Materials and Methods**

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4 Aquamin (Food and Drug Administration (FDA) GRAS 000028) was prepared from the
5 mineral-rich red marine algae, *Lithothamnion corallioides* harvested off the Atlantic
6 coasts of Ireland and Iceland under approved licenses. The calcified seaweed was
7 separated from extraneous materials, sterilized, dried and milled under ISO and HACCP
8 certification. Approximately 0.5mg/ml of Aquamin is equivalent to a physiological
9 level of extracellular Ca²⁺. The concentrations of Aquamin were determined from
10 previous experiments using low passage human dermal fibroblasts, calcium sensitive
11 and calcium resistant colon carcinoma cell lines (CBS, Moser, Fet, HCT-116 and
12 SW480) (Aslam *et al.*, 2009), the murine macrophage cell line RAW 264.7, and the pre-
13 osteoblastic cell line MC3T3-E1 (unpublished data). Aquamin demonstrated no signs of
14 toxicity at any of the concentrations used in the current study. Glial-enriched cultures
15 were prepared from cortical tissue isolated from seven separate postnatal day 2 Sprague-
16 Dawley rat pups (Biological Services Unit, University College Cork) as previously
17 described (Long-Smith *et al.*, 2010). All scientific procedures were performed under a
18 license issued by the Department of Health and Children (Ireland) and in accordance
19 with the European Communities Council Directive (86/609/EEC). After 7-10 days *in*
20 *vitro* (DIV), glia were incubated in the presence or absence of LPS (50 ng/ml) and
21 Aquamin (0.05, 0.1, 0.5, 1 and 2 mg/ml). Twenty-four hours later, supernatant was
22 removed for analysis of TNF α and IL-1 β by ELISA (R&D Systems, UK). Untreated
23 culture media was used as a control. The cells were assessed for morphological signs of
24 apoptosis and necrosis (loss of cell membrane asymmetry and attachment, cell
25 shrinkage, cell blebbing, nuclear fragmentation and chromatin condensation) prior to,
26 during and after 24 hours incubation with Aquamin. All cells were considered viable
27 throughout the experiment. ANOVA with *post hoc* Student Newman-Keuls was used to
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4 determine which conditions were significantly different from each other. Results were
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6 expressed as means with standard error of the mean (SEM) and deemed significant
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8 when $p < 0.05$.
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10 11 12 13 14 **Results and Discussion** 15

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18 The data presented demonstrate that LPS induced a significant increase in TNF α (Figure
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20 1A) and IL-1 β (Figure 1B) secretion from glial-enriched cortical cultures 24 hours after
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22 LPS treatment ($p < 0.001$). Treatment with all doses of Aquamin significantly attenuated
23
24 the LPS-induced increase in TNF α and IL-1 β secretion ($p < 0.01$). Analysis of
25
26 extracellular concentrations of TNF α revealed that Aquamin prevented its release from
27
28 LPS-stimulated cortical glial cells in a dose-dependent manner and that all doses of
29
30 Aquamin significantly attenuated the LPS-induced release of IL-1 β . These data suggest
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32 that the anti-inflammatory effects of Aquamin may be reliant on blockade of the pro-
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34 inflammatory effects of both TNF α and IL-1 β .
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43 Seaweed extracts have previously been reported to inhibit pro-inflammatory cytokine
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45 release. Fucoidans from brown algae have been shown to inhibit TNF- α and IL-1
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47 production in cerebrospinal fluid in a rabbit meningitis model (Granert *et al.*, 1999) and
48
49 an extract from the seaweed *Hizikia fusiform* reduced TNF α production in LPS-
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51 stimulated murine microglial cells (Jung *et al.*, 2007). Aquamin is a seaweed-derived
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53 multi-mineral rich in calcium and magnesium. It has been shown that IL-1 β reduced
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55 Ca $^{2+}$ channel activity and influx in rat cortical neurons (MacManus *et al.*, 2000)
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57 suggesting that Ca $^{2+}$ supplementation may be beneficial in situations of excessive IL-
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4 1 β concentration in the brain such as occurs with aging. Increased magnesium in the
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6 diet may influence inflammation through reducing the serum level of the pro-
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8 inflammatory C-reactive protein. Copper, zinc and manganese are essential cofactors of
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10 the antioxidant enzyme superoxide dismutase and selenium is a vital constituent of the
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12 antioxidant glutathione peroxidase. A compromised antioxidant defense has been
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14 observed in the cortex of aged rats in parallel with increased IL-1 β concentration
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16 (McGahon *et al.*, 1999). Accumulation of reactive oxygen species in the rat cortex and
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18 hippocampus has been demonstrated after LPS treatment (Nolan *et al.*, 2003) while an
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20 antioxidant-enriched diet has been shown to reverse age-related and inflammatory-
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22 induced neuronal deficits (McGahon *et al.*, 1999). Consequently, many of the
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24 antioxidant-related minerals that compose Aquamin may be anti-inflammatory and may
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26 directly or indirectly have neuroprotective properties.
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35 The current study provides clear evidence that Aquamin exerts anti-inflammatory
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37 effects by preventing LPS-induced TNF α and IL-1 β release from rat cortical glia. This
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39 evidence for an anti-neuroinflammatory activity of the food supplement Aquamin may
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41 have significant implications for brain health
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Table 1: Typical Mineral Composition of Aquamin.

Mineral	Dry Salt Weight
Calcium	141,200ppm
Magnesium	18,580ppm
Phosphorous	436ppm
Potassium	81.5ppm
Sulphur	3620ppm
Iron	234ppm
Boron	8.45ppm
Sodium	1780ppm
Manganese	9.71ppm
Cobalt	<0.05ppm
Copper	0.191ppm
Zinc	10.7ppm
Selenium	1.75ppm

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Figure Legends:

Figure 1: Aquamin prevents LPS-induced release of TNF α and IL-1 β . TNF α (A) and IL-1 β (B) levels from LPS-stimulated glial-enriched cortical cultures. Data are expressed means \pm SEM of seven independent experiments, each performed in triplicate. ***p<0.001 vs. control; ++p<0.01; +++p<0.001 vs. LPS (ANOVA).

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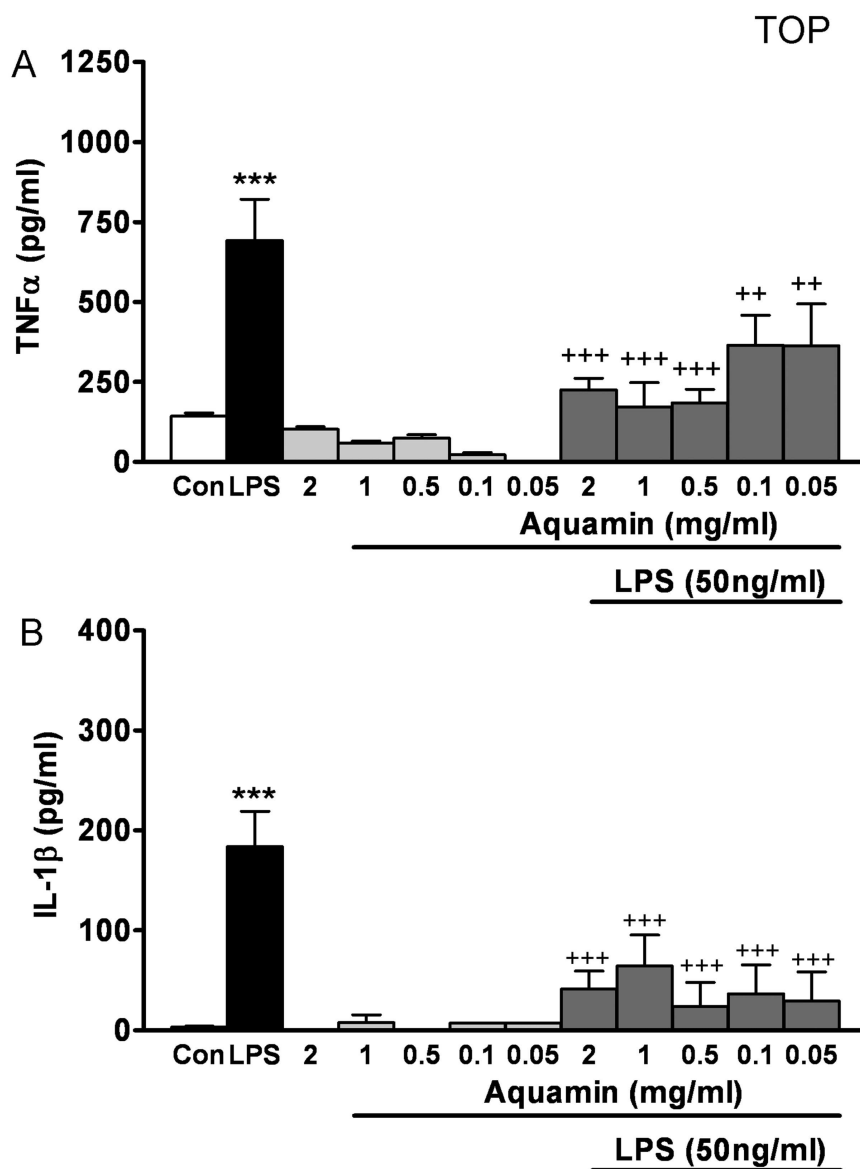


Figure 1: Aquamin prevents LPS-induced release of TNF α and IL-1 β . TNF α (A) and IL-1 β (B) levels from LPS-stimulated glial-enriched cortical cultures. Data are expressed means \pm SEM of seven independent experiments, each performed in triplicate. *** p <0.001 vs. control; ++ p <0.01; +++ p <0.001 vs. LPS (ANOVA).
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