Colostrinin™ Alleviates Amyloid-β Induced Toxicity in Rat Primary Hippocampal Cultures

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ABSTRACT
Colostrinin™ (CLN), a complex mixture of proline-rich polypeptides derived from colostrums, can alleviate cognitive decline in early Alzheimer’s disease patients. The molecular basis of the action of CLN has been studied in vitro using human neuroblastoma cell lines. The aim of the present study was to use quantitative immunocytochemistry and immunoblotting to investigate the ability of CLN to relieve amyloid-β (Aβ)-induced cytotoxicity in rat primary hippocampal neuronal cells. Our data confirm that CLN alleviates the effect of Aβ-induced cytotoxicity and causes a significant reduction in the elevated levels of the antioxidant enzyme SOD1.

Keywords: Alzheimer’s disease, amyloid-β, colostrinin, hippocampus, oxidative stress, superoxide dismutase

Alzheimer’s disease is the most common form of dementia, affecting 18 million people worldwide [1]. It is characterized by extracellular senile plaques consisting mainly of aggregated amyloid-β (Aβ) and intracellular neurofibrillary tangles, containing the cytoskeletal protein tau [2]. Colostrinin™ (CLN) has been shown to alleviate symptoms of Alzheimer’s disease [3]. The possible mechanism of action of CLN has been tested in vitro using cell lines, where it has been shown to protect against oxidative stress [12]. In particular, CLN has been found to prevent increases in the levels of reactive oxygen species (ROS) in response to hydrogen peroxide (H2O2) or Aβ in the SH-SY5Y human neuroblastoma cell line [4], although the precise mechanism by which these effects occur remains unclear. A key defense against ROS-induced damage is provided by the antioxidant enzyme superoxide dismutase 1 (SOD1) [5], and in AD patients increased levels of SOD1 have been found associated with plaques [6]. In the present study, we have investigated the mechanism of action of CLN using, for the first time, cultured primary rat hippocampal cells.

Sprague Dawley rats of both sexes were used at embryonic day 18 (E18). All animal experimentation was carried out under UK Government Home Office guidelines. A protocol for the culture of dissociated rat hippocampal cells similar to that of Banker and Cowan [7] was used. Live cells were plated at a density of 20,000 cells/coverslip or 400,000 cells/well in BME (Basal Medium Eagle) containing 0.5% glucose, 1 mM sodium pyruvate, N2 supplement, and 10% horse serum (Gibco/Invitrogen) on either poly-lysine coated 19 mm coverslips (VWR) for immunocytochemistry, or poly-lysine coated plates, for Western blotting. On the third day in vitro (DIV3), medium was replaced with BME including the supplement B27 (Gibco/Invitrogen). Bovine CLN was isolated from bovine colostrum and was supplied by ReGen Therapeutics. All the CLN used for the experiments reported in the present study was from the same batch. CLN was dissolved in phosphate buffered saline (PBS) at 2 mg/ml and diluted as appropriate. Aβ1-42 (Biosource) was dissolved in saline,
diluted to 1 mg/ml in PBS, and incubated for 48 h at 37°C/5% CO\textsubscript{2} with or without bovine CLN. Cultures were treated on DIV3 for 24 h for Western blotting experiments and for 48 h for immunocytochemistry experiments.

Primary hippocampal cultures were fixed in 4% paraformaldehyde/PBS pH 7.4 for 1 h at room temperature (RT) and washed with PBS [8,9]. Rabbit polyclonal anti-GFAP (glial fibrillary acidic protein) antibody raised against cow GFAP (Dako-Cytomation 20334, 1:1000) or mouse monoclonal anti-rat brain MAP-2 (microtubule associated protein) (Abcam ab11267, 1:1000) were applied overnight or for 48 h at RT, respectively. After washing in PBS, cultures were incubated with anti-rabbit biotinylated antibody (GE Healthcare RPN1004V1, 1:250), and strepavidin fluorescein (GE Healthcare RPN123V1, 1:100) for GFAP, or goat-anti-mouse amino-methyl-coumarin-acetate (AMCA) conjugated secondary antibody (Millipore AP130M, 1:100) for MAP-2. Immunopositive cells were counted using a square grid system on the stage manager function on an Olympus BX61 microscope at x400 magnification. One hundred grid squares (347.64 µm by 260.73 µm) covering 9 mm\textsuperscript{2} of the coverslip were counted for each coverslip with at least three replicates for each condition per experiment.

For Western blotting, treated cells were lysed with radio-immunoprecipitation assay lysis buffer containing protease inhibitors, sonicated and spun. Protein content was measured using a standard assay (Biorad), and samples were run at 100V for 2 h on 15% SDS polyacrylamide gels, which were transferred to nitrocellulose membranes. Rabbit anti-SOD1 antibody (Abcam ab51254) was applied at 1:25,000 for 1 h at RT before incubation in anti-rabbit horseradish peroxidise conjugated secondary (Thermo Scientific-Pierce 31460) for 1 h at RT. Enhanced chemiluminescent reagent (ECL, from Perkin-Elma) was used to detect horseradish peroxidase and developing film was exposed to the blot. A second blocking step was carried out before rabbit anti-actin antibody (Rabbit monoclonal antibody against modified β-cytoplasmic actin Nterminal peptide, Sigma-Aldrich A1978) was applied overnight at 4°C. Developed films were scanned as greyscale images and Image J software was used to carry out densitometric analysis. Quantitative data were obtained in the form of cell counts or semi-quantitative densitometry values expressed as percentage of control for Western blots. Data were analyzed using one-way ANOVAs with a Bonferroni post-hoc test, or for non-parametric data using a Mann-Whitney test for comparisons between two groups. Data are expressed as means ±SEM.

Treatment of cultures for 48 h with 25 µM A\textsubscript{β}1-42 resulted in MAP-2 positive cells (neurons) with shrunken appearance and reduced processes. These changes were much less apparent when 5 µg/ml bovine CLN was pre-incubated and co-administered with A\textsubscript{β}1-42. Densitometric quantification of Western blots (Figure 1A and 1B ) showed that 24 h treatment of primary rat hippocampal cultures with 10 µM A\textsubscript{β}1-42 caused a significant increase in the SOD1 protein levels (28%, P=0.037, Figure 1B) compared to untreated control cells. Pre-incubation and coadministration of 5 ng/ml or 5 µg/ml bovine CLN with A\textsubscript{β}1-42 reduced significantly the A\textsubscript{β}1-42- induced increase in SOD1 protein levels to control levels (P=0.05, Figure 1B). Quantification of MAP-2 cells (Figure 2A) revealed significant cell loss (44%, P=0.003) following treatment of dissociated hippocampal cultures with A\textsubscript{β}1-42. The reduction in MAP-2 positive cells was alleviated when CLN at 5 µg/ml was pre-incubated and co-administered with A\textsubscript{β}1-42 (20%), compared to A\textsubscript{β}1-42 alone (Figure 2A) but this reduction was just below the 0.05 significance level.
The morphology of GFAP positive astrocytes was unaltered by treatment with A\(\beta\)1-42 compared to control. While treatment of cultures with 25 \(\mu\)M A\(\beta\)1-42 for 48 h led to a large reduction (54%) in the number of GFAP positive cells present in the cultures (Figure 2B), CLN at 5 \(\mu\)g/ml did not significantly reduce this loss (Figure 2B).

The efficacy of CLN as a potential treatment for neurodegenerative disorders has been reviewed by Rattray [10], Boldogh and Kruzel [4], and Stewart [11]. A\(\beta\)1-42 is known to have a toxic effect on SHSY-5Y cells in culture when pre-incubated and co-administered with A\(\beta\)1-42 [12], an effect which is significantly alleviated by CLN. Moreover, 24 h pre-treatment of SHSY-5Y cells with 5 \(\mu\)g/ml CLN was shown to reduce A\(\beta\)1-40-induced apoptosis in human neuronal cells [13]. Our data confirm the efficacy of CLN in alleviating the adverse effects of A\(\beta\)1-42, in the present case its ability to protect primary hippocampal cells. Treatment of these cells with 25 \(\mu\)M A\(\beta\)1-42 caused a significant (44%) decrease in the number of MAP-2 positive neurons present in these primary cell cultures compared to controls. When A\(\beta\)1-42 was pre-incubated with bovine CLN before application to cultured cells, there was a reduction of this A\(\beta\)1-42-induced loss of MAP-2 positive neurons, and Western[11] blot data demonstrated that pre-treatment with CLN also produced a significant reduction in the elevated levels of the antioxidant enzyme SOD1 seen in response to A\(\beta\)1-42 treatment. These data confirm that CLN may prevent oxidative stress and hence that it could have prophylactic potential to alleviate neurodegenerative disorders.

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REFERENCES


**FIGURE LEGENDS**

**Figure 1.** A) A representative Western blot for SOD1 for densitometric quantification of SOD1 from primary hippocampal cell line lysate after treatment with 5 ng/ml or 5 µg/ml bovine CLN, 10 µM Aβ1-42 or CLN pre-incubated and co-administered with Aβ1-42. B) SOD1 protein levels in cultures treated with CLN at 5 ng/ml or 5 µg/ml. 10 µM Aβ1-42 increased SOD1 protein levels (P=0.037) and pre-incubation and co-administration of bovine CLN prevented this increase (P=0.05). n=3. * represents P<0.05. Error bars ±SEM.

**Figure 2.** A) Quantification of the number of MAP-2 positive neurons and B) GFAP positive astrocytes in dissociated hippocampal cultures at 20,000 cells/well treated with Aβ1-42 and CLN. The number of MAP-2 positive cells (neurons) (A), and GFAP positive cells (glial cells) (B) decreased when cultures were treated with 25 µM Aβ1-42 alone (but was significant only for MAP-2 positive cells; P=0.03). The decrease in MAP-2 cells was alleviated when with Aβ1-42 and was co-incubated with CLN but much less so for GFAP. n=4 with at least 3 replicates per condition in each experiment, error bars ±SEM. * indicates P=0.003.

Fig 1A
|                | 5ng/ml | 5μg/ml | 10μM Aβ1-42 | 10μM Aβ1-42 | 10μM Aβ1-42  
|----------------|--------|--------|-------------|-------------|---------------
| Control        | CLN    | CLN    | CLN         | CLN         | CLN           
| 5ng/ml Aβ1-42  | + CLN  | + CLN  | + CLN       | + CLN       | + CLN         
| 5μg/ml Aβ1-42  | + CLN  | + CLN  | + CLN       | + CLN       | + CLN         

Fig 1B
**Fig 2A**

The figure shows a bar graph representing SOD1 protein corrected for actin as a percentage of control after different treatments. The treatments include control, 5ng/ml CLN, 5μg/ml CLN, 10μM Aβ1-42, 10μM Aβ1-42 + 5ng/ml CLN, and 10μM Aβ1-42 + 5μg/ml CLN. The p-values indicate statistical significance: *P=0.037* and *P=0.05*.
Fig 2B

Number of MAP-2 positive cells in 9 mm²

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>300</td>
</tr>
<tr>
<td>5μg/ml CLN</td>
<td>330</td>
</tr>
<tr>
<td>25μM Aβ1-42</td>
<td>220</td>
</tr>
<tr>
<td>25μM Aβ1-42 + 5μg/ml CLN</td>
<td>250</td>
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* P=0.003