

## Protective Activity of the Extracts from Japanese eel (*Anguilla japonica*) Against Zinc-induced Neuronal Cell Death: Carnosine and an Unknown Substance

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### Summary

The Japanese eel, *Anguilla japonica*, is the traditional staple food in summer in Japan. We have previously reported that both carnosine and an aqueous extract from eel muscle prevent zinc-induced GT1-7 cell death, which is associated with neuronal death after transient global ischemia. In this study, we found that these protective activities were exhibited by the heat-treated extracts from both eel muscle and skin. We developed a high-performance liquid chromatography system for quantifying carnosine; this system demonstrated that the muscle extract contained carnosine in an amount sufficient for preventing zinc-induced GT1-7 cell death, which reveals that carnosine is the substance in the muscle extract that is responsible for the protective activity. The analysis of the expression of zinc-inducible gene indicates that carnosine acts as an intracellular protective substance in GT1-7 cells. We also demonstrated that the eel skin contained unknown substance(s) that prevent zinc-induced cell death.

Zinc -an essential trace element in the body- is concentrated in the central nervous system and is released during synaptic activity or membrane depolarization<sup>1-3</sup>). Recent studies have suggested that endogenous zinc plays the role of an ionic mediator of neuronal cell death<sup>4</sup>). Following brain injury such as global ischemia, presynaptic zinc specifically accumulated in the degenerating neurons of the hippocampal CA1 as well as other vulnerable neurons in the cortex, amygdala, striatum, and thalamus, thus resulting in neurodegeneration<sup>5, 6</sup>). An experiment using a metal chelator revealed that the inhibition of such a Zn<sup>2+</sup> accumulation drastically reduced ischemic neuronal cell death, and strongly suggests that the inter-neuronal movement of Zn<sup>2+</sup> is a key mechanism involved in ischemic neuronal death<sup>6</sup>). In other words, zinc-induced neuronal cell death is a good phenomenon for examining the neurodegenerative damage following brain injury such as transient ischemia<sup>4</sup>). Thus, protective agents against zinc-induced neuronal cell death may prove to be good candidates for the development of a medicine for reducing neurodegenerative damage following transient ischemia<sup>4</sup>).

We have established a convenient and rapid screening system for substances with protective activity against zinc-induced neurodegeneration by using GT1-7 cells, the immortalized hypothalamic neurons<sup>7-9</sup>), which are very sensitive to zinc. Additionally, we have previously reported that both carnosine and eel muscle extract inhibit zinc-induced GT1-7 cell death<sup>10</sup>). In this study, we further investigated the protective activities in both muscle and skin of the Japanese eel, *Anguilla japonica*. We quantitatively analyzed carnosine and demonstrated that it was the substance in the eel muscle extract responsible for prevention against zinc-induced GT1-7 cell death; we found that the eel skin contained unknown substance(s) that prevented zinc-induced cell death. We also investigated the mechanism of the protective activity of carnosine in GT1-7 cells.

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## Materials and Methods

### 1. Preparation of eel extracts

We obtained Japanese eels (*Anguilla japonica*) from local commercial sources and separated their skin and muscles. An appropriate amount of water was added to the specimens, and the mixture was homogenized in a juicer. The supernatants were obtained by successive centrifugations at 12,000 *g* for 20 min and at 20,000 *g* for 30 min. The aliquots of extracts filtered through a 0.22- $\mu$ m-pore filter were used as unheated samples. The remainder sample was heated at 95 °C for 30 min and then centrifuged at 20,000 *g* for 30 min. The clear supernatants were filtered through a 0.22- $\mu$ m-pore filter and then lyophilized.

### 2. Cell culture and cell viability assay

GT1-7 cells were cultured and the cell viabilities were determined as described previously<sup>9)</sup>. The cells were resuspended in serum-free Dulbecco's modified Eagle's medium and plated on 96-well formatted culture dishes at a concentration of  $5 \times 10^5$  cells/well. The viability of the cells was measured by the WST-1 assay (Cell Counting Kit; Dojindo Chemicals, Japan) after a 24 h exposure to zinc. The eel extracts were preadministered to the culture medium immediately before exposure to zinc.

### 3. Morphological observation

The GT1-7 cells cultured on the plates were treated with 30  $\mu$ M ZnCl<sub>2</sub> and/or eel extract for 24 h. The phase-contrast images of the cells were observed under a microscope (IX71; Olympus, Japan).

### 4. High-performance liquid chromatography analysis for carnosine and mass spectrometry

A reverse-phase high-performance liquid chromatography (HPLC) system using a SHIMADZU CLASS-VP HPLC system (Shimadzu Co. Ltd., Kyoto, Japan) with a Hypercarb column (4.6 mm i.d.  $\times$  100 mm; Thermo Electron Corp., Waltham, MA, USA) was used. Carnosine was analyzed using a 10-min linear gradient of 2–10 % acetonitrile in the presence of 0.05 % trifluoroacetic acid at a flow rate of 1 mL/min, and monitored at 215 nm. Mass analyses were performed using an LCQ advantage ion-trap mass spectrometer equipped with an electrospray ion source (Thermo Electron Corp.). The mass spectrometer was operated in the positive ion mode. Mass spectra for mass analyses were acquired over *m/z* 100 to 2000.

### 5. Real-Time polymerase chain reaction

The GT1-7 cells grown in 12-well culture plates ( $5 \times 10^6$  cells/well) were treated with 50  $\mu$ M ZnCl<sub>2</sub> with or without the eel extract for 2 h. Total RNA was extracted, and first-strand cDNA was synthesized using SuperScript<sup>TM</sup> III First-Strand Synthesis System for reverse transcription-polymerase chain reaction (RT-PCR) (Invitrogen, Carlsbad, CA, USA) by priming with oligo-(dT)<sub>20</sub>. Real-time PCR was performed on the LightCycler (Roche Diagnostics, Mannheim, Germany) using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) with the following primers: 5'-GGC CAA CAC CAG CAA TTC CAA CG-3' (forward) and 5'-AAG GCA TTC ACG ACC ACG ATC ACG-3' (reverse) for ZNT1; 5'-CGC ATC CTC TTC CTC CCT GG-3' (forward) and 5'-CCT AGA AGC ACT TGC GGT GCA C-3' (reverse) for  $\beta$ -actin.

### 6. Statistical analysis

A statistical analysis of cell viability was carried out using the Student's *t* test. Data are expressed as means  $\pm$  SD. The value of *p* < 0.001 was considered significant.

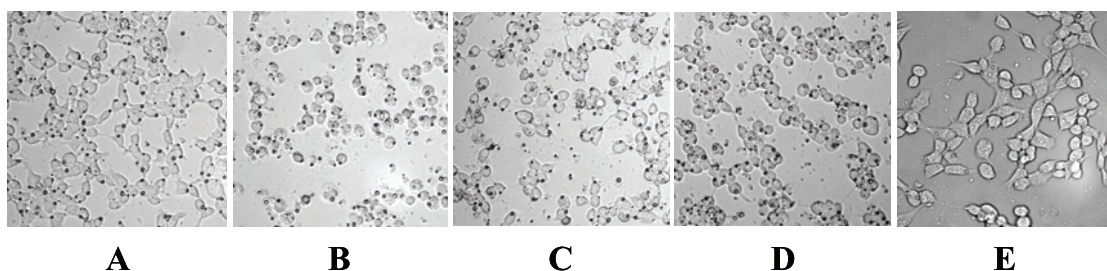
## Results

### 1. Heat-treated extracts from both eel muscle and skin prevent zinc-induced GT1-7 cell death

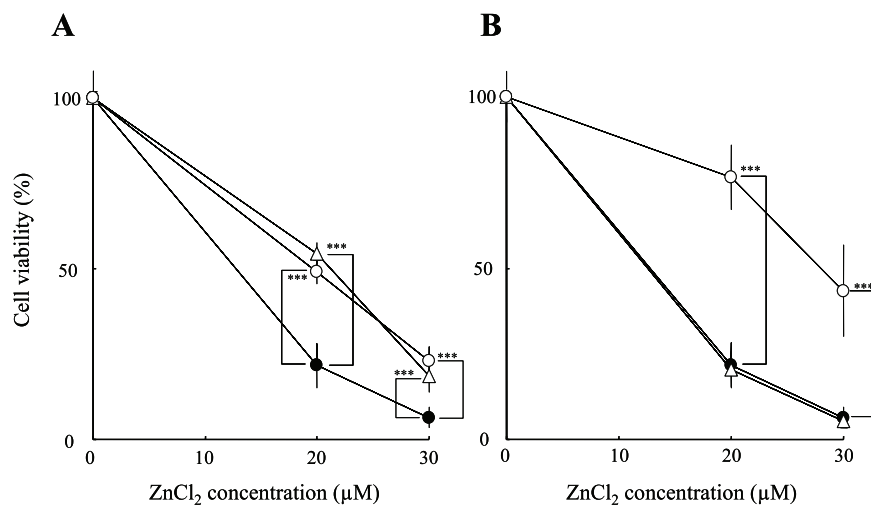
The aqueous supernatants obtained from both the muscle and skin of the Japanese eel were heated to reduce the

viscosity of the samples. The heat-treated extracts were administered to GT1-7 cells that were then observed by phase-contrast microscopy (Fig. 1). The GT1-7 cells not treated with zinc exhibited spherical or neuron-like shapes (A). The exposure to zinc caused morphological changes in the cells, such as shrinkage and formation of apoptosis-like shapes (B). However, the administration of the heat-treated extracts from the eel muscle (C) or skin (D) resulted in little change in cell morphology even after exposure to zinc. We used carnosine as a positive control for preventing the zinc-induced morphological changes in the cell shapes (E).

We further examined the effect of the heat treatment of the extract on the protective activity against zinc-induced GT1-7 cell death (Fig. 2). Heat treatment did not affect the protective activity of the muscle extract (Fig. 2A); however, heat treatment was necessary to demonstrate the protective activity of the skin extract (Fig. 2B). These results show that both eel muscle and skin have protective activity against zinc-induced GT1-7 cell death, and the substances responsible for such protection are heat-stable.



**Fig. 1** The protective activity of the eel extracts against a zinc-induced GT1-7 cell death. The GT1-7 cells were treated with the 30  $\mu\text{M}$   $\text{ZnCl}_2$  with or without the extracts, and observed under a phase-contrast microscope after a 24-h exposure to zinc. (A): Control (without  $\text{ZnCl}_2$ ), (B): treated with  $\text{ZnCl}_2$  alone (30  $\mu\text{M}$ ), (C):  $\text{ZnCl}_2$  in the presence of the eel extract from the muscle, (D):  $\text{ZnCl}_2$  in the presence of the eel extract from the skin, (E):  $\text{ZnCl}_2$  in the presence of 10 mM carnosine.

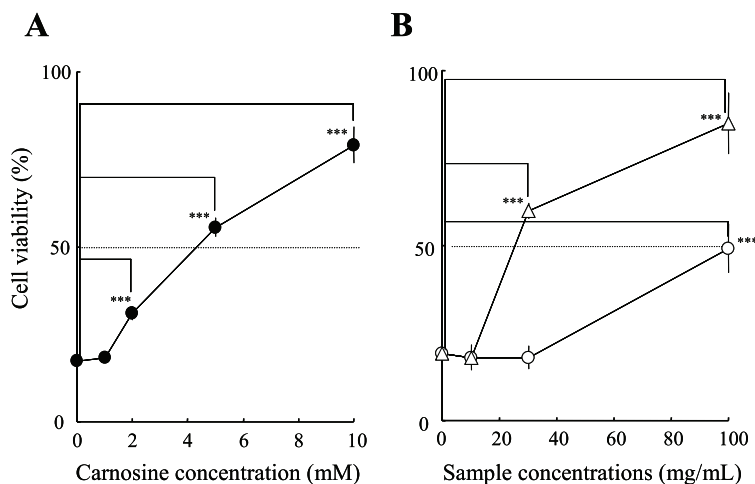


**Fig. 2** The effect of heat treatment of the eel extracts obtained from the muscle (A) and skin (B) on zinc-induced cell death. The viability of the GT1-7 cells was determined after exposure to 20 or 30  $\mu\text{M}$   $\text{ZnCl}_2$  with the unheated extracts (open triangles), heat-treated extracts (open circles), and without the extract (closed symbols). The vertical axes of both graphs are expressed as the percentage of viability in the cells treated without  $\text{ZnCl}_2$ , which is assumed to be 100 %. Data are expressed as mean  $\pm$  SD,  $n = 6$ . \*\*\*: indicates the respective significances at  $p < 0.001$  vs. the cells treated with  $\text{ZnCl}_2$  alone.

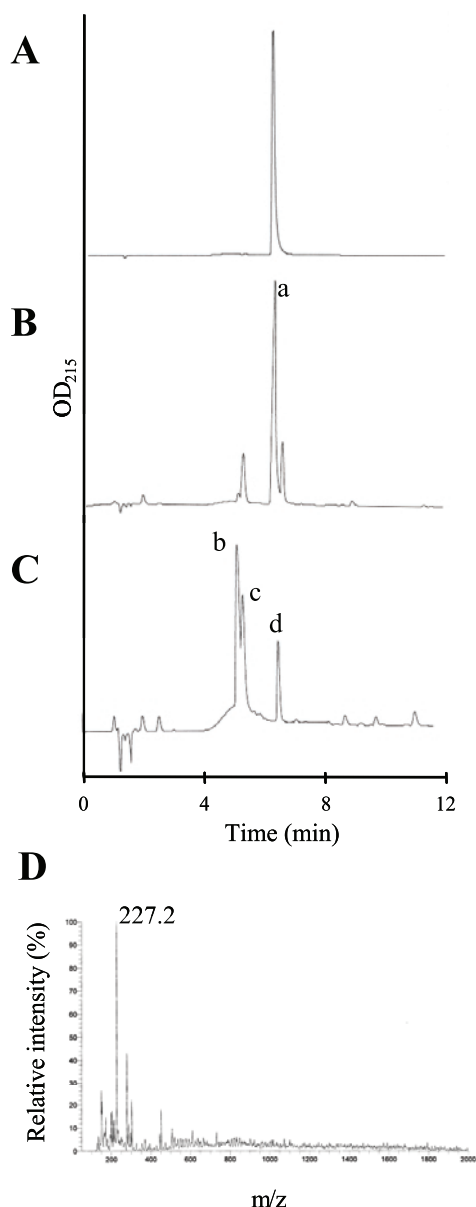
## 2. Protection against zinc-induced GT1-7 cell death is provided by carnosine in the eel muscle, and an unknown substance(s) in the eel skin

The protective activities of the eel extracts and carnosine were compared quantitatively (Fig. 3). The freeze-dried eel extracts were dissolved in an appropriate amount of water, and the viabilities of GT1-7 cells were determined after exposing them to zinc in the presence of carnosine or the extracts. Carnosine demonstrated its protective activity in a concentration-dependent manner (Fig. 3A), which indicates that 50 % cells survived as a result of the administration of approximately 4 mM carnosine. Figure 3B shows that 50 % cells survived due to the administration of 1 mg/mL muscle extract or 0.3 mg/mL skin extract. This result shows that the protective activity of the skin extract is stronger than that of the muscle extract.

We developed an HPLC system for analyzing and quantifying carnosine, which has the ability to separate carnosine derivatives, including anserine, homocarnosine, or alanylhistidine, from each other (data not shown). In this system, carnosine appeared at 6.1 min (Fig. 4A). The peaks that had the same retention time as carnosine appeared in extracts from both eel muscle (B, peak a) and eel skin (C, peak d). Electrospray-ionization (ESI)-mass spectrometry demonstrated that a major signal appeared at  $227.2 [M+H]^+$  in both the peaks, which corresponds to the calculated mass of carnosine, i.e., 226.24 (Fig. 4D). Using this system, the concentrations of carnosine in both the extracts were determined; the concentrations were determined to be 3.5 mM in 1 mg/mL muscle extract and 0.15 mM in 1 mg/mL skin extract. These quantitative results show that the substance responsible for the protective activity in the eel muscle extract is carnosine because the concentration of carnosine in the 1 mg/mL muscle extract almost corresponded to the carnosine concentration (4 mM) in the surviving 50 % cells. On the other hand, the quantitative result indicates that the eel skin extract contains unknown substance(s) that is/are responsible for the protective activity against the zinc-induced GT1-7 cell death; this is because the carnosine content in the eel skin extract was too low to exhibit any protective activity.



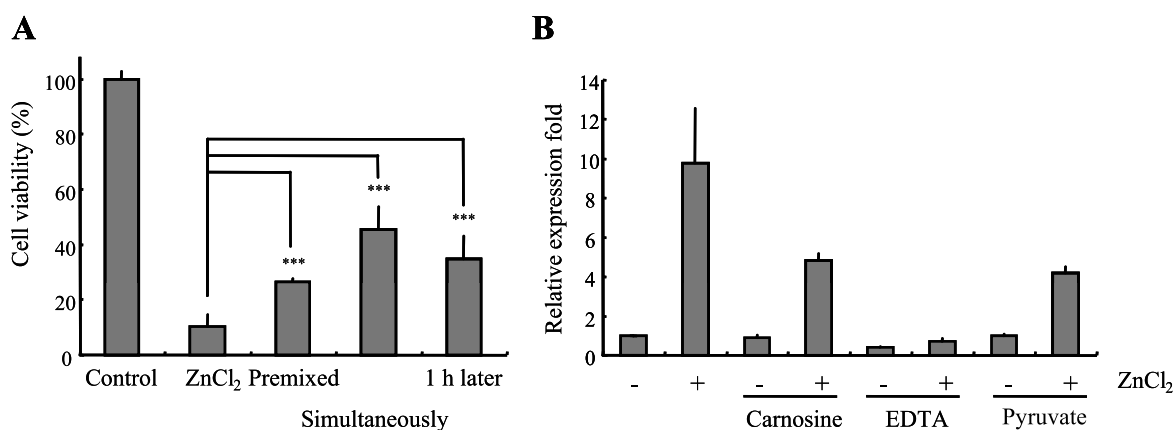
**Fig. 3** The effect of carnosine (A) and the eel extracts (B) on the zinc-induced GT1-7 cell death. The viabilities of the GT1-7 cells were determined after exposure to  $30 \mu\text{M}$   $\text{ZnCl}_2$  at various concentrations of carnosine (closed circles) and the heat-treated extracts from eel muscle (open circles) and skin (open triangles). The vertical axes of both graphs express the percentage of the viability of the cells not treated with  $\text{ZnCl}_2$ , which is assumed to be 100 %. The data are expressed as mean  $\pm$  SD,  $n = 6$ . \*\*\* indicates the respective significances at  $p < 0.001$  vs. Control.



**Fig. 4** The HPLC profiles of carnosine (A), the heat-treated extract from eel muscle (B) and eel skin (C). The peaks indicated by the marks (a-d) were isolated and analyzed by mass spectrometry. Mass spectrum of the peak marked by “a” is shown (D).

### 3. Carnosine acts as an intracellular protective substance against the zinc-induced GT1-7 cell death

To investigate the molecular mechanisms through which carnosine protects against zinc-induced GT1-7 cell death, we first examined whether the reduction in extracellular zinc concentration was the main reason for the protection because carnosine was reported to have the ability to bind to zinc<sup>11</sup>. We first examined the effect of the timing of administering carnosine on zinc-induced GT1-7 cell death. The protection activities in the experiments in which ZnCl<sub>2</sub> was premixed with carnosine were slightly lower than those in the experiments in which ZnCl<sub>2</sub> and carnosine were simultaneously administered and when carnosine was added 1 h after ZnCl<sub>2</sub> administration (Fig. 5A). The protective activity of carnosine was retained even when it was administered 1 h after zinc exposure. We further examined zinc-induced gene expression as a marker for the elevation of intracellular zinc concentration using a real-time PCR technique (Fig. 5B). The expression of intracellular zinc transporter protein 1 (ZNT1), which was induced by zinc to export the intracellular zinc out of the cells<sup>12</sup>, increased approximately 10-fold as a result of the exposure to 50 μM ZnCl<sub>2</sub> for 2 h. In the presence of carnosine, the expression of the ZNT1 gene was increased approximately 5-fold by zinc. The induction fold in the presence of carnosine was almost the same as that of pyruvate, which is reported to act as intracellular protective substance<sup>13</sup>. On the other hand, ethylenediamine tetraacetic acid (EDTA)



**Fig. 5** Effect of the timing of administering carnosine on zinc-induced GT1-7 cell death (A) and effect of the protective substances on zinc-induced ZNT1 gene expression in the GT1-7 cells (B). (A) “Premixed”: ZnCl<sub>2</sub> (30 μM) and carnosine (5 mM) were premixed and incubated for 1 h in an appropriate buffer; the mixture was then administered to the cells. “Simultaneously”: ZnCl<sub>2</sub> and carnosine were administered simultaneously. “1 h later”: carnosine was administered after 1-h exposure to ZnCl<sub>2</sub>. The vertical axis of the graphs is expressed as the percentage of the viability of the cells not treated with ZnCl<sub>2</sub>, which is assumed to be 100 %. Data are expressed as mean ± SD, n = 6. \*\*\* indicates the respective significances at *p* < 0.001 vs. the cells treated with ZnCl<sub>2</sub> alone. (B) We administered 50 μM of ZnCl<sub>2</sub> in the presence of 10 mM carnosine, 0.25 mM EDTA, and 5 mM pyruvate. The vertical axis of the graphs is expressed as the relative fold to the ZNT1 expression level of the control cells, which is assumed to be 1. The level of expression of ZNT1 gene in each treatment was normalized to that of β-actin. Data are expressed as mean ± SD, n = 3.

(chelating agent for metal ion, including Zn<sup>2+</sup>) blocked zinc-induced ZNT1 expression. These results suggest that carnosine acts as an intracellular protective substance against zinc-induced GT1-7 cell death.

## Discussion

We observed that the heat-treated extracts from both eel muscle and skin exhibited protective activities against zinc-induced neuronal cell death. Carnosine was the substance in the muscle responsible for the protective activity, and eel skin contained an unknown substance(s) that prevented zinc-induced cell death because the carnosine content in the extract of eel skin was too low to exhibit any protective activity. The protective substance(s) in the eel skin is/are heat-stable and water-soluble. Analysis using reverse-phase HPLC with Hypercarb column revealed that compared to carnosine, the extract contained compounds with higher polarity (Fig. 4C, peaks b and c). However, the analysis of peaks b and c by ESI-mass spectrometry did not reveal any significant peak (data not shown). Further studies are required to identify the substance(s) in the eel skin that are responsible for preventing zinc-induced GT1-7 cell death. Using an HPLC system to quantify carnosine, we confirmed that carnosine is the major substance in eel muscle that is responsible for preventing zinc-induced GT1-7 cell death. Carnosine is a small dipeptide reported to be present abundantly in the muscles of fishes, chickens, and mammals<sup>14)</sup>. Carnosine is also present in the brain and is reported to be secreted in the synaptic clefts along with the excitatory neurotransmitter glutamate during neuronal excitation<sup>15)</sup>. Carnosine is particularly abundant in the neurons of the olfactory bulb<sup>15)</sup>; compared to hippocampal neurons, those of the olfactory bulb are less sensitive to damages after ischemia. This observation increases the possibility that carnosine plays a physiological role in preventing zinc-induced neurodegeneration, and that specific receptor for carnosine may exist on the membrane of neuronal cells.

Unlike EDTA, a strong chelating agent for zinc, carnosine did not inhibit the influx of zinc into the cell even though carnosine is reported to have the ability to bind to zinc<sup>11)</sup>. The analyses of zinc-induced gene expression revealed that the induction fold of the ZNT1 gene in the presence of carnosine is almost the same as that in the presence of pyruvate; it is reported that pyruvate prevents zinc-induced neuronal cell death through a mechanism of recovering the energy deficiency that is attributable to the loss of NAD<sup>+</sup> and inhibition of glycolysis<sup>13)</sup>. It was reported that uptake of carnosine was found to be mediated by a high affinity, energy-dependent dipeptide transport system, subsequently identified as the peptide transporter PepT2<sup>16)</sup>. These results indicate that carnosine acts as an intracellular protective substance. The details of the molecular mechanisms of how carnosine prevents the zinc-induced cell death is under investigation.

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