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# Zinc Neurotoxicity and the Pathogenesis of Vascular-Type Dementia: Involvement of Calcium Dyshomeostasis and Carnosine

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## Abstract

Zinc (Zn) is an essential trace element that is abundantly present in the brain. Despite its importance in normal brain functions, excess Zn is neurotoxic and causes neurodegeneration following transient global ischemia and plays a crucial role in the pathogenesis of vascular-type dementia. We found that GT1-7 cells (immortalized hypothalamic neurons) are more vulnerable to zinc-induced neurotoxicity than other cultured neuronal cells are. Further, we investigated the molecular mechanisms of Zn-induced neurotoxicity *in vitro* using GT1-7 cells. Pharmacological evidence based on our own results and those of numerous other studies has indicated the significance of calcium dyshomeostasis in the mechanisms of Zn-induced neuronal injury. We developed a screening system for substances, which prevent Zn-induced neurotoxicity, with the aim of identifying a treatment for vascular-type dementia. Here, we review in detail the characteristics and mechanisms of Zn-induced neuronal death in relation to calcium homeostasis. The potential role of carnosine (ß-alanyl histidine), a dipeptide present in the brain as an endogenous protective substance, in neuronal injury is also discussed.

**Keywords:** Calcium homeostasis; Vascular-type dementia; Ischemia; Excitotoxicity; Aluminum

## Introduction

Senile dementia is a serious problem in a rapidly aging world. Its prevalence increases with age, and approximately 25% of elderly individuals are affected [1]. In Japan, 3 million people have been estimated to be affected by senile dementia by 2025, and the number continues to grow annually. Senile dementia is mainly divided to Alzheimer's disease (AD) and vascular-type dementia (VD). AD is characterized by pathological hallmarks such as the deposition of senile plaques and neurofibrillary tangles in the patient's brain [2]. The abnormal accumulation of ß-amyloid protein (AßP) and subsequent neuronal loss are implicated in the pathogenesis of AD [3,4]. Meanwhile, VD is a degenerative cerebrovascular disease. Its risk factors include aging, sex difference (male), diabetes, and high blood pressure. The most common type of VD is caused by a series of small strokes or ischemia [5-7]. Following transient global ischemia or stroke, the interruption of blood flow and the resulting oxygen-glucose deprivation induce long-lasting membrane depolarization and cause an excessive release of glutamate into synaptic clefts. Thereafter, the excess glutamate causes over-stimulation of its receptors, namely, *N*-methyl-D-aspartate (NMDA)-type receptors, amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type receptors, and kainate receptors. Finally, $Ca^{2+}$ dyshomeostasis, i.e., the entry of large quantities of $Ca^{2+}$ occurring in glutamate-responsive neurons, triggers the delayed death of vulnerable populations of neurons such as pyramidal neurons in the hippocampus —an area associated with learning, memory, and language. Thereafter, the development of an infarct and the subsequent cognitive dysfunction mark the pathogenesis of VD in elderly people. Approximately 30% of stroke patients show symptoms of dementia within 3 months of the initial stroke [8,9].

Increasing evidence has supported the hypothetical idea that zinc (Zn) as a key mediator and modulator in delayed neuronal death after ischemia, and that Zn neurotoxicity is central to the pathogenesis of VD [10-12]. It is widely known that Zn is coexist with glutamate in synaptic vesicles and excess Zn is secreted to synaptic clefts following an ischemic insult in the range of up to 300 μM [13].

We found that GT1-7 cells (immortalized hypothalamic neurons) are more vulnerable to Zn than are other cultured neuronal cells [14]. Further, we employed GT1-7 cells to investigate the molecular mechanisms of Zn-induced neuronal death *in vitro* [15-17]. Moreover, we developed a screening system for substances that protect neurons against Zn by using GT1-7 cells based on the idea that such substances may be potential candidates for the treatment of VD [18-21]. Among various food products or agricultural products we tested, we found that carnosine (ß-alanyl histidine) was markedly effective at preventing the neuronal death induced by Zn [22]. Carnosine has previously been reported as effective for the treatment of other neurodegenerative diseases including AD [23] and prion diseases [24], or for the ageing-related disorders including cataracts [25].

In this article, we review the current understanding of the molecular mechanisms of Zn-induced neurotoxicity and the link between the pathogenesis of VD. We also discuss carnosine as a possible therapeutic agent for VD and other neurodegenerative diseases.

## Zn in the Brain

Zn is an essential trace element for most organisms. It plays important roles in various physiological functions such as mitotic cell division, and in the synthesis of proteins, DNA, and RNA as a co-factor of more than 300 enzymes or metalloproteins [26]. Zn is also

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important in the immune system, with Zn signaling playing crucial roles in biological systems of humans [27].

The human body contains approximately 2 g of Zn, mostly in the testes, muscle, liver, and brain tissues. In the brain, Zn is found at the highest concentrations in the hippocampus, amygdala, cerebral cortex, thalamus, and olfactory cortex [28,29]. The total Zn content of the hippocampus is estimated to be 70–90 ppm (dry weight). Although some Zn in the brain binds firmly to metalloproteins or enzymes, a substantial fraction (approximately 10% or more) either forms free Zn ions ($Zn^{2+}$) or is loosely bound, and is histochemically detectable by staining using chelating reagents. The chelatable Zn is stored in presynaptic vesicles of specific excitatory glutamatergic neurons and is secreted from these vesicles into synaptic clefts along with glutamate during neuronal excitation. Recent studies have suggested that this secreted $Zn^{2+}$ plays crucial roles in information processing, synaptic plasticity, learning, and memory [30]. Ueno et al. showed that $Zn^{2+}$ can provide spatiotemporal information regarding neuronal plasticity in the hippocampus using the newly developed high-sensitivity $Zn^{2+}$ indicator ZnAF-2 [31]. Moreover, $Zn^{2+}$ in the hippocampus is essential for the induction of long-term potentiation (LTP), a form of synaptic information storage that has become a well-known paradigm for the mechanisms underlying memory formation [32]. Removal of $Zn^{2+}$, or Zn deficiency, was shown to inhibit the induction of LTP [33,34]. Zn deficiency in childhood is known to cause the dwarfism, the retardation of mental and physical development, and the learning disabilities in humans [35]. Furthermore, Zn deficiency in adults causes odor disorders, the taste disorders, disorders of fear-conditioning and related emotional activity [36].

However, despite its importance, excess Zn is neurotoxic and is central to ischemia-induced neuronal death. It is widely known that a considerable amount of Zn (up to 300μM) is co-released with glutamate into synaptic clefts by membrane depolarization in ischemic conditions. In 1996, Koh et al. reported that Zn caused the apoptotic death of primary cultured cortical neurons [37]. They also revealed that Zn accumulates within the cell bodies of degenerating neurons following transient global ischemia. The movement of chelatable Zn from presynaptic terminals into postsynaptic neuronal cell bodies, and the increase in intracellular $Zn^{2+}$ levels ($[Zn^{2+}]_i$), namely, "Zn translocation," occurs in vulnerable neurons in the CA1 or CA3 regions of the hippocampus prior to the onset of the delayed neuronal death after transient global ischemia [38]. This Zn translocation is reported to enhance the appearance of the infarct. Administration of calcium EDTA (Ca EDTA), a membrane-impermeable chelating agent that chelates cations with the exception of calcium, blocked the Zn translocation, protected the hippocampal neurons after transient global ischemia, and reduced the volume of the infarct [39].

It is also revealed that Zn enhances $Ca^{2+}$-permeability, which is central for the molecular mechanism of neurodegeneration after ischemia. Most hippocampal neurons express the AMPA receptors, which is composed of 4 subunits (GluR1 to GluR4) and is poorly permeable to divalent cations including $Ca^{2+}$ and $Zn^{2+}$, under normal conditions. However, under ischemic conditions, an acute reduction in the expression of GluR2 subunits occurs in the hippocampus, and the AMPA-receptors lacking the GluR2 subunit, which becomes more $Ca^{2+}$ permeable (Ca-AMPA/kainate channels), appear in vulnerable neurons [40]. Although NMDA-type glutamate receptors are present in most neurons, and gate highly $Ca^{2+}$-permeable channels, the permeability of $Ca^{2+}$ through AMPA/kainate channels is greater than that through NMDA-receptor channels. Thus, the appearance of Ca-AMPA/kainite channels increases permeability to $Ca^{2+}$ and enhances toxicity following ischemia. Zn is implicated in the transcriptional regulation of Ca-AMPA/kainite channels and Ca EDTA has been shown to attenuate the ischemia-induced downregulation of the GluR2 gene [39].

These results strongly implicate Zn is a key player in delayed neuronal death after transient global ischemia, a process that might be involved in the pathogenesis of VD [11,41]. The accumulation of Zn has also been observed following head trauma and epilepsy [42], implying that Zn neurotoxicity might underlies the pathological mechanisms of various neuronal injuries. Moreover, the disruption of Zn homeostasis has also been implicated in other neurodegenerative diseases, including AD [43-45], prion diseases [46], amyotrophic lateral sclerosis (ALS) [47], and Wilson's disease [48]. Thus, Zn might play a role like that of Janus, an ancient Roman god of doorways with two different faces, in the brain: both Zn depletion and excess Zn cause severe damage to neurons.

## Mechanism of Zn-Induced Neuronal Death

### GT1-7 cells: A model system for investigating Zn neurotoxicity *in vitro*

Understanding the molecular mechanism of Zn-induced neuronal death is of great importance for the treatment of VD. Numerous studies have been undertaken to elucidate the mechanism of Zn-induced neuronal death. To this end, many researchers have investigated Zn neurotoxicity *in vitro*, mainly using primary cultured neurons from rat cerebral cortex or hippocampus [49], or PC-12 cells, a pheochromocytoma cell line [50]. However, the roles of Zn are highly complex. For example, the most established effect of Zn in the central nervous system is the inhibition of NMDA-type glutamate receptors and regulation of the excitability of glutamatergic neurons [51]. Considering that both glutamate and Zn are neurotoxic, distinguishing of the effects of Zn and glutamate by using neuronal cells, which possess glutamate receptors, has proved difficult.

We investigated the effects of Zn and AßP by using GT1-7 cells [44] and found that GT1-7 cells are much more sensitive to Zn than other neuronal cells are [14,15]. Figure 1 shows the viability of GT1-7 cells, PC-12 cells, B-50 cells (a neuroblastoma cell line), primary cultured neurons of the rat cerebral cortex, and primary cultured neurons of the rat hippocampus, following exposure to identical concentrations of Zn. Among these neuronal cells, GT1-7 cells exhibited the highest rates of cell death. Zn caused the apoptotic death of GT1-7 cells in a dose-dependent and time-dependent manner. The degenerated GT1-7 cells were terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labeling (TUNEL) positive and exhibited DNA fragmentation (Figure 2).

The GT1-7 cells were originally developed by Mellon et al. by genetically targeting tumorigenesis of mouse hypothalamic neurons [52]. The cells possess neuronal characteristics such as the extension of neurites, secretion of gonadotropin-releasing hormone (GnRH), and expression of neuron-specific proteins or receptors including microtubule-associated protein 2 (MAP2), tau protein, neurofilament, synaptophysin, $GABA_A$ receptors, dopamine receptors, and L-type $Ca^{2+}$ channels. Additionally, the GT1-7 cells either lack or possess low levels of ionotropic glutamate receptors and do not exhibit glutamate toxicity [53]. These properties make the GT1-7 cell line an excellent model system for the investigation of Zn-induced neurotoxicity.

### Implications of energy failure in Zn-induced neurotoxicity

We investigated the detailed characteristics of Zn-induced death in

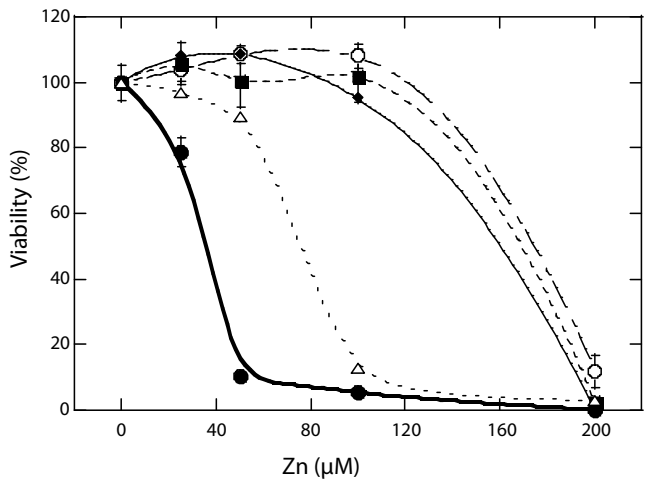


**Figure 1: Zn-induced death of GT1-7 cells and other neuronal cells.** Various concentrations of $ZnCl_2$ were administered to B-50 cells (open circle;○), PC-12 cells (closed square; ■), GT1-7 cells (closed circle; ●), and primary cultured neurons from rat cerebral cortex (closed diamond; ♦), or hippocampus (open triangle; △). After 24-h exposure, cell viability was measured using WST-1 method. Data are presented as mean ± S.E.M., *n*=6.

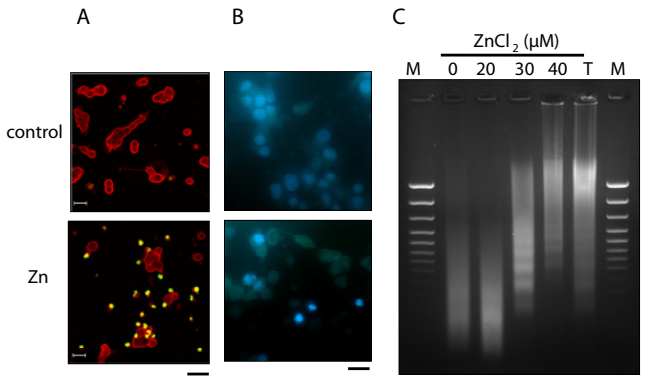


**Figure 2: Apoptotic characteristics of the Zn-induced death of GT1-7 cells.**
A: GT1-7 cells were exposued to $ZnCl_2$ (50 μM) and were then fixed using paraformaldehyde after 24 h. The GT1-7 cells were observed with TUNEL staining by fluorescent microscopy. Scale bar represents 50 μm.
B: The GT1-7 cells were observed with Hoechst staining by fluorescent microscopy after 24 h of exposure to Zn.
C: Gel electrophoresis of DNA extracted from GT1-7 cells. M: marker, T: thapsigargin as a positive control.

GT1-7 cells and its mechanisms [14,15]. First, we tested the effects of various pharmacological agents prior to Zn treatment of GT1-7 cells. We demonstrated that neither antagonists nor agonists of excitatory neurotransmitters (D-APV, glutamate, and CNQX), or those of inhibitory neurotransmitters (bicuculline, muscimol, baclofen, and GABA) attenuated the viability of GT1-7 cells after Zn exposure. It was reported that agonists of glutamate receptors, such as NMDA or AMPA, enhance Zn-induced neurotoxicity in cultured cortical neurons [49]; however, our findings in GT1-7 cells, which lack such glutamate receptors, are inconsistent with these findings.

We also showed that the administration of sodium pyruvate, an energy substrate, significantly inhibited the Zn-induced death of GT1-7 cells [14]. The results are consistent with findings of other studies using primary cultured cortical neurons, oligodendrocyte progenitor cells, or retinal cells [54-56]. Furthermore, the administration of pyruvate attenuated the neuronal death after ischemia *in vivo* [57]. Shelline and his colleagues reported that Zn exposure decreased the levels of $NAD^+$ and ATP in cultured cortical neurons, and that treatment with pyruvate restored the $NAD^+$ level [58]. An imaging study using a Zn-sensitive fluorescent dye and a mitochondrial marker revealed that Zn is localized within mitochondria [59]. Zn is reported to inhibit various mitochondrial enzymes, such as mitochondrial complex I, aconitase, cytochrome *c* oxidase, α-ketoglutarate dehydrogenase, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), and monoamine oxidase. Zn also inhibits the intracellular trafficking of mitochondria [60]. It has also been reported that Zn produced ROS and caused oxidative damage resulting from mitochondrial impairments [61]. Therefore, energy failure and the inhibition of glycolysis in mitochondria may be involved in Zn neurotoxicity.

## Routes of Zn influx and efflux

It is widely recognized that Zn translocation is the primary event in the pathway of Zn-induced neuronal death. Sensi et al. observed a temporal change of $[Zn^{2+}]_i$ in cultured cortical neurons by using a Zn-sensitive fluorescent dye, mag-fura-5; their results revealed that $[Zn^{2+}]_i$ increases after 15 sec of exposure to Zn under a depolarization condition by high $K^+$ [62]. The $[Zn^{2+}]_i$ increase was attenuated by blockers of voltage-gated $Ca^{2+}$ channel, including $Gd^{3+}$, verapamil, and nimodipine. Activation of glutamate-related channels by the application of NMDA or kainite also enables the entry of $Ca^{2+}$. Furthermore, it has been shown that $Zn^{2+}$, as well as $Ca^{2+}$, is translocated through Ca-AMPA/kainate channels. Hence, at least 3 major routes of $Zn^{2+}$ entry have been identified; voltage-gated $Ca^{2+}$ channels, NMDA-type glutamate receptors, and AMPA/kainite-type glutamate receptors [41].

The permeability of $Zn^{2+}$, as well as $Ca^{2+}$, through Ca-AMPA/kainate channels is greater than that through NMDA-receptor channels. The intracerebral administration of 1-naphtyl acetyl spermine, a blocker of Ca-AMPA/kainate channels, protected hippocampal neurons from ischemia-induced neurodegeneration and attenuated the accumulation of Zn in vulnerable neurons [63]. Therefore, the expression of $Zn^{2+}$-permeable Ca-AMPA/kainite channels and the entry of $Ca^{2+}$ and/or $Zn^{2+}$ through these channels are mediators of delayed neuronal death after ischemia [41]. Zn-specific membrane transporter proteins (Zn transporters) also play a role in the influx and the efflux of Zn. Zn transporters primarily control Zn homeostasis; they facilitate Zn influx when there is a deficiency, and Zn efflux during Zn excess. Therefore, disorders of Zn transporters cause various diseases [64].

## Implication of Ca dyshomeostasis in Zn-induced neurotoxicity

To evaluate the involvement of other metal ions in Zn neurotoxicity, we investigated the viability of GT1-7 cells, with or without various metal ions, after exposure to Zn (Figure 3), and found that the equimolar addition of $Al^{3+}$ and $Gd^{3+}$ significantly inhibited Zn-induced neurotoxicity [16,17]. Moreover, overloading of $Ca^{2+}$ and $Mg^{2+}$ inhibited the Zn-induced death of GT1-7 cells; Zn protected GT1-7 cells from neurotoxicity induced by $Ca^{2+}$ overload, and *vice versa* (Figure 3b). It is widely known that $Gd^{3+}$ is a blocker of voltage-gated $Ca^{2+}$channels [61], and that $Al^{3+}$ inhibits various types of $Ca^{2+}$ channels [65,66]. Furthermore, Kim et al. reported that Zn neurotoxicity in PC-12 cells was attenuated by an L-type $Ca^{2+}$ channel blocker, nimodipine, and enhanced by the L-type $Ca^{2+}$ channel activator, S(-)-Bay K 8644

[50]. Additionally, Zn neurotoxicity was attenuated by aspirin, which prevents $Zn^{2+}$ entry through voltage-gated $Ca^{2+}$ channels [67].

These results suggest that Ca dyshomeostasis is involved in the mechanism of Zn-induced neurotoxicity. To address this issue, we employed a high-resolution multi-site video imaging system with fura-2 as the cytosolic free calcium reporter fluorescent probe for the observation of temporal changes in $[Ca^{2+}]_i$ after exposure to Zn (Figure 4). This multisite fluorometry system enables the simultaneous long-term observation of temporal changes in $[Ca^{2+}]_i$ of more than 50 neurons [68,69]. The elevations in $[Ca^{2+}]_i$ were observed among GT1-7 cells after 3-30 min of the exposure to Zn. Detailed analysis of Zn-induced $[Ca^{2+}]_i$ revealed that pretreatment of $Al^{3+}$ significantly blocked the Zn-induced $[Ca^{2+}]_i$ elevations. Thus, it is possible that $Al^{3+}$, a blocker of various types of $Ca^{2+}$ channels, attenuate Zn-induced neurotoxicity by blocking Zn-induced elevations in $[Ca^{2+}]_i$.

## Carnosine as an Endogenous Protective Substance against Zn Neurotoxicity

Considering the implication of Zn in transient global ischemia, substances that protect against Zn-induced neuronal death could be potential candidates for the prevention or treatment of neurodegeneration following ischemia, and ultimately provide a lead to treatments for VD. With the aim of exploring this idea, we developed a rapid, sensitive, and convenient assay system for the mass-screening of such substances by using GT1-7 cells. We examined the potential inhibitory effects of various agricultural products such as vegetable extracts, fruits extracts, and fish extracts, and found that extracts from eel muscles significantly protected against Zn-induced neurotoxicity [20]. To identify the active substances in the eel extract, we separated the extract by reversed-phase high performance liquid chromatography (HPLC) and analyzed its structure, considering that the active substances were water-soluble, heat-stable small compounds [21]. Finally, we demonstrated that carnosine, a small hydrophilic peptide abundant in eel muscles, protected GT1-7 cells from Zn-induced neurotoxicity in a dose-dependent manner [22]. Additionally, we revealed that citrate from mango (*Mangifera indica L.*) fruit was effective in protecting against Zn-induced neurotoxicity [18]. The administration of carnosine was reportedly effective in the prevention of ischemia-induced neuronal death *in vivo* [70-72]. Therefore, we applied for the patent on carnosine as a drug for the treatment of VD or for slowing the progress of cognitive decline after ischemia (the application No. 2006-145857; the publication No. 2007-314467 in Japan) [22].

Carnosine is a naturally occurring dipeptide and is commonly present in vertebrate tissues, particularly within the skeletal muscles and nervous tissues [73-75]. It is found at high concentrations in the muscles of animals or fish which exhibit high levels of exercise, such as horses, chickens, and whales. The concentration of carnosine in the muscles of such animals is estimated to be 50–200 mM [76]. Thus, it is believed that carnosine plays important roles in the buffering capacities of muscle tissue [77]. During high-intensity anaerobic exercise, proton accumulation causes a decrease in intracellular pH, which influences various metabolic functions. The p*K*a value of carnosine is 7.01, close to intracellular pH. Therefore, carnosine contributes to physicochemical non-bicarbonate buffering in skeletal muscles, and the administration of carnosine has been reported to induce hyperactivity in animals [78].

The physiological role of carnosine within brain is still under investigation. In the brain, a considerable amount of carnosine is localized in the neurons of the olfactory bulb. It is secreted into synaptic clefts along with the excitatory neurotransmitter glutamate during neuronal excitation. Considering the olfactory pathway is the primary gateway to the external environment, it is interesting to note that olfactory bulb neurons are less sensitive to damage after ischemia than are hippocampal neurons in spite of an equivalent accumulation of Zn [79].

Carnosine has also been reported to have antioxidant activity

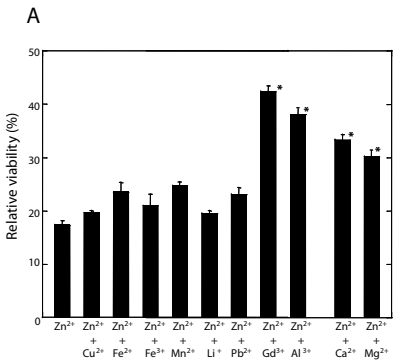


**Figure 3: Metal-metal interactions in Zn-induced neurotoxicity.**
A: Effects of various metals on Zn neurotoxicity in GT1-7 cells
Various metal ion solutions containing 50 μM of $CuCl_2$ ($Cu^{2+}$), $FeCl_2$ ($Fe^{2+}$), $FeCl_3$ ($Fe^{3+}$), $MnCl_2$ ($Mn^{2+}$), $LiCl_3$ ($Li^{+}$), $PbCl_2$ ($Pb^{2+}$), $GdCl_3$ ($Gd^{3+}$), $AlCl_3$ ($Al^{3+}$), or 2 mM of $CaCl_2$ ($Ca^{2+}$), or $MgCl_2$ ($Mg^{2+}$) were administered to GT1-7 cells prior to treatment with $ZnCl_2$ (50 μM). After 24 h, cell viability was measured using the WST-1 method. For the compensation of endogenous toxicity of the metal, the difference was calculated between cell viability with the metal alone and viability with Zn and metal, and was described as the relative viability. Data are presented as mean ± S.E.M., *n*=6. * p<0.01.
B: Effects of calcium overload
The viability of GT1-7 cells was compared among control (culture media containing 1.8 mM of $Ca^{2+}$), $Zn^{2+}$ (50 μM of $ZnCl_2$ was pre-administered), $Ca^{2+}$ (5 mM of $CaCl_2$ was pre-administered), and $Zn^{2+}$+ $Ca^{2+}$ (50μM of $ZnCl_2$ and 5 mM of $CaCl_2$ were co-administered) treatments. Both $Zn^{2+}$ and $Ca^{2+}$ caused marked death of GT1-7 cells, but co-administration of $Zn^{2+}$ and $Ca^{2+}$ protected GT1-7 cells. Data are presented as mean ± S.E.M., *n*=6. * p<0.01.

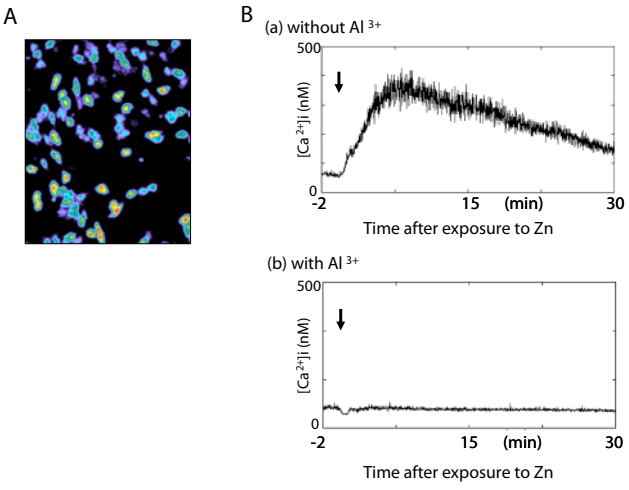


**Figure 4: $Ca^{2+}$ imaging of Zn-intoxicated GT1-7 cells.**
A: Pseudocolor images of $[Ca^{2+}]_i$ of GT1-7 cells
B: A solution of $ZnCl_2$ (250 μM) was applied onto fura-2-loaded GT1-7 cells without (a) or with preadministration of 100 μM $Al^{3+}$ (b). Temporal changes of fluorescence intensities corresponding to increases in $[Ca^{2+}]_i$ before and after the exposure to $Zn^{2+}$ were depicted. Arrows indicate the time of exposure.

[80], having the ability to chelate metal ions including $Zn^{2+}$ and $Cu^{2+}$ [81]. Carnosine inhibits the Maillard reaction that involves reducing sugars and proteins, providing a multitude of end-products, most notably advanced glycation end-products (AGEs) [82]. AGEs can contribute to the pathogenesis of various senile diseases such as AD, vascular stiffening, atherosclerosis, osteoarthritis, inflammatory arthritis, and cataracts [83]. Furthermore, carnosine is reported to have anti-crosslinking properties [84]. Attanasio et al. reported that carnosine inhibited the fibrillation of alpha-crystallin [85]. Thus, *N*-acetylcarnosine (NAC), which is more resistant to enzymatic digestion than carnosine, has been proposed as a therapeutic agent for cataract and skin care [86]. It is widely recognized that crosslinking and conformational changes in disease-related proteins (e.g., AßP, prion protein) are central in the pathogenesis of various neurodegenerative diseases termed "conformational diseases" including AD and prion diseases [87,88]. We showed that carnosine attenuated the neuronal death induced by prion protein fragment peptide (PrP106-126) by changing its conformation [24]. It was also demonstrated that carnosine inhibited the aggregation and subsequent neurotoxicity of AßP [89]. Corona et al. showed that dietary supplementation of carnosine attenuated mitochondrial dysfunction and the accumulation of AßP in Alzheimer's model mice [23]. Carnosine level is significantly reduced in the serum of AD patients [89]. These results suggest possible beneficial effects of carnosine as a treatment for AD and prion diseases. Analogues of carnosine such as anserine or homocarnosine may also proved to be useful owing their similar antioxidant activities. Figure 5 exhibits the structure of carnosine and it's reported various effects [90].

All of these functions of carnosine (e.g., antioxidant, anti-glycating, anti-crosslinking, scavenging toxic aldehydes) are related to the ageing processes. The level of carnosine varies during development and is low in the aged animals [91]. Therefore, it is highly possible that carnosine protects against external toxins and acts as an endogenous protective substance against neuronal injury, senescence, and ageing.

## Zinc Hypothesis and the Pathogenesis of VD

Considering the accumulated evidence presented in this paper, we inferred a scheme for Zn neurotoxicity and the role of carnosine (Figure 6). Under normal conditions, neuronal excitation causes the release of glutamate and Zn. The certain subunits of NMDA-type glutamate receptors such as NR2A and NR2B form binding sites for the exogenous Zn [92]. Zn inhibits the NMDA-type channels, and attenuate glutamate-induced neuronal death by blocking the increase of $[Ca^{2+}]_i$. Moreover, low concentration of Zn reportedly enhanced LTP [93]. In this condition, Zn acts as an endogenous modulator of neuronal excitability and neuronal plasticity, and is implicated in the memory formation and in the information processing [94].

However, under pathological conditions such as ischemia, oxygen-glucose deprivation induces the release of excess glutamate as well as Zn into the synaptic clefts. Excess Zn enhances the expression of Ca-AMPA/kainite channels, and is translocated through the Ca-AMPA/kainite channels or through other pathways into the target neuron, where Zn acts to inhibit various enzymes, inhibit mitochondrial respiration, cause energy depletion, and produce ROS. Excess glutamate induces elevation of intracellular $Ca^{2+}$ levels in the target neuron. Elevated levels of intracellular $Ca^{2+}$ then trigger various apoptotic pathways such as the activation of calpain, caspases or other enzymatic pathways related to apoptosis; ultimately this leads to neuronal death. Zn also influences intracellular $Ca^{2+}$ levels and enhances effects of glutamate.

Zn in the synaptic clefts is either re-absorbed or binds to carnosine. Carnosine, an endogenous blocker of Zn neurotoxicity, is synthesized

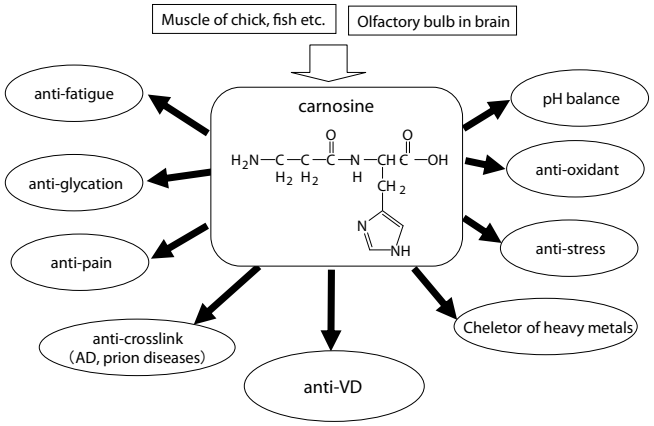


**Figure 5: Structure and roles of carnosine.**
The various effects of carnosine in mammals are depicted. Details are shown in the text.

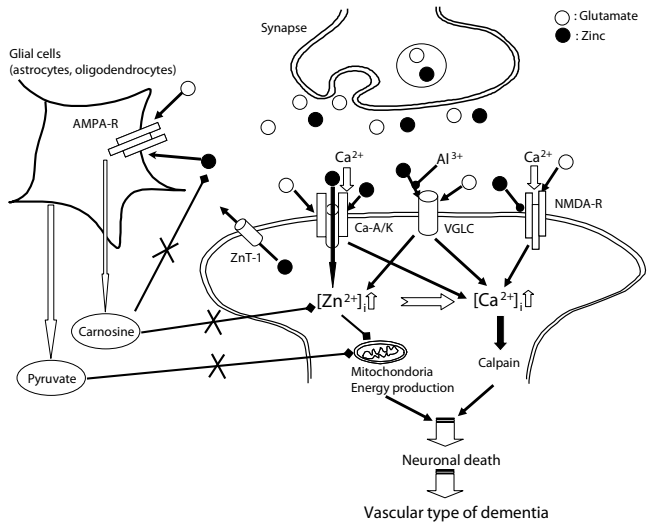


**Figure 6: Hypothetical scheme of Zn neurotoxicity.**
Zn (Zn: closed circle) coexists with glutamate (Glu: open circle) in presynaptic vesicles and is secreted upon neuronal excitation. Under normal conditions, secreted Zn binds to NMDA-type glutamate receptors (NMDA-R) and modulates postsynaptic excitability. Carnosine is synthesized in glial cells and is secreted in response to stimulation by glutamate and Zn; it protects neurons from glutamate-Zn neurotoxicity. This feedback pathway contributes to Zn homeostasis. Concentrations of intracellular Zn ($[Zn^{2+}]_i$) are also maintained by ZnT-1, a zinc transporter. Pyruvate is also released from glial cells and protects neurons from mitochondrial energy deficits caused by Zn.
However, under pathological conditions such as transient global ischemia, large amounts of both glutamate and Zn are released into synaptic clefts. Zn potentiates the expression of $Ca^{2+}$-permeable AMPA/kainate-type glutamate receptor (Ca-A/K) channels. Zn is translocated into postsynaptic target neurons through Ca-A/K channels or other pathways such as voltage-gated L-type $Ca^{2+}$ channels (VGLC) or NMDA-R. Increased $[Zn^{2+}]_i$ inhibits numerous enzymes, including mitochondrial respiratory enzymes, and thus causes energy depletion. Meanwhile, excess glutamate also increases $[Ca^{2+}]_i$. The increase of Ca-A/K channels and the increased $[Zn^{2+}]_i$ both contribute to the increase in $[Ca^{2+}]_i$. Increased $[Ca^{2+}]_i$ consequently triggers various apoptotic pathways, including the activation of calpain. Carnosine levels are decreased in aged bodies. Therefore, the protective effects of carnosine in the hippocampus or cerebral cortex might be insufficient in cases of pathological conditions under the aged brain. Dyshomeostasis of Zn and Ca eventually causes delayed neuronal death after transient global ischemia; it ultimately leads to the pathogenesis of VD.

and stored in glial cells, such as astrocytes and oligodendrocytes, in the hippocampus and other brain regions [95]. It was reported that glutamate and Zn caused the release of carnosine from glial [96,97]. Thus, this feedback pathway of carnosine-Zn protects neurons against both glutamate toxicity and Zn toxicity.

In conclusion, our new approach to ischemia-induced neurodegeneration from the perspective of the Zn hypothesis will lead to new therapeutic tools for the treatment and/or prevention of VD. Considering the advantageous properties of carnosine (relatively non-toxic, heat-stable, and water-soluble), the dietary supplementation of carnosine might be an effective strategy for the prevention or treatment of neurodegenerative diseases such as ischemia, VD, AD, and prion diseases [98]. Indeed, the content of carnosine in the brain increased after the oral administration of carnosine-rich chicken breast extract [99]. Further research into the role of Zn in neuronal injury and determining the significance of Zn and Ca homeostasis might lead to the development of new treatments for neurodegenerative diseases.

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