Review

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Review

The Role of Zinc in Neurodegenerative Diseases and the Potential of Carnosine as Their Therapeutic Agent

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Abstract: Synaptic zinc ions (Zn^{2+}) play an important role in the development of vascular dementia (VD) and Parkinson's disease (PD). In this article, based on our study and many others, we review the molecular pathways by which Zn^{2+} causes neurotoxicity. Zn^{2+} influences calcium homeostasis, energy production pathway, production of reactive oxygen species, endoplasmic reticulum stress pathway, and activated protein kinase/c-Jun amino terminal kinase (SAPK/JNK) pathway and consequently exerts neurotoxicity. Furthermore, we searched various crops for substances that protect neurones from neurotoxicity caused by Zn^{2+} and clarified that carnosine (β-alanylhistidine) may be a therapeutic drug for VD and PD. Here, we also review the molecular mechanisms underlying the role of carnosine as an endogenous protector and its protective effect against Zn^{2+}-induced cytotoxicity and discuss prospects for future neurodegenerative diseases therapeutic applications of this dipeptide.

Keywords: apoptosis; carnosine; endoplasmic reticulum stress; synapse; vascular dementia; zinc

1. Introduction

Zinc (Zn) plays an important role in a variety of physiological functions, including cell mitosis, immune system, and protein and nucleic acid synthesis, and acts as a cofac-tor for over 300 enzymes or metalloproteins, contributing to normal brain function [1]. Nevertheless, Zn is widely known to play an important role in the development of post-ischaemic neurodegeneration and vascular dementia (VD) [2]. Recently, it has been reported that Zn is involved in the mechanism of Parkinson's disease (PD) pathogenesis [3].

Senile dementia is characterised by severe memory loss and inability to form new memories in the elderly, and its prevalence increases with age. A fact sheet published by the World Health Organization in 2023 states that there are 55 million people with dementia worldwide, an increase of nearly 10 million each year [4]. Dementia is a serious social problem in rapidly aging societies. Senile dementia is classified into Alzheimer's disease (AD), VD, and dementia with Lewy bodies (DLB). VD is considered the second most common type of dementia, accounting for approximately 20%-40% of senile dementias [5]. Both AD and DLB are characterised by abnormally accumulated protein deposits (β-amyloid protein (AβP) in AD and α-synuclein in DLB), whereas VD is a degenerative cerebrovascular disease with a series of strokes or ischaemia [6–8]. PD is a progressive neurodegenerative disease that presents with motor deficits, such as resting tremors, muscle rigidity, akinesia, and impaired postural reflexes and develops in people over the age of 60 years at a rate of approximately 1 in 100 [9,10]. Although its pathogenesis is unclear, it is caused by the loss of dopamine neurones in the substantia nigra and promotion of inflammatory responses by microglia at the lesion site [11,12].

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We revealed the influence of energy production pathways, disruption of calcium (Ca) homeostasis, and endoplasmic reticulum (ER) stress pathways on the molecular mechanisms of Zn^{2+}-induced neurotoxicity [13]. Based on our recent findings regarding the involvement of the stress-activated protein kinase/c-Jun amino terminal kinase (SAPK/JNK) pathway in the generation of reactive oxygen species (ROS) [14], we hypothesised that these molecular pathways are related to Zn^{2+}-induced neurotoxicity.

Substances that reduce Zn^{2+}-induced neurotoxicity are potential agents for the treatment or prevention of VD [15]. During the search for a protective substance against Zn^{2+}-induced neurotoxicity, we found that carnosine (β-alanylhistidine) exhibited a marked inhibitory effect on Zn^{2+}-induced neurotoxicity and proposed that it is a candidate drug for the treatment of VD [16]. Carnosine is an endogenous dipeptide with various useful properties such as antioxidant, anti-glycation and anti-cross-linking (Figure 1) [17,18]. Carnosine accumulates abundantly in the skeletal muscle and olfactory bulb. As the olfactory bulb is the gateway for external information and substances, it likely protects neurones from external toxins and acts as an endogenous protector against damage and aging. Carnosine levels change during development and decrease with age [19]. This may be associated with an increased risk of neuropathy with aging. Carnosine supplementation in older adults may reduce this risk. In this article, based on our own and other previous studies, we focus on the molecular mechanisms of Zn^{2+}-induced neurotoxicity and the properties of carnosine against this disease and discuss potential agents for VD.

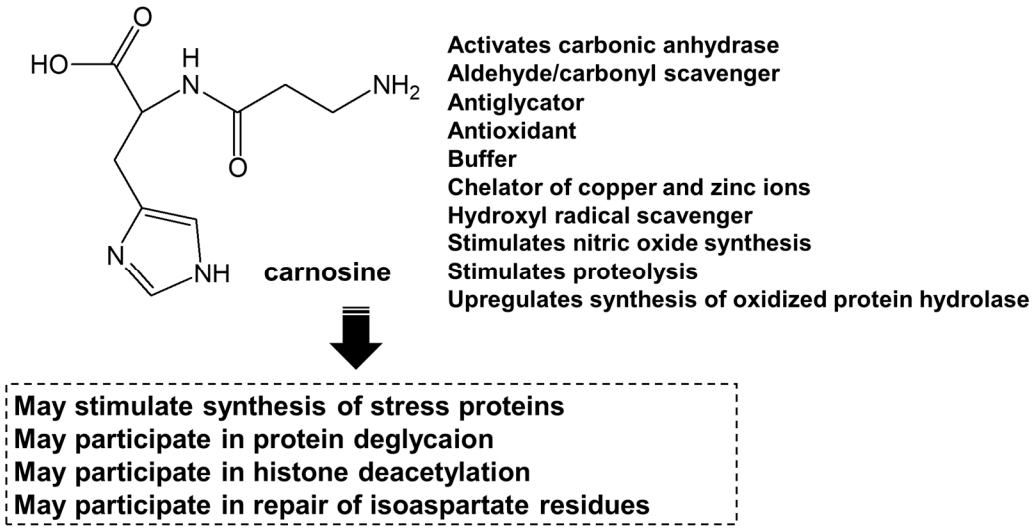

Figure 1. Structures of carnosine and its related compounds, and roles of carnosine. The various functions of carnosine in mammals.

2. Zn neurotoxicity

2.1. Usefulness of GT1-7 cells in the study of Zn^{2+}-induced neurotoxicity

It has been recognised that abnormalities in Zn homeostasis (e. g., excess or deficiency) are involved in neurological diseases such as VD [2]. Understanding the molecular mechanism of neuronal cell death induced by Zn^{2+}, which accounts for a large amount of Zn in the brain, is important for elucidating the pathogenesis of VD and developing therapeutic agents. However, it is difficult to examine Zn^{2+}-induced neuronal cell death using primary cultured neurones of the cerebral cortex and hippocampus, which are used to study cell death in the brain, or PC-12 cells, a pheochromocytoma cell line. Zn^{2+} is released along with glutamate upon glutamatergic neuronal excitation [20]. As glutamate also causes neurotoxicity, it is difficult to distinguish between the effects of Zn^{2+} and glutamate in cells with glutamate receptors. We have shown that Zn^{2+} causes cell death in immortalised hypothalamic neurone GT1-7 cells [21,22]. In these studies, GT1-7 cells were more

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vulnerable to Zn^{2+} cytotoxicity than other neuronal cells, including primary cultures of rat cortical and hippocampal neurones, PC-12 cells, and B-50 cells. (Figure 2). GT1-7 cells possess neuronal characteristics such as neurite extension, secretion of gonadotropin-releasing hormone (GnRH), and expression of neurone-specific proteins and receptors, including microtubule-associated protein 2, tau protein, neurofilament, synaptophysin, $GABA_A$ receptors, dopamine receptors, and L-type Ca^{2+} channels [23]. In contrast, GT1-7 cells lack or possess low levels of ionotropic glutamate receptors and exhibit little cytotoxicity to glutamate [24]. These properties suggest that the GT1-7 cell line is an excellent model for investigating Zn^{2+}-induced neurotoxicity.

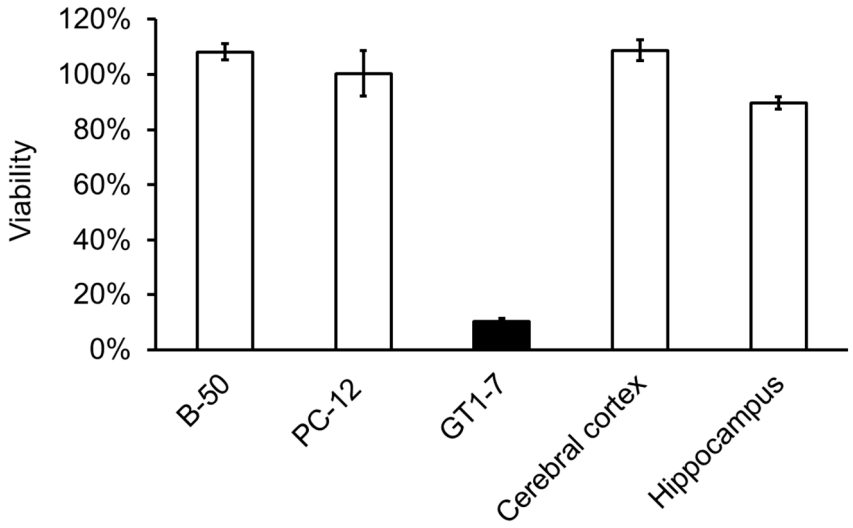

Figure 2. Apoptotic death of GT1-7 cells after exposure to Zn^{2+}. Viability of various neuronal cells after exposure to Zn^{2+}. Cultured neuronal cells (GT1-7, PC-12, and B-50 cells [a neuroblastoma cell line], primary cultured neurones of the rat cerebral cortex, and primary cultured neurones of the rat hippocampus) were administered 50 μM of $ZnCl_2$. After 24 h, cell viability was analysed by the WST-1 method.

2.2. Molecular mechanism of Zn^{2+}-induced GT1-7 cytotoxicity

Zn^{2+}-treated GT1-7 cells are positive for transferase-mediated biotinylated UTP nick-end labelling, indicating that Zn^{2+} induces apoptosis in GT1-7 cells [21,22]. Studies using DNA microarray analysis and real-time PCR (RT-PCR) have revealed that the administration of Zn^{2+} to GT1-7 cells induces the expression of various genes, including in metal-related genes (Zn transporter 1 [*ZnT-1*], metallothionein [*MT*]*1*), and *MT2*), ER stress-related genes, and Ca^{2+} signalling transmission-related genes [25]. The administration of Zn^{2+} to GT1-7 cells also increased intracellular Ca^{2+} levels ($[Ca^{2+}]_i$). Apoptosis is inhibited by pyruvate, citrate, and Ca^{2+} channel antagonists (nifedipine, conotoxin, and Al^{3+}) [21,22,26]. Using a high-resolution multisite video imaging system with Fura-2 as a fluorescent probe for cytosolic Ca^{2+}, we observed the changes in $[Ca^{2+}]_i$ after exposure to Zn^{2+} [22]. This revealed that pretreatment with Al^{3+}, which acts as various kinds of Ca^{2+} channel blocker [27], suppressed the elevation of the $[Ca^{2+}]_i$ levels in Zn^{2+}-treated GT1-7 cells. Al has difficulty entering cells without a membrane-permeable chelator, and Al^{3+} does not affect the viability of GT1-7 cells under these experimental conditions [28]. Zn^{2+}-induced GT1-7 cell death may be attenuated by Al^{3+}, which suppress the elevation of the $[Ca^{2+}]_i$ level. Therefore, Ca^{2+} homeostasis is likely to be involved in Zn^{2+} neurotoxicity.

We previously showed that the energy substrates pyruvate and citrate salts inhibit Cu^{2+}- and Zn^{2+}-induced GT1-7 cell death [29]. The coexistence of pyruvate and citrate did not affect the $[Ca^{2+}]_i$, intracellular Zn^{2+} levels ($[Zn^{2+}]_i$), or *MT* mRNA levels. Therefore, it is unlikely that pyruvate and citrate attenuated Cu/Zn-induced neurotoxicity by chelating Cu^{2+} and Zn^{2+}. Shelline et al. reported that Zn exposure decreased the nicotinamide adenine dinucleotide (NAD^+) and ATP levels in

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cultured cortical neurones, and treatment with pyruvate restored the NAD^+ levels [30,31]. Pyruvate administration also attenuated post-ischaemic neuronal cell death *in vivo* [32]. Imaging studies using Zn^{2+}-sensitive fluorescent dyes and mitochondrial markers have revealed that Zn^{2+} is localised within mitochondria [33]. Zn^{2+} has been reported to inhibit various mitochondrial enzymes and intracellular trafficking of mitochondria. Taken together, these results suggest that energy deficiency and inhibition of mitochondrial glycolysis are involved in Zn^{2+} neurotoxicity [28].

DNA microarray analysis revealed that Zn^{2+} markedly increased the expression of ER stress-related genes, including CCAAT enhancer-binding protein homologous protein (*CHOP*) and growth arrest and DNA damage-induced gene 34 (*GADD34*) [34,35]. The ER acts as an intracellular Ca^{2+} reservoir and is involved in the regulation of $[Ca^{2+}]_i$. Therefore, an increase in the $[Ca^{2+}]_i$ is associated with a decrease in the Ca^{2+} level in the ER. A decreased Ca^{2+} level in the ER is thought to cause ER stress because it leads to the decreased function of molecular chaperones and enzymes that bind Ca^{2+} [36]. The increase in the $[Ca^{2+}]_i$ in GT1-7 cells induced by Zn^{2+} administration appears to be closely related to the upregulation of these ER stress-related factors. ER stress is associated with the accumulation of unfolded and misfolded proteins and is involved in various neurological diseases such as cerebral ischaemia, AD, and prion disease (PD) [37]. Three signalling proteins (ER stress sensors) —inositol-requiring enzyme 1α (IRE1α), protein kinase R-like ER kinase (PERK), and activating transcription factor (ATF) 6— are activated by sensing ER stress [38]. IRE1α, PERK, and ATF6 activate diverse signalling pathways. Phosphorylation of the α-subunit of eukaryotic translation initiation factor 2α regulates ATF4 translation via PERK. ATF4 is a transcription factor that promotes *CHOP* and *GADD34*. In addition to these factors, whose expression was confirmed to be induced by Zn^{2+} administration in DNA microarray studies, we examined the expression of other ER stress-related genes such as immunoglobulin binding protein (*Bip*), ER degradation-enhancing α-mannosidase-like protein (*EDEM*), spliced X-box binding protein-1 (*sXBP1*), glucose-regulated protein 94 (*GRP94*), and protein disulfide isomerase (*PDI*) by Zn^{2+} using RT-PCR. RT-PCR confirmed that Zn^{2+} administration to GT1-7 cells induced the expression of activity-regulated cytoskeleton (*Arc*), *CHOP*, *GADD34*, and *ATF4*. The induction of the expression of metal-related genes *ZnT-1*, *MT1*, and *MT2* was also confirmed, whereas other ER stress-related genes, including *Bip*, *EDEM*, *sXBP1*, *GRP94*, and *PDI*, showed no significant changes. Furthermore, dantrolene, an inhibitor of ER stress, attenuated Zn^{2+}-induced GT1-7 cytotoxicity [25]. These results strongly suggested that PERK-related pathways are involved in Zn^{2+}-induced ER stress.

In addition to Zn, trace elements such as iron (Fe), copper (Cu), and manganese (Mn) are distributed at different levels in various parts of the brain and maintain the normal structure and function [39,40]. Among these metals, we showed that the presence of Cu^{2+} markedly exacerbated Zn^{2+}-induced GT1-7 cytotoxicity [25]. Cu^{2+} alone did not affect the gene expression levels of *Arc*, *CHOP*, and *GADD34* but significantly enhanced the induction of these factors by Zn^{2+}. Furthermore, western blotting showed that the co-administration of Zn^{2+} and Cu^{2+} significantly increased the amount of CHOP protein. CHOP is involved in the initiation of the apoptotic cascade [41] and the activation of GADD34, which reportedly increases after traumatic brain injury [42]. We also found that the antioxidant thioredoxin-conjugated human serum albumin (HSA-Trx) attenuated Cu^{2+}- and Zn^{2+}-induced neuronal cell death [43]. Zn exists only as Zn^{2+}, whereas Cu is a redox-active metal that exists as oxidised Cu^{2+} and reduced Cu^+. Cu^{2+} administration induces ROS generation in GT1-7 cells, whereas Zn^{2+} alone does not induce ROS generation or affect Cu^{2+}-induced ROS generation [14]. It is widely known that oxidative stress is involved in various neurodegenerative diseases, and ROS that cause oxidative stress adversely affects many signalling pathways, such as SAPK/JNK-related and ER-related pathways [44–47]. The co-administration of Cu^{2+} and Zn^{2+} to GT1-7 cells activated SAPK/JNK, phospho-c-Jun, and phospho-ATF2 downstream of the SAPK/JNK pathway. Furthermore, SP600125, an inhibitor of the SAPK/JNK signalling pathway, significantly suppresses Cu^{2+}- and Zn^{2+}-induced SAPK/JNK signalling pathway activation and neuronal cell death [14]. In addition, suppression of Cu^{2+} and Zn^{2+} cytotoxicity by HSA-Trx inhibits SAPK/JNK signalling pathway activation and ROS production [43]. Furthermore, the endogenous selenium (Se)-containing amino acid selenomethionine (Se-Met) induces glutathione peroxidase, blocks ROS production,

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significantly inhibited CHOP induction, and inhibites Cu^{2+}- and Zn^{2+}-mediated cytotoxicity [48]. Cu^{2+} triggers ROS production, which may enhance Zn^{2+} cytotoxicity by inducing the SAPK pathway and ER stress. These findings indicated that Zn^{2+} may be significantly involved in the ER stress pathway. Figure 3 shows our hypotheses regarding Zn^{2+}-induced neuronal death (and the protective effect of carnosine, which will be discussed later).

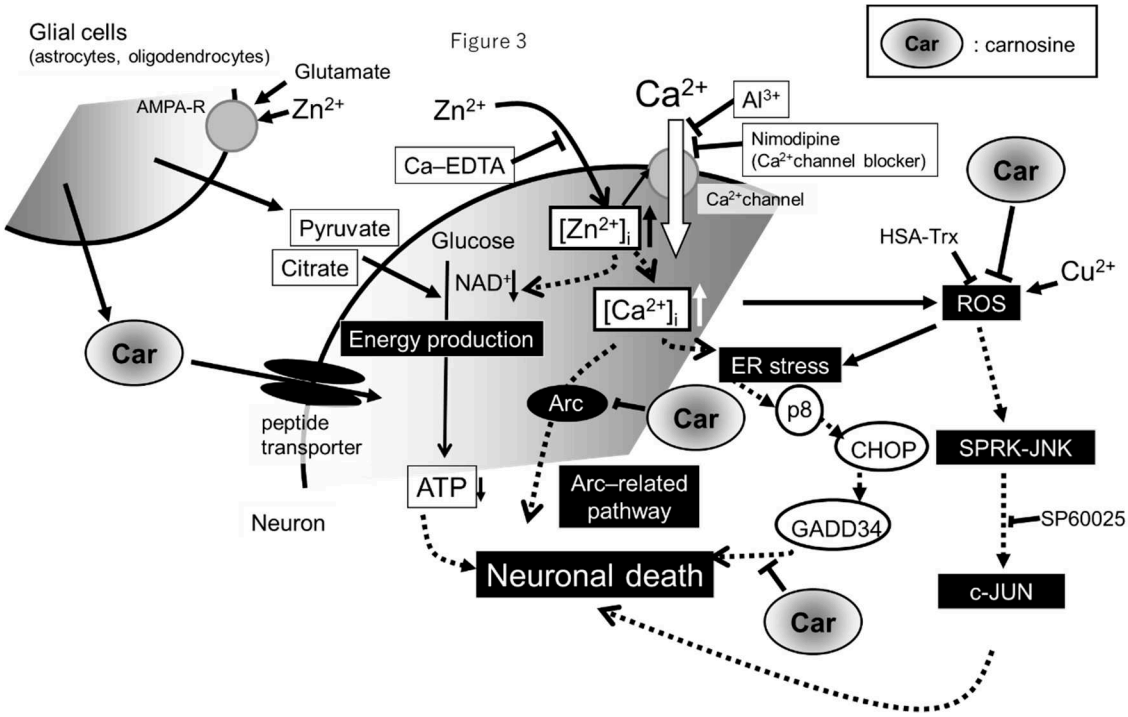

Figure 3. Hypothetical illustration of the molecular mechanism underlying the protective effect of carnosine in preventing zinc-induced neuronal death. Zn^{2+} is stored in presynaptic vesicles and is secreted into the synaptic cleft during ischaemia. Excess secreted Zn^{2+} translocates into the cell and can cause disruption of Ca^{2+} homeostasis, mitochondrial energy failure, endoplasmic reticulum (ER), and oxidative stress and consequently apoptotic neuronal death. Co-exposure to Zn^{2+} and Cu^{2+}, which are stored in presynaptic vesicles and secreted during ischaemia, potentiates these effects. These pathways are inhibited by Zn^{2+} chelators (Ca-EDTA), Ca^{2+} channel blockers (Al^{3+} and nimodipine), energy substrates (pyruvate and citrate), SAPK/JNK signalling pathway inhibitor (SP600125), and antioxidants (HSA-Trx). Carnosine inhibits the ER stress-related, Arc-related apoptotic, and ROS pathways. Carnosine is synthesised in glial cells, secreted in response to glutamate and Zn^{2+} stimulation, and protects neurones from Zn^{2+} neurotoxicity.

2.3. neurodegenerative diseases and Zn

Based on the aforementioned results, the hypotheses regarding Zn^{2+}-related neurotoxicity are as follows (Figure 3). Normally, Zn^{2+} and Cu^{2+} are released into the synaptic cleft upon neuronal excitation and regulate signal transduction [20]. Secreted Zn^{2+} and Cu^{2+} undergo rapid reuptake into presynaptic neurones via the Zn transporter or CTR1, thereby maintaining the level of these ions in the synaptic cleft. However, under conditions such as transient global cerebral ischaemia, prolonged neuronal excitation occurs in major parts of the brain, and Zn^{2+} and Cu^{2+} are released from synaptic vesicles into the synaptic cleft and translocate to the same neurones in large amounts. Increased $[Zn^{2+}]_i$ inhibit of mitochondrial energy production mechanisms and increases the $[Ca^{2+}]_i$. Impaired cellular protein folding due to energy depletion causes the accumulation of defective proteins in the ER. An increase in the $[Ca^{2+}]_i$ induces ROS generation. This potentiates ER stress and/or the SAPK/JNK pathways leading to apoptotic neuronal cell death.

There is increasing evidence that Zn^{2+}-mediated postischaemic neuronal cell death is involved in neurodegeneration after stroke or ischaemia [39,49–51]. VD is a disease associated with such

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neurodegeneration [8,52]. After transient global ischaemia, blockage of blood flow and concomitant oxygen and glucose deprivation induce abnormal neuronal excitation in most parts of the brain, followed by an excessive release of glutamate into synaptic clefts. A continuous influx of large amounts of Ca^{2+} is triggered, causing delayed cell death of vulnerable neurones in the hippocampus or cerebral cortex, leading to the development of infarcts and, ultimately cognitive impairment and VD. Epidemiological studies have reported that approximately 30% of patients with stroke develop symptoms of dementia 3 years later [53]. Under ischaemic conditions, significant amounts of Zn^{2+} (approximately 300 μM) have been reported to be released into the synaptic cleft along with glutamate after membrane depolarisation [54]. Furthermore, there is a report showing that Zn accumulates in apoptotic neurones in the hippocampus after ischaemia [55]. The administration of calcium ethylenediaminetetraacetate (Ca-EDTA), a membrane-impermeable Zn^{2+} chelator, protected hippocampal neurones and reduced infarct volume after transient global ischaemia in experimental animals [56]. Kitamura et al. revealed an increase in the extracellular Zn^{2+} levels in rats with transient middle cerebral artery occlusion using microdialysis [57]. Additionally, Zn^{2+} contributes to increased blood-brain barrier (BBB) permeability following ischaemia [58].

Zn^{2+}-mediated neuronal cell death may be involved in the pathogenesis of PD [3]. Dopaminergic neuronal shedding and microglial activation, which are implicated in the pathogenesis of PD, have been observed in animal models of PD established using 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [59]. ROS derived from 6-OHDA, which are taken up through dopamine transporters, intraneuronal 6-OHDA autoxidation, extracellular 6-OHDA autoxidation, and microglial activation are the molecular mechanisms responsible for 6-OHDA-induced dopaminergic degeneration [60]. 6-OHDA is readily oxidised and generates several reactive and cytotoxic products, such as quinones, H_2O_2, 5,6-dihydroxyindole, superoxide anion radicals, hydroxyl radicals, and singlet oxygen [61]. ROS derived from paraquat, an herbicide that is also taken up by dopamine transporters, lead to glutamate exocytosis via transient receptor potential melastatin 2 cation channel activation in the substantia nigra, followed by nigral dopaminergic degeneration via intracellular Zn^{2+} dysregulation [62,63]. From the intracellular ROS derived from 6-OHDA and PQ, H_2O_2 readily passes through cell membranes via aquaporin channels [64]. H_2O_2 elevation in the extracellular compartment excites glutamatergic neurone terminals [65], and Zn^{2+} is released along with glutamate. Additionally, we showed that treatment with 6-OHDA induces integrated stress-related genes, such as *CHOP*, *GADD34*, and *ATF4* in GT1-7 cells [66]. Increase of Zn^{2+} release by 6-OHDA may be related to induction of these factors.

Zn^{2+} entry and an increased $[Zn^{2+}]_i$ in other words 'Zn translocation' are key events in Zn^{2+}-induced neurotoxicity. There are three major pathways for Zn^{2+} entry: voltage-gated Ca^{2+} channels, N-methyl-D-aspartic acid (NMDA)-type glutamate receptors, and AMPA/kainate-type glutamate receptors (A/K-R) [28]. Under normal conditions, most hippocampal neurones express AMPA receptors containing GluR2 subunits that are poorly permeable to Ca^{2+} and Zn^{2+}. However, after ischaemia, there is a sharp decrease in GluR2 expression, and neurones express Ca^{2+}-permeable AMPA receptors (Ca-A/KR). As the permeability of Zn^{2+} and Ca^{2+} through the Ca-A/KR channels is greater than that through the NMDA receptor channels, the increased expression of Ca-A/KR channels enhances Ca^{2+} and Zn^{2+} toxicity. Zn^{2+} is also thought to be involved in the transcriptional regulation of Ca-A/KR channels, as Ca-EDTA attenuates ischaemia-induced downregulation of the *GluR2* gene [56]. These Zn^{2+}-mediated neuronal cell death events can be explained by the Zn^{2+}-mediated neurotoxicity hypothesis. Considering the involvement of Zn^{2+} in transient global ischaemia, substances that prevent Zn^{2+}-induced neuronal cell death may be potential candidates for the prevention or treatment of postischaemic neurodegeneration and, ultimately, the treatment of VD and PD. We used extracts of various agricultural products, such as vegetables, fruits and fish, and found that extracts of the Japanese eel (*Anguilla japonica*), mango fruit (*Mangifera indica L.*), and round herring (*Etrumeus teres*) protected GT1-7 cells from Zn^{2+}-induced neurotoxicity. The active fractions were separated from these extracts using high-performance liquid chromatography (HPLC) and the structures of the components were analysed by LC mass spectrometry. The active compounds included carnosine, citric acid, and histidine [16,34,67].

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3. Carnosine can be a therapeutic agent for cerebrovascular dementia

3.1. Carnosine

Carnosine is a natural dipeptide composed of β-alanine and L-histidine (His). Carnosine and its analogues (anserine [1-methylcarnosine] and homocarnosine) are present in most vertebrate tissues, including those of birds, fish, and mammals including humans [17,18]. In particular, carnosine is present in high levels in animals and fish that exercise frequently, such as horses, chickens, bonitos, and whales. In humans, the carnosine levels have been reported to be higher in males, decrease with age, and be diet dependent, with a vegetarian diet reducing carnosine levels in the skeletal muscle [68,69]. Similar to creatine and ATP, this dipeptide is also found in some muscle net at 50-200 mM [70,71]. In animals, factors such as trauma, shock, starvation, and injection adversely affect the carnosine levels in muscle tissue. Infection and trauma may be associated with dysregulation of cellular Ca and myocardial depression. Carnosine may also play a role in the regulation of cardiac cell contractility and $[Ca^{2+}]_i$ [72].

Carnosine is abundant in animals and fish that exercise frequently, such as horses, chickens, bonito, and whales. For example, intramuscular carnosine concentrations are 6–10 times higher in horses than in humans [73]. We analysed the carnosine content in thoroughbred horse muscle and found that carnosine content was associated with the muscle fibre type [74]. Among the five equine muscle tissues (radius flexor, gill triceps brachii, masseter, gluteus medius, and sternocleidomastoid), the gluteus medius had the highest carnosine concentration. The glutaeus medius is enriched with type IIa (fast-twitch oxidative glycolytic muscle fibres) and IIx (fast-twitch glycolytic muscle fibre) fibres [75]. As these muscle fibres are primarily used during high-intensity exercise, carnosine may play an important role in high-intensity exercise. Due to the alkaline nature of carnosine (pKa = 7.01), it is thought to play a significant role in intracellular buffering [76]. This buffering action is thought to play an important role in maintaining pH balance against the production of lactic acid, which causes muscle contraction fatigue due to acidosis during high-intensity anaerobic exercise and a decrease in intracellular pH. Therefore, the muscle carnosine concentration may be positively related to exercise performance [73,77]. Highly trained athletes have higher carnosine levels compared to untrained individuals [78]. Furthermore, dietary supplementation with carnosine or β-alanine increases the concentration of muscle carnosine and delays fatigue during high-intensity exercise [78].

In addition, carnosine possesses various functions, such as anti-oxidation, anti-glycation, anti-cross-linking, and metal chelation, which mediate its beneficial effects *in vivo* [18]. Carnosine scavenges both reactive oxygen- and nitrogen-containing unpaired electrons and inhibits lipid oxidation through a combination of free radical scavenging and metal chelation. It also inhibits the Maillard reaction, which produces many end-products, especially advanced glycation end-products, that contribute to the development of various senile diseases, such as AD, vascular sclerosis, atherosclerosis, and osteoarthritis. In addition, carnosine exhibits anti-crosslinking properties that inhibit protein oligomerisation. N-acetylcarnosine is used as a treatment for cataracts because carnosine inhibits α-crystal fibrosis of the lens [79]. Polaprezinc, a Zn-carnosine complex, is effective in repairing gastrointestinal ulcers and other lesions [80]. Polaprezinc is also used in Zn supplementation therapy and shows protective effects against cadmium-induced lung injury [81].

3.2. Carnosine in the brain

Carnosine and homocarnosine have been detected in the mammalian brain, but anserine has not yet been detected [82]. β-alanine is readily transported throughout the brain by Na^+-dependent-amino acid transport system(s) and acts as a neuromodulator/neurotransmitter or might, in theory, form carnosine [83]. Additionally, the carnosine transporter (peptide transporter 2) is expressed in some rat neuronal cells [84,85]. This suggests the putative ability of carnosine to cross the BBB. In the brain, carnosine is present in the olfactory bulb [86] and has been reported to be secreted from oligodendrocytes upon stimulation with glutamate [87]. Boldyrev et al. reported that carnosine is mainly present in the neurones or glial cells of the olfactory bulb, with levels in the olfactory bulb

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exceeding 1,000 μmol/kg [18]. We developed a quantitative analysis method for carnosine and its analogues using an HPLC system equipped with a carbon column (Hypercarb™) [88] and investigated the distribution of these compounds in the rat brain [89]. The rat brain contains significant amounts of carnosine in the olfactory bulb but less carnosine in the cerebral cortex and cerebellum, and anserine was not detected in any region tested. It has also been revealed that the carnosine levels in the olfactory bulb increase from the foetal stage to maturity. In contrast, the homocarnosine levels show no change with postnatal age. These results are similar to those of the previous studies described earlier. Biffo et al. showed that carnosine is located in olfactory receptor neurones, specifically in the perinuclear membrane and neurites, including the axons and synaptic terminals of the olfactory bulb [90]. Carnosine is rapidly synthesised and transported to the olfactory bulb via axonal transport [91]. In primary olfactory neurones, carnosine synthase activity is decreased by denervation and is restored by regeneration [92,93]. Carnosine in the olfactory bulb may be localised mainly in the sensory neurones [19].

Because carnosine forms complexes with Ca^{2+}, Cu^{2+}, and Zn^{2+} [94,95], it plays an important role in regulating Zn^{2+} homeostasis at synapses in neural tissues, especially in the carnosine- and Zn-rich olfactory lobes [18]. Disease-associated proteins (e.g., AβP, prion protein and α-synuclein) are thought to be central to the pathogenesis of various neurodegenerative diseases known as 'structural diseases' including AD, DLB, and PD. Carnosine interferes with cross-linking and subsequent conformational changes through its anti-cross-linking action [40]. Corona et al. reported that the administration of carnosine inhibited AβP deposition and improved the learning ability of AD model mice [96]. Carnosine prevents oxidative stress and inflammation induced by AβP [97]. We previously reported that carnosine alleviates neuronal cell death by changing the conformation of the prion protein fragment peptide (PrP106-126) [98]. In addition, carnosine has been reported to reduce Mn-induced neurotoxicity [99]. Based on these beneficial properties, carnosine is thought to act as a 'gatekeeper' or a 'neuroprotectant' in the brain [100].

3.3. Carnosine suppresses Zn-induced neuronal death

We found that carnosine has a protective effect on neurones from Zn^{2+}-induced neurotoxicity (Figure 4) and are investigating the mechanism. Our previous study showed that carnosine did not affect $[Zn^{2+}]_i$ or the expression of metal-related genes, such as *ZnT-1* (Figure 5a,b) [35]. Although carnosine can chelate Zn^{2+}, it does not inhibit Zn^{2+} translocation by binding to extracellular Zn^{2+}. In contrast, we found that carnosine inhibited the Zn^{2+}-induced expression of ER stress-related genes, such as *GADD34* and *CHOP*, and the Ca^{2+}-related gene *Arc* (activity-related cytoskeletal protein) (Figure 5b) [35]. ER stress has been implicated in the development of various neurodegenerative diseases such as AD, PD, and ischaemia-induced neurodegeneration [34,101]. *GADD34*, a gene encoding a sensor protein for ER stress, is induced by DNA damage and is thought to be involved in DNA repair and tumorigenesis [102]. *Arc* resides in the dendrites and encodes a protein that plays an important role in synaptic plasticity and memory consolidation. *Arc* expression is induced by increased neuronal activity in response to learning and brain-derived neurotrophic factor [103]. Carnosine attenuates neurodegeneration induced by ER stressors such as thapsigargin and tunicamycin [35]. Thus, carnosine may protect neurones from Zn^{2+}, not by inhibiting Zn^{2+} translocation, but by affecting ER stress and Arc-related pathways (Figure 3). Studies in experimental animals have suggested that carnosine protects against ischaemia-induced neurodegeneration *in vivo* [104–107]. Carnosine reduced 6-OHDA-induced neuronal cell death and inflammatory responses in GT1-7 cells. Specifically, carnosine markedly inhibited 6-OHDA-induced upregulation of the stress-related genes, such as *Chop*, *GADD34*, and *Atf4*. Furthermore, it suppresses 6-OHDA-induced activation of the SAPK/JNK signalling pathway by inhibiting ROS production [66]. Therefore, it may effectively prevent the onset and/or exacerbation of PD.

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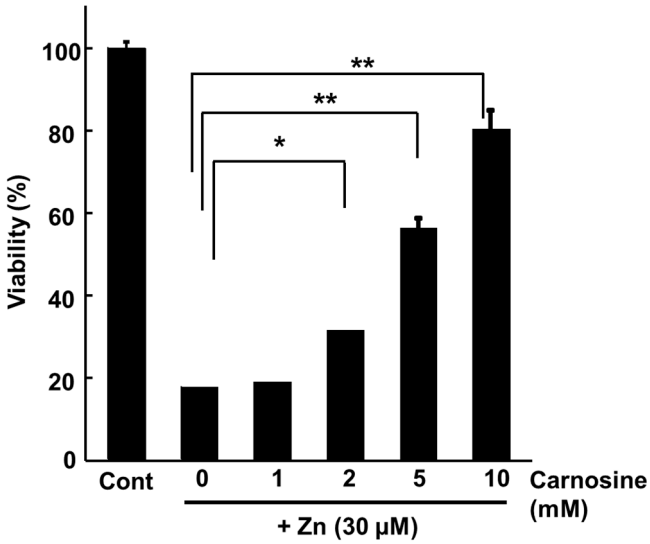
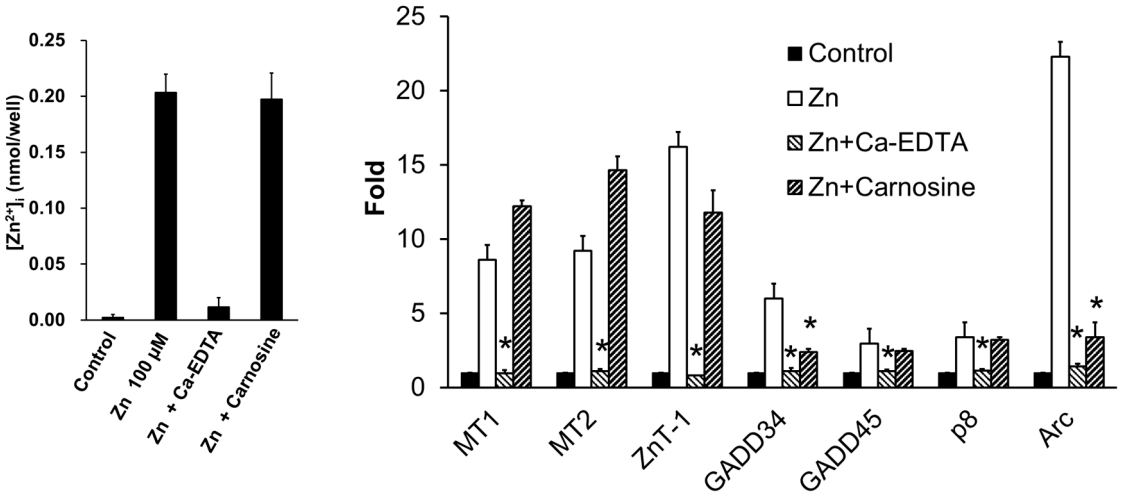


Figure 4. Protective activity against Zn^{2+}-induced neurotoxicity in GT1-7 cells. GT1-7 cells are treated with $ZnCl_2$ (30 μM) with or without various carnosine levels. After 24 h, the viability of GT1-7 cells is measured using the WST-1 assay. The data are presented as means ± S.E.M., $n = 6$. * $p < 0.01$, ** $p < 0.005$.



(**a**) (**b**)

Figure 5. Effects of carnosine on the expression of Zn-induced factors. (**a**) Effects of carnosine and anserine on Zn influx into GT1-7 cells. GT1-7 cells are treated with 100 μM of $ZnCl_2$ for 30 min in the presence or absence of Ca-EDTA (0.2 mM) or carnosine (2.0 mM), and $[Zn^{2+}]_i$ was measured using a Metallo Assay Zinc LS kit (Metallogenics, Chiba, Japan) according to the manufacturer's instructions. Data are presented as means ± S.E.M., $n = 3$. (**b**) Effects of carnosine on Zn-induced gene expression. GT1-7 cells are treated with 50 μM $ZnCl_2$ for 6 h in the presence or absence of Ca-EDTA (0.5 mM) or carnosine (5.0 mM). The expression of metal-, ER stress-, and Arc-related genes is analysed by RT-PCR, and the gene expression levels are normalised to β-actin. Data are presented as the mean ± S.E.M., $n = 3$. ** $p < 0.01$ versus the Zn group.

3.4. Potential uses of carnosine and its derivative as supplements

Orally administered carnosine is widely believed to be rapidly degraded to β-alanine and histidine by circulating carnosinases (CN1). However, carnosine or β-alanine supplementation has been reported to increase the carnosine levels in the brain [108,109]. Carnosine supplementation (40 mg/day) has been shown to be effective in treating patients with severe depressive disorder [110]. In addition, carnosine/anserine supplementation (750 mg of anserine and 250 mg of carnosine per day)

has been reported to improve episodic memory [111] and mild cognitive impairment [112] in older adults. Therefore, dietary carnosine or related amino acids may be synthesised into carnosine in the brain and taken up into cells by oligopeptide transporters such as PEPT2, PHT1, and PHT2 [113]. A recent epidemiological study reported an inverse correlation between the serum β-alanine levels and aetiology of dementia [114]. Considering these factors and the fact that the carnosine levels in the body decrease with age [115], carnosine replacement therapy may be beneficial for VD, AD, and other neurological disorders. Finding derivatives or analogues of carnosine that are resistant to degradation by CN1 is important for exploiting its full potential. Pharmacological variants of carnosine, such as carnosinol, a reduced carnosine derivative that is resistant to CN1, have been developed and show promise for use in the treatment of metabolic diseases, such as obesity and diabetes [116]. Additionally, balenine, found in marine mammals and reptiles, is a more stable natural analogue than carnosine in vivo and has potential uses as a dietary or ergogenic supplement [117].

4. Conclusions

Our hypothesis regarding the molecular pathways involved in Zn^{2+}-induced neurotoxicity may aid in the development of preventive and therapeutic agents for VD and PD. Based on the activity of carnosine, we published two patents for carnosine and its related compounds (D-histidine) as drugs or supplements for the prevention and/or treatment of VD [118,119]. Carnosine has many beneficial properties such as water solubility, heat inactivation, and non-toxicity, making it an excellent neuroprotective drug or supplement that benefits human health. Further studies are required to elucidate the molecular mechanisms by which carnosine prevents neurotoxicity.

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