Protective activity of mango (*Mangifera indica* L.) fruit against a zinc-induced neuronal cell death is independent of its antioxidant activity

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Summary

Mango, *Mangifera indica* L., is widely used in traditional medicine. We now report the novel activity of mango fruits protecting the zinc-induced neuronal cell death. Among five kinds of fruits including mango, apple, cherry, kumquat, and loquat, the aqueous extract of the mango fruit alone prevented the zinc-induced apoptosis of GT1-7 cells, which originated from hypothalamic neuron cells. This activity was observed both in the sarcocarp and the peel of the mango fruits. Because the effect of reactive oxygen species is implicated in neuronal cell death following transient ischemia, the antioxidant activities of the mango fruits were also examined. A protective activity against H_2O_2-induced GT1-7 cell death was observed in the peel extract, but not in the sarcocarp one. The scavenging activity assays of the superoxide anion and 1-1-diphenyl-2-picrylhydrazyl radical showed that both activities in the peels were higher than those in the sarcocarps. The features of the antioxidant activity in the mango fruits did not correspond to those of the protective activity against zinc-induced cell death. This indicates that the protective activity of the mango fruit against a zinc-induced neuronal cell death is independent of its antioxidant activity.

Mango, *Mangifera indica* L., is commonly grown in many parts of the world and the plant is widely used in traditional medicines. The extracts obtained from the fruits and stem bark contains vitamins, polyphenols, terpenoids, steroids, fatty acid, and trace elements, and have been reported to possess antitumor, antineoplastic, antioxidant, and anti-inflammatory activities[1-6]. Recently, it has been reported that an ischemia-induced neuronal loss and oxidative damage in the gerbil brain were reduced by the aqueous extract obtained from the stem bark of mango[7]. This report led us to examine whether mango fruits as a food possesses the activity protecting the neurodegenerative damage induced by transient ischemia.

Zinc, which is an essential trace element in the body, is concentrated in the central nervous system and is released with synaptic activity or membrane depolarization[8-10]. Recent studies have suggested that endogenous zinc play the role as an ionic mediator of neuronal cell death[11]. Following brain injury such as global ischemia, presynaptic zinc was specifically accumulated in the degenerating neurons of the hippocampal CA1 as well as other vulnerable neurons in the cortex, amygdala, striatum, and thalamus, which caused neurodegeneration[12, 13]. The experiment using a metal chelator showed that the inhibition of such Zn^{2+} accumulation drastically reduced ischemic neuronal cell death, and strongly suggested that the inter-neuronal movement of Zn^{2+} is a key mechanism of ischemic neuronal death[13]. In other words, the zinc-induced neuronal cell death is a good phenomenon for examining the neurodegenerative damage following brain injury such as transient ischemia[11].

We have developed an *in vitro* screening system using zinc-induced cell death, which would determine the compounds preventing ischemic neuronal death. We chose the immortalized hypothalamic neuronal GT1-7 cells[14, 15],

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which are widely used as the model neurons for neuroendocrine studies[16]. We found that the apoptosis-like death of GT1-7 cells occurred by exposure to lower concentrations of zinc than the primary cultured neurons of the rat cerebral cortex and hippocampus[14] as well as other immortalized neuronal cells such as PC12 did[17]. Using this system, we have already identified the protective agents for cell death[14, 18]. The candidates include pyruvate, which has also been reported to inhibit the zinc-induced death of primary cultured neuronal cell[19], α-tocopherol (an antioxidant agent), and gadolinium (a widely known channel blocker).

In this report, we investigated the protective activity against zinc-induced neuronal cell death in five kinds of fruits including mango, apple, cherry, kumquat, and loquat. The aqueous extract obtained from mango fruits alone was found to attenuate the cell death among five extracts. Because reactive oxygen species are implicated in neuronal cell death following transient ischemia, we further investigated antioxidant activities of mango fruits. The protective and antioxidant activities were examined and compared in two portions, sarcocarp and peel, of three different ripening mango fruits.

Materials and Methods

Preparation of fruit extracts Five fruits, mango (*Mangifera indica* L. Irwin ver.) (Miyazaki, Japan), apple (*Malus* × *domestica* Borkh. cv. Fuji) (Aomori, Japan), cherry (*Prunus avium* L.) (Miyazaki, Japan), kumquat (*Fortunella japonica*) (Miyazaki, Japan), and loquat (*Eriobotrya japonica*) (Nagasaki, Japan) were obtained from local commercial sources. The fruit samples were homogenized by a fruit juicer, and the final concentrations of the juices were adjusted to 0.5 g fresh weight of fruit per mL with the appropriate amount of water. They underwent two freeze-thaw cycles. The clear supernatants were obtained by centrifugation at 20,000 g for 20 min, and then filtered through a 0.22 μm-pore filter. The fruit aqueous extracts were stored at -20℃ until use.

Cell culture GT1-7 cells were cultured as previously described[15]. Briefly, the cells were grown in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 Ham (DMEM/F-12) supplemented with 10 % fetal calf serum. Upon reaching confluence, the cells were dissociated by incubation for 5 min at 37℃ in trypsin-EDTA solution. After enzymatic dissociation, the cells were resuspended in serum-free DMEM and plated on 96-well formatted culture dishes at the concentration of 5×10^5 or 10^6 cells / well. The cells were incubated for 24 hr in a humidified atmosphere of 93 % air and 7 % CO_2 at 37℃.

Cell viability assay GT1-7 cells were plated on 96-well formatted culture dishes at the concentration of 5×10^5 cells / cm^2 for assaying zinc-induced cell death or 10^6 cells / cm^2 for assaying H_2O_2-induced cell death. After 24 hr *in vitro*, the solutions of $ZnCl_2$ were added to the cell culture medium at the final concentrations of 30 μM, or the diluted H_2O_2 were added to the medium at the final concentrations of 250 μM for inducing neuronal cell death. The viability of the cells was measured by the WST-1 assay (Cell Counting Kit, Dojindo Chemicals, Japan) after a 24-hr exposure to zinc or H_2O_2. The WST-1 assay is a modification of the MTT assay, which is commonly used for assaying the cell viability, and measured the activity of the cellular mitochondorial dehydrogenase using a microplate spectrophotometric reader. The OD_{450} - OD_{620} value represented the cell viability. To examine the effects of the extract obtained from the various fruits on the zinc or H_2O_2-induced cell death, the extracts were preadministered to the culture medium just prior to exposure to the zinc or H_2O_2.

Antioxidant activity assay The free radical scavenging activity of the extract was measured by both the SOD Assay Kit-WST-1 (Dojindo Chemicals, Japan) and 1,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay[20]. In the SOD Assay Kit-WST, the highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium) produces a water-soluble formazan dye upon reduction with a superoxide anion. The experiments were performed according to manufacturer's instructions. Briefly, 2 μL fruit extracts (1 mg fresh weight of fruit / well) and superoxide anion-generating solution were put into the wells on a 96-well formatted plate, then the plate was incubated for 30 min at 37℃ after addition of the WST-1 solution. The absorbance of the WST-1 formazan was measured at 450 nm using a spectrophotometric microplate reader. The superoxide anion scavenging activity was calculated according to the following formula.

$$\text{Activity (\%)} = 1 - [(\text{Abs}_{\text{sample (+)}} - \text{Abs}_{\text{sample (-)}}) / (\text{Abs}_{\text{control (+)}} - \text{Abs}_{\text{control (-)}})] \times 100$$

where (+) and (-) indicate the presence and absence of the superoxide anion-generating solution, respectively. In the DPPH radical scavenging assay, 200 μL of DPPH (200 μM) was added to 2 μL extracts (1 mg fresh weight of fruit / well) on the 96-well formatted plate, and incubated for 30 min at 37℃. The absorbance of the DPPH radical was measured at 540 nm using a spectrophotometric microplate reader. The DPPH radical scavenging activity was calculated according to the following formula.

$$\text{Activity (\%)} = 1 - (\text{Abs}_{\text{sample}} / \text{Abs}_{\text{control}}) \times 100$$

Statistical analysis A statistical analysis of cell viability was carried out by the Student's t test. Data are expressed as means +/- SD. The value of $p < 0.05$ is determined to be significant.

Results

The 24-hr treatment of the $ZnCl_2$ induced the apoptosis-like cell death, which had been confirmed by a terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling[15]. The cell morphology was changed after the treatment of zinc; the cell became round and their cell surface blebbing occurred (Fig. 1A). Aqueous extracts from the edible portion of mango fruit partially prevented the zinc-induced cell death, whereas the kumquat extract had no effect (Fig. 1B and C). Protective activity against zinc-induced neuronal cell death was also examined in aqueous extracts from the edible portion of various fruits. The WST-1 assay also showed that the mango extract alone significantly attenuated the zinc-induced cell death among the five fruit-extracts (Fig. 2A). Furthermore, the aqueous extracts obtained from two portions, the sarcocarp and the peel, of the mango fruits during three different ripening stages were examined (Fig. 2B). The mango fruits change their peel colors in the order of green, purple and red during fruit ripening. The sarcocarp of the green-colored mango had the highest protective activity among them. The protective activity significantly decreased as the mango fruits ripened in the sarcocarps. In contrast, the red-colored mango peel extract alone showed the protective activity. The direction of the activity changes is opposite between the sarcocarps and peels in the mango fruits.

Because the effect of reactive oxygen species is implicated in neuronal cell death following transient ischemia, the antioxidant activities of mango fruits were also examined by the assay of H_2O_2-induced GT1-7 cell death (Fig. 3). The aqueous sarcocarp extracts obtained from purple-colored and green-colored mango fruits significantly reduced the H_2O_2-induced cell death, whereas the extract obtained from the red ones did not (Fig. 3A). The purple-colored mango extract showed the highest protective activity among them. The peel extracts obtained from the mango fruits of all ripening stages showed a protective activity against the H_2O_2-induced cell death, and the activity in the peel of the purple-colored mango is the highest (Fig. 3B). The scavenging activities for superoxide anion and DPPH

radical was examined in the aqueous extracts obtained from two portions, the sarcocarp and the peel, of the mango fruits during three different ripening stages (Fig. 4). In both assays with the superoxide anion and DPPH radical, the peel extracts showed a higher scavenging activity than the sarcocarps in every ripening mango fruit. The sarcocarp extracts of the red-colored mango fruit showed the highest scavenging activity for both the superoxide anion and DPPH radical.

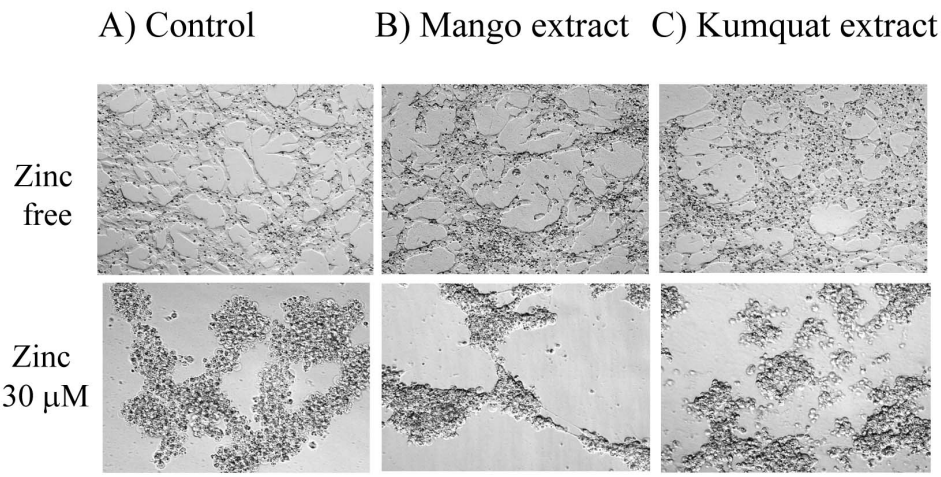

Fig. 1 Effect of aqueous mango and kumquat extracts on zinc-induced GT1-7 cell death. The GT1-7 cells were administered with the extracts, then treated with (lower) or without (upper) zinc (30 μM), and phase-contrast was taken after a 24-hr exposure of zinc.

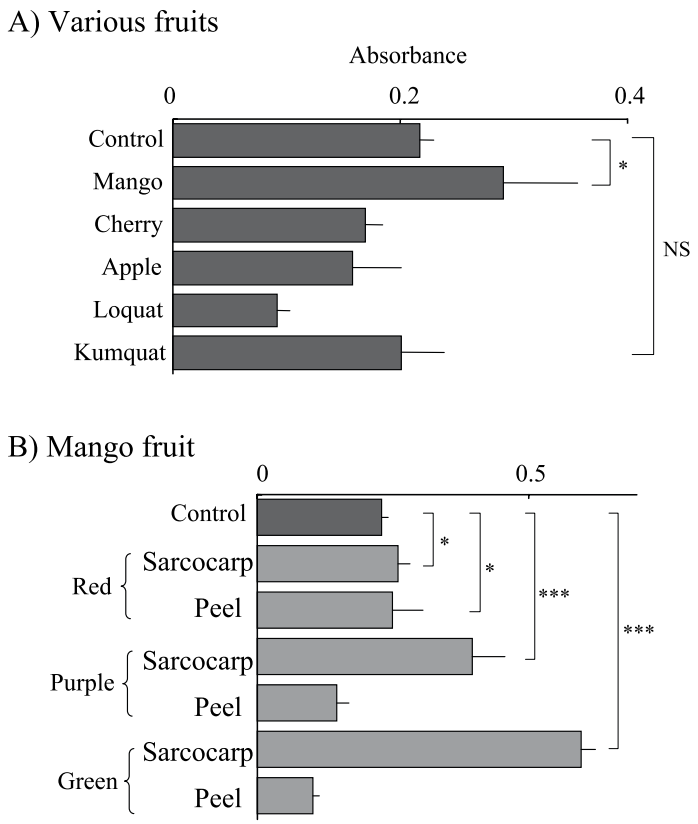

Fig. 2 Effect of aqueous extract obtained from five kinds of fruits (A) and from the sarcocarps and peels (B) of three different ripening mango fruits on zinc-induced GT1-7 cell death. Viabilities were determined by the WST-1 absorbance using the cells after a 24-hr exposure of zinc at the final concentration of 30 μM in the presence of the fruit extracts. Data are expressed as mean +/- SD, n = 6. * and *** indicate significances at $p < 0.05$ and $p < 0.001$ vs. controls, respectively.

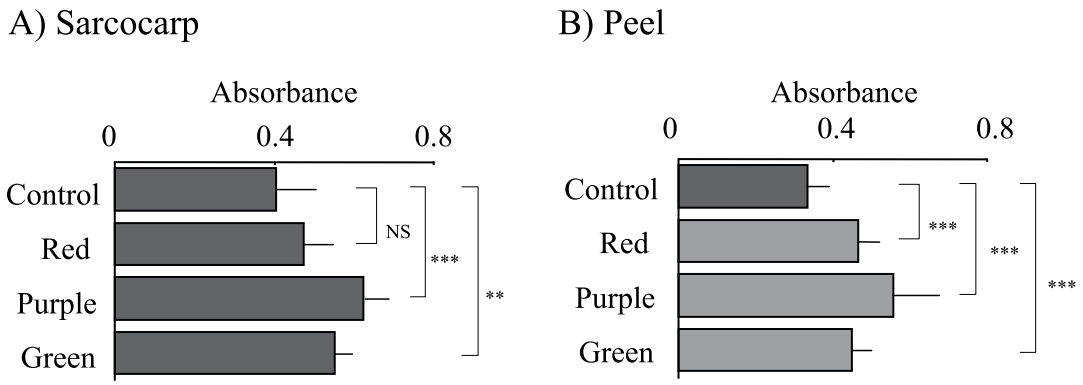

Fig. 3 Effect of aqueous extract obtained from the sarcocarps (A) and peels (B) of three different ripening mango fruits on H_2O_2-induced GT1-7 cell death. Viabilities were determined by the WST-1 absorbance using the cells after a 24-hr exposure of H_2O_2 at the final concentration of 250 μM in the presence of the fruit extracts. Data are expressed as mean +/- SD, n = 6. ** and *** indicate significances at $p < 0.01$ and $p < 0.001$ vs. controls, respectively. "NS" indicates no significance.

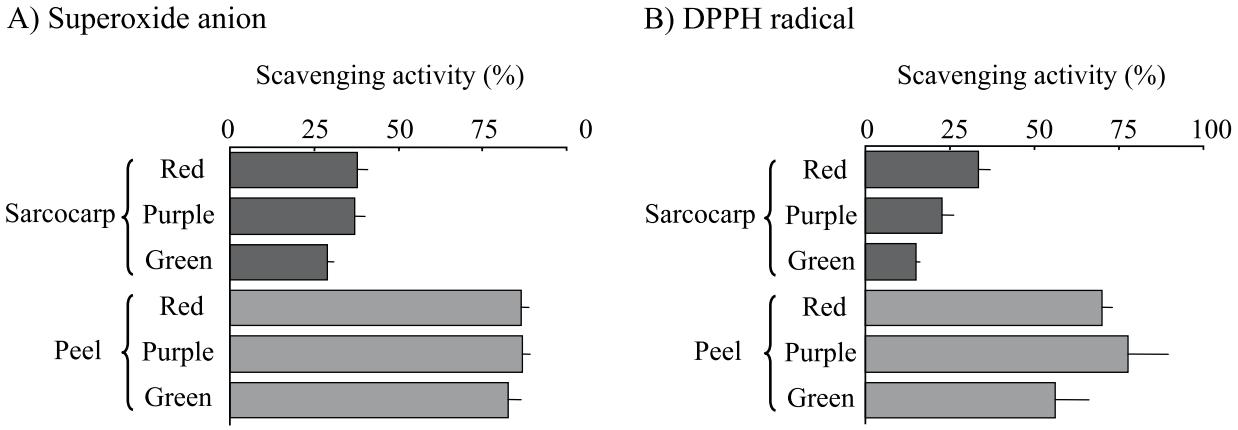

Fig. 4 Scavenging activities for superoxide anion (A) and DPPH radical (B) in aqueous extracts obtained from the sarcocarps and peels of three different ripening mango fruits. Data are expressed as mean +/- SD, n = 4.

Discussion

It has been reported that endogenous zinc plays the role as an ionic mediator of neuronal cell death[11], and the inhibition of such Zn^{2+} by metal chelator accumulation drastically reduced neuronal cell death[13]. Furthermore, brain injury, such as transient ischemia, induces the accumulation of zinc in the presynaptic region, which causes the subsequent neurodegeneration[12, 13]. Thus, protective agents for the zinc-induced neuronal cell death would be good seeds for developing a medicine for reducing neurodegenerative damage following the transient ischemia[11]. In this study, we determined the novel activity of mango fruits protecting the zinc-induced neuronal cell death. Among five kinds of fruits, the mango fruit alone showed the most significant protective activity. The eatable mature mango has a protective activity against zinc-induced cell death both in its edible portion, sarcocarp, and peel even though the highest activity is found in the sarcocarp of the immature green-colored mango fruits. These results suggest that the tropical fruit, mango, contains compound(s) for preventing ischemia-induced neuronal cell death, which some-

times causes vascular dementia.

It is generally assumed that free radical damage under ischemia is induced by the enhanced production of the superoxide anion and OH radical, which are toxic and contribute to neuronal death[21]. Sánchez et al.[7] reported that the aqueous extract obtained from the mango stem bark reduces both the ischemia-induced neuronal cell death and oxidative damage in the gerbil brain *in vivo*, and suggests that these protective activities are due to the antioxidant activity of the extract. We examined the antioxidant activities of the aqueous extracts obtained from two portions, the sarcocarp and the peel, of mango fruits during three different ripening stages. Assays of the scavenging activity for the superoxide anion and DPPH radical showed that the activity in the peel is higher than that in the sarcocarps in every ripening mango fruit. This result does not correspond to that in assays of the zinc-induced cell death because the protective activities against zinc-induced cell death are not different between the sarcocarp and the peel extracts. In the sarcocarp extracts, the red-colored mango fruit showed the highest scavenging activity in both assays. The protective activity against H_2O_2-induced cell death is higher in the sarcocarp extracts of the purple-colored mango fruits than the green-colored ones. Because the aqueous sarcocarp extract of the green-colored mango fruits shows the highest protective activity against zinc-induced cell death among the three different ripening mangos, the assay results of the H_2O_2-induced cell death did not correlate with that in the assays of the zinc-induced cell death. As a result, the features of the antioxidant activity in the mango fruits did not correspond to those of the protective activity against zinc-induced cell death. This indicates that the protective activity of the mango fruit against a zinc-induced neuronal cell death is independent of its antioxidant activity. We have shown that both pyruvate and heavy metal ions (Al^{3+} and Gd^{3+}) prevent the zinc-induced cell death[15, 18]. However, they may not be the effective ingredient for inhibiting zinc-induced cell death because the pyruvate concentration in mango aqueous extract is too low to inhibit (data not shown). We need further studies to identify the component responsible for protecting against zinc-induced neuronal cell death.

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