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Research Article

Cytoprotective effects of phenolic acids on methylglyoxal-induced apoptosis in Neuro-2A cells

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In the process of glycation, methylglyoxal is a reactive dicarbonyl compound physiologically generated as an intermediate of glycolysis, and is found in high levels in blood or tissue of diabetic models. Biological glycation has been commonly implicated in the development of diabetic microvascular complications of neuropathy. Increasing evidence suggests that neuronal cell cycle regulatory failure followed by apoptosis is an important mechanism in the development of diabetic neuropathy complication. Naturally occurring antioxidants, especially phenolic acids have been recommended as the major bioactive compounds to prevent chronic diseases and promote health benefits. The objective of this study was to investigate the inhibitory abilities of phenolic acids (chlorogenic acid, syringic acid and vanillic acid) on methylglyoxal-induced mouse Neuro-2A neuroblastoma (Neuro-2A) cell apoptosis in the progression of diabetic neuropathy. The data indicated that methylglyoxal induced mouse Neuro-2A neuroblastoma (Neuro-2A) cell apoptosis via alternation of mitochondria membrane potential and Bax/Bcl-2 ratio, activation of caspase-3, and cleavage of poly (ADP-ribose) polymerase. Furthermore, the results demonstrated that activation of mitogen-activated protein kinase signal pathways (JNK and p38) participated in the methylglyoxal-induced Neuro-2A cell apoptosis process. Treatment of Neuro-2A cells with phenolic acids markedly suppresses cell apoptosis induced by methylglyoxal, suggesting that phenolic acids possess cytoprotective ability in the prevention of diabetic neuropathy complication.

Keywords: Cytoprotection / Glycation / Methylglyoxal / Neuro-2A cells / Phenolic acids

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1 Introduction

Biological glycation has been commonly investigated in the development of diabetic microvascular complications, including neuropathy, retinopathy, and atherosclerosis [1]. In the process of glycation, methylglyoxal is a reactive dicarbonyl compound physiologically generated as an intermediate of glycolysis. Glycation is a process of post-translational modification of proteins, in which reducing sugars such as glucose react non-enzymatically with amino groups in proteins through a series of Maillard reactions forming reversible Schiff base and Amadori compounds, producing finally advanced glycation end products (AGEs) [2]. Glucose-derived dicarbonyl intermediate, such as methylglyoxal or 3-deoxyglucosone (3-DG), is a potent precursor of AGEs formation, and is found in high levels in blood or tissue of experimental models of diabetes. Diabetic complications generally develop at a slow rate, and the long-term effects of methylglyoxal on the formation of AGEs have been well established and demonstrated [3]. AGEs have been known to accumulate in various tissues at an accelerated rate under diabetic condition [4] and are implicated in the development of diabetic complications [5, 6], suggesting that these protein deposits have been long exposed to AGEs precursors such as the reactive dicarbonyl methylglyoxal compound. This process of methylglyoxal accumulation often occurs under hyperglycemic conditions, impaired glucose metabolism and oxidative stress [7, 8]. Methylglyoxal is most likely to participate in intracellular AGEs formation, due to its enormous reactivity and con-

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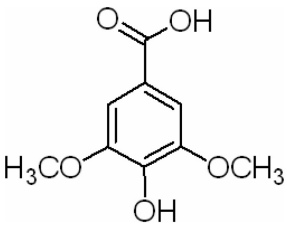
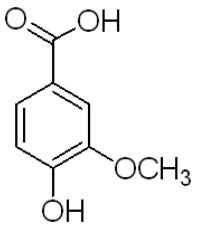
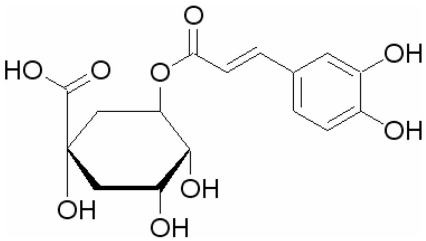
Abbreviations: AGEs, advanced glycation end products; **ΔΨm**, mitochondrial membrane potential; **LDH**, lactate dehydrogenase; **MAPK**, mitogen-activated protein kinase; **MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; **Neuro-2A**, mouse Neuro-2A neuroblastoma; **PARP**, poly (ADP-ribose) polymerase; **PI**, propidium iodide



Syringic acid



Vanillic acid



Chlorogenic acid

Figure 1. Chemical structures of syringic acid, vanillic acid and chlorogenic acid.

stant production as a consequence of the degradation of triosephosphates [9]. Methylglyoxal has received considerable attention as it is converted from triosephosphates, and must be detoxified by well-know catabolism pathway, the glutathione-dependent glyoxalase system, a metabolic pathway that catalyzes the detoxification of methylglyoxal to D-lactose [10]. It is important to note that excess methylglyoxal presents serious toxicological effects since it depletes glutathione via covalent bonding between methylglyoxal and glutathione.

The cytotoxicity of methylglyoxal to tissue or cells is mediated through induction of apoptosis. Some previously published studies have demonstrated that reactive methylglyoxal is capable of induction of apoptosis in Jurkat leukemia T cells by activating c-Jun N-terminal kinases, which further decreases the mitochondrial membrane potential ($\Delta\Psi m$), and followed caspase-3 activation [11]. The methylglyoxal has been implicated in nerve damage with much work done on the etiology of diabetic neuropathy. There are many complex interactions involved in the progression of diabetic neuropathy including oxidative stress, biological advanced glycation, reduced nerve conduction volume and blood, axonal degeneration, fiber demyelination, and neuronal cell apoptosis [12]. Recent study indicated that dicarbonyl compound has been linked to central nervous system complication, and the cytotoxic effect of methylglyoxal is due to its ability to augment intracellular oxidative stress and decrease cell viability [13]. Mechanistically, methylglyoxal-induced peripheral nerve-derived Schwann cell apoptosis via the p38 mitogen-activated protein kinase (MAPK) pathway suggested that glucose-derived methylglyoxal contributes to the development of diabetic neuropathy by damaging nerve system via cell apoptosis in hyperglycemia milieu [14].

Phenolic acids, are widely distributed in various vegetables and fruits, and possess many physiological and pharmacological functions [15]. Many studies have suggested that phenolic acids in plants could inhibit oxidative stress induced by free radicals and protect photooxidation. *In vitro* and *in vivo* experiments have shown that phenolic acids exhibit powerful effects on biological responses by scavenging free radicals and eliciting antioxidant capacity. Additionally, phenolic acids were also found to exhibit anti-inflammatory, antiallergic, antimutation effects, and inhibit cardiovascular diseases [16, 17]. In our previous studies, we reported that phenolic acids showed biological functions in the protection against adverse effects related to oxidative stress damage [18, 19]. However, the cytoprotective effect of phenolic acids on methylglyoxal accumulation leading to apoptosis of neuronal cells remains unclear. In our preliminary experiments, we tested 14 types of phenolic acids, finding that chlorogenic, syringic, and vanillic acids showed the most inhibitory effects on methylglyoxal-induced Neuro-2A cell death by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The objective of this study was to investigate the biological effects of phenolic acids (chlorogenic, syringic and vanillic acid) (Fig. 1) on methylglyoxal-induced Neuro-2A cell apoptosis in the progression of diabetic neuropathy and to understand, which of the phenolic acids exhibits cytoprotective effects and contributes to diabetic complications.

2 Materials and methods

2.1 Antibodies and chemical reagents

Chlorogenic acid, methylglyoxal, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, propidium iodide (PI), syringic acid and vanillic acid were purchased from Sigma Chemical (St. Louis, MO). α-MEM medium, trypsin-EDTA (TE) and PSN solution (penicillin-streptomycin) were purchased from Gibco BRL (Grand Island, NY). Polyvinylidene fluoride (PVDF) membrane was purchased from Millipore (Bedford, MA). Anti-caspase-3, anti- poly (ADP-ribose) polymerase (PARP), anti-BCL-2, anti-Bax, anti-Bad, anti-Bak, anti-JNK, anti-phospho-JNK, anti-ERK, anti-phospho-ERK, anti-p38, anti-phospho-p38, and anti-β-actin were purchased from Cell Signal Technology (Beverly, MA). All other chemicals used were of the highest purity available.

2.2 Cell culture and treatment

The Neuro-2A neuroblastoma cell line was obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsin Chu, Taiwan). Cells were grown in DMEM medium, supple-

mented with 10% heat-inactivated fetal bovine serum (FBS), glutamine (2 mM), streptomycin/penicillin (100 mg/mL/ 100 U/mL), and L-glutamine (2 mM) at 37°C, in a humidified atmosphere of 95% air and 5% CO_2. For experiment, the neuronal cells were co-cultured with 400 μM of methylglyoxal and 20 μM of phenolic acids (chlorogenic acid, syringic acid and vanillic acid).

2.3 Cell viability assay

The cell viability was determined with the MTT assay. Neuro-2A cells were co-cultured with 400 μM of methylglyoxal and with the indicated concentrations of phenolic acids (chlorogenic, syringic and vanillic acids) for 72 h. Then, Neuro-2A cells were seeded onto 96-well plates at a concentration of 1×10^6 cells/well in DMEM plus 10% FBS. Dye solution (10 μL) specific for the MTT assay was added to each well for additional incubation for 4 h at 37°C. After the addition of DMSO (100 μL/well), the absorbance at 570 nm (formation of formazan) and 630 nm (reference) was recorded with a FLUOstar Galaxy plate reader (BMG Lab Technology, Offenburg, Germany). The percent viability of the treated cells was calculated as follows:
$(A_{570\,nm} - A_{630\,nm})$ sample/$(A_{570\,nm} - A_{630\,nm})_{control} \times 100$.

2.4 Cytotoxicity assay

Lactate dehydrogenase (LDH) leakage was measured as indicator of cytotoxicity. Neuro-2A cells were co-cultured with 400 μM methylglyoxal and 20 μM of phenolic acids (chlorogenic, syringic and vanillic acid) for 72 h. Cells were seeded onto 96-well plates at a concentration of 1×10^6 cells/ well in DMEM plus 10% FBS and then analyzed for LDH leakage into the culture media by using the commercial kits (Sigma Chemical). The total LDH activity was determined after cells were thoroughly disrupted by sonication. The percentage of LDH leakage was then calculated to determine membrane integrity. The LDH leakage was expressed as a percentage of total activity: (activity in the medium)/(activity in the medium + ctivity of the cells) × 100.

2.5 Analysis of cell-cycle distribution by PI staining

To investigate the effects of phenolic acids (chlorogenic, syringic and vanillic) on the cell-cycle distribution of Neuro-2A, cells (2×10^6 cells/mL) treated with various concentrations of phenolic acids and cultured for 72 h were harvested, washed with PBS, and fixed in 75% of ethanol at 4°C overnight. After washing twice with cold PBS, cells were re-suspended in PBS containing 40 μg/mL PI and 0.1 mg/mL RNase followed by shaking at 37°C for 15 min. Cells were analyzed with flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) and the data were consequently evaluated by CELL Quest software.

2.6 Annexin V-FITC/PI double staining analysis by flow cytometry

Annexin V-FITC/PI double staining of the cells was evaluated using an Annex V-FITC kit (ANNEX100F, SEROTEC, UK). To determine the effects of phenolic acids (chlorogenic, syringic and vanillic) on early apoptosis, late apoptosis and necrosis state induced by methylglyoxal in Neuro-2A cells, Neuro-2A cells (1×10^6 cells/dish) were seeded to each well of a 6-cm dish and incubated for 72 h at 37°C in 1 mL culture medium containing testing agent at suitable concentrations to give a final concentration of 0, 50, 100, and 250 μM. Approximately 1×10^5 cells were stained for 10 min at room temperature with Annexin V-FITC and PI in a Ca^{2+}-enriched binding buffer (Annex V-FITC kit) and analyzed by FACScan flow cytometry, using emission filters of 525 and 575 nm, respectively. Approximately 1×10^4 counts were made for each sample. The percentages of distribution of normal (Annexin V-FITC-/PI-), early apoptotic (Annexin V-FITC+/PI-), late apoptotic (Annexin V-FITC+/PI+) and necrotic cells (Annexin V-FITC-/PI+) were calculated by CELL Quest software.

2.7 Mitochondrial membrane potential assay

The $\Delta\Psi m$ was analyzed with the J-aggregate forming lipophilic compound 5,50,6,6 tetrachloro-1,1,3,3 tetraethylbenzamidazolocarbocyanin iodide (JC-1), which has been incorporated into the MitoPT 100 dye kit (Immunochemistry Technologies, LLC) used for reproducible detection of the mitochondrial permeability transition (PT) events in apoptotic cells. Neuro-2A cells (1×10^5 cells/mL) were seeded onto 96-well plate and treated with 20 μM of phenolic acids (chlorogenic, syringic and vanillic) for indicated times followed by incubation of the cells stained with the MitoPTTM dye reagent at 37°C for 15 min in the CO_2 incubator. The aggregate red form was detected by FLUO star galaxy spectrophotometer (BMG Labtechnologies, Offenburg, Germany) after excitation at 485 and emission at 520 nm. The apoptotic cells were identified by the accumulation of green fluorescence compared to the control.

2.8 Measurement of caspase-3 activity

For the determination of caspase activity, the downstream executor enzyme caspase-3 was evaluated. Cells were pretreated with methylglyoxal and the indicated phenolic acids (chlorogenic, syringic and vanillic) for 48 h. Then, the caspase-3 activity was measured by proteolytic cleavage of the fluorogenic substrate using the CaspaTagTM Caspase-3 (DEVD) Activity kit (BioTech, Santa Cruz, CA). Cells (1×10^6 cells/mL) from different treatments were collected and incubated with the Working Dilution reagent at 37°C for 1 h. After washing with the wash buffer, the cells were resuspended with PBS and the fluorescent intensity was

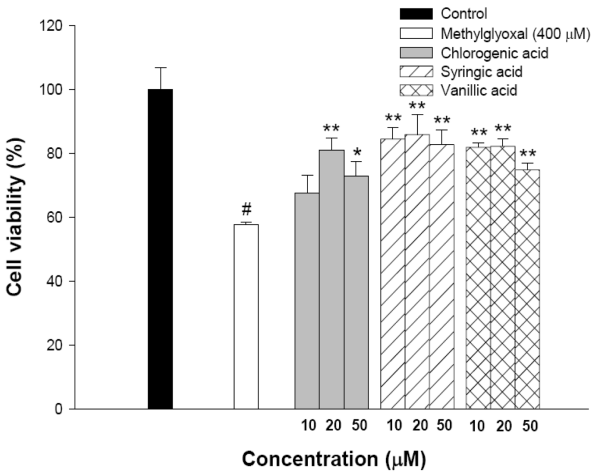


Figure 2. Effects of phenolic acids on cell viability of methylglyoxal-treated Neuro-2A cells. Neuro-2A cells were co-cultured with the indicated phenolic acid (10, 20 and 50 μM) and methylglyoxal (400 μM) for 72 h, and cell viability was evaluated by MTT assay. Values are mean ± SD of three independent experiments. #, $p < 0.05$ versus vehicle control. *, $p < 0.05$ versus methylglyoxal treatment only. **, $p < 0.01$ versus methylglyoxal treatment only.

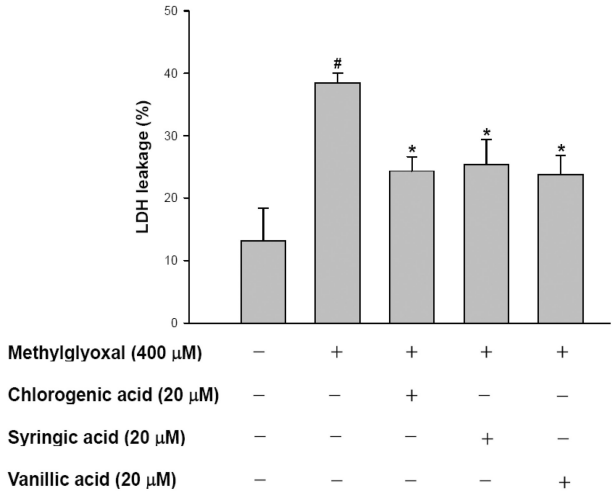


Figure 3. Effects of phenolic acids on cell cytotoxicity of methylglyoxal-treated Neuro-2A cells. Neuro-2A cells were co-cultured with the indicated phenolic acid (20 μM) and methylglyoxal (400 μM) for 72 h. LDH leakage was determined. Values are mean ± SD of three independent experiments. #, $p < 0.05$ versus vehicle control. *, $p < 0.05$ versus methylglyoxal treatment only.

detected by the fluorescence spectrophotometer with an excitation wavelength of 485 and an emission wavelength of 520 nm.

2.9 Western blot

The cytosolic proteins were isolated from Neuro-2A cells (1×10^6 cells/mL) after the treatment with methylglyoxal and the indicated phenolic acids (chlorogenic, syringic and vanillic). The total proteins were extracted by adding 500 μL of lysis buffer (50 mM Tris-HCl, pH 8.0; 1 mM NaF; 150 mM NaCl; 1 mM EGTA; 1 mM PMSF; 1% NP-40; and 10 μg/mL leupeptin) to the cell pellets on ice for 30 min, followed by centrifugation at $10\,000 \times g$ for 306 min at 4°C. The cytosolic fraction (supernatant) proteins were measured by Bradford assay (Bradford, 1976) with BSA as a standard. Total cytosolic extracts (250 μg of protein) were separated on 10% SDS-polyacrylamide mini-gels for indicated antibody detection (caspase-3, PARP, BCL-2, Bax, Bad, Bak, JNK, ERK, p38 and β-actin), and then transferred to Immobilon polyvinylidene difluoride membrane (PVDF; Millipore, Bedford, MA) with transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membrane was blocked in 5% BSA solution for 1 h at room temperature and then incubated overnight at 4°C with indicated primary antibodies (1:1000 dilutions). After hybridization with primary antibodies, the membrane was washed with Tris-buffered saline Tween-20 (TBST) three times, incubated with HRP-labeled secondary antibody for 45 min at room temperature, and washed with TBST three times. Final detection was performed with ECL (Enhance Chemiluminescence) Western blotting reagents (Amersham Pharmacia Biotech, UK). The relative expression of proteins was quantified using the software LabWorks 4.5 (Cambridge, UK) and calculated according to the reference bands of β-actin.

2.10 Statistical analysis

Each experiment was performed in triplicate and repeated three times. The results were expressed as means ± SD. Statistical comparisons were made by one-way analysis of variance (ANOVA), followed by a Duncan multiple-comparison test. Differences were considered significant when the p values were < 0.05.

3 Results

3.1 Effects of phenolic acids on cell viability of methylglyoxal-treated Neuro-2A cells

In preliminary experiments, the cell viability assay was performed with MTT photometric analysis. Neuro-2A cells were co-cultured with various doses of phenolic acids and 400 μM of methylglyoxal for 72 h, and then cell viabilities were measured. As shown in Fig. 2, methylglyoxal at a dose of 400 μM significantly reduced cell viability ($p < 0.05$). However, 20 μM of the indicated phenolic acids (chlorogenic, syringic and vanillic) showed inhibitory effects on methylglyoxal-induced Neuro-2A cell death ($p < 0.01$).

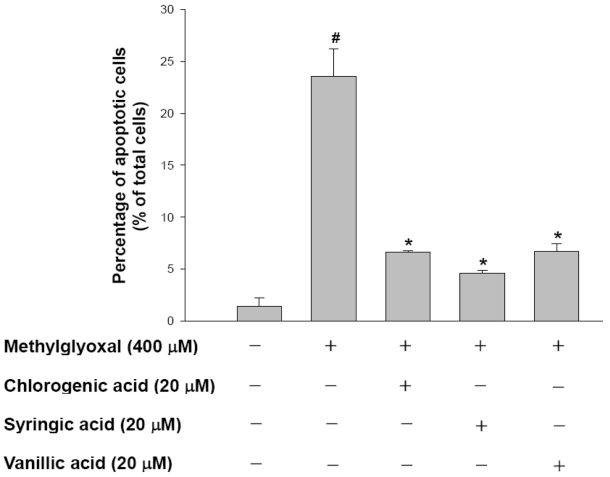


Figure 4. Effects of phenolic acids on cell apoptosis of methylglyoxal-treated Neuro-2A cells. Neuro-2A cells were co-cultured with the indicated phenolic acid (20 μM) and methylglyoxal (400 μM) for 72 h. Values are mean ± SD of three independent experiments. #, $p < 0.05$ versus vehicle control. * $p < 0.05$ versus methylglyoxal treatment only.

3.2 Effects of phenolic acids on cell cytotoxicity of methylglyoxal-treated Neuro-2A cells

The activity of LDH leakage was measured in the culture medium of Neuro-2A cells after treatments with 20 μM of phenolic acids and 400 μM of methylglyoxal. As shown in Fig. 3, methylglyoxal treatment of Neuro-2A cells for 72 h showed cytotoxicity and led to significant release of LDH as compared with control ($p < 0.05$). However, significant inhibition of LDH leakage was observed with a treatment with 20 μM of the indicated phenolic acids (chlorogenic, syringic and vanillic acid) ($p < 0.05$).

3.3 Effects of phenolic acids on Neuro-2A cell apoptosis induced by methylglyoxal

To quantify the degree of apoptosis, the amount of sub-G_1 DNA was analyzed by flow cytometry. As illustrated in Fig. 4, methylglyoxal treatment significantly increased the percentage of apoptotic cells at sub-G1 phase for 72 h ($p < 0.05$). Thus, treatment of Neuro-2A cells with methylglyoxal caused cell apoptosis. However, treatment with 20 μM of the indicated phenolic acids (chlorogenic, syringic and vanillic acid) of methylglyoxal-induced Neuro-2A cells significantly decreased the percentage of cells at sub-G1 phase ($p < 0.05$).

3.4 Effects of phenolic acids on Neuro-2A cell apoptosis/necrosis induced by methylglyoxal

The cell populations were further examined by Annexin V-FITC-binding assay to identify apoptosis, and PI staining was performed to exclude necrotic cells. Referring to the results shown in Table 1, the numbers of apoptotic cells including early and late apoptotic cells increased after the treatment with 400 μM of methylglyoxal for 72 h. The percentages of apoptotic cells (including early and late apoptotic cells) increased from 1.6% (control) to 27.9% (methylglyoxal, 400 μM) ($p < 0.05$). Methylglyoxal-induced Neuro-2A cell apoptosis was significantly reduced by the treatment with 20 μM of the indicated phenolic acids (chlorogenic, syringic and vanillic acid) ($p < 0.05$).

Table 1. Effects of phenolic acids on Neuro-2A cell apoptosis/necrosis induced by methylglyoxal

	Percentage of cells		
	Normal	Apoptotic	Necrotic
Control	97.7 ± 0.2	1.6 ± 0.3	0.7 ± 0.05
Methylglyoxal (MGO)	69.9 ± 0.3#	27.9 ± 0.1#	2.5 ± 0.2#
MGO + chlorogenic acid	85.8 ± 0.3*	12.1 ± 0.2*	2.1 ± 0.1*
MGO + syringic acid	91.3 ± 0.3*	6.9 ± 0.3*	1.8 ± 0.05*
MGO + vanillic acid	92.7 ± 0.4*	6.5 ± 0.5*	0.7 ± 0.06*

Cells were co-cultured with or without the indicated phenolic acids (20 μM) and methylglyoxal (MGO, 400 μM) for 72 h. Annexin V-FITC/PI double stained cells were analyzed by flow cytometry. The ratio of apoptotic cells was calculated by CELL Quest software. Values are means ± SD of three independent experiments.

\# $p < 0.05$ versus vehicle control.

* $p < 0.05$ versus methylglyoxal treatment only.

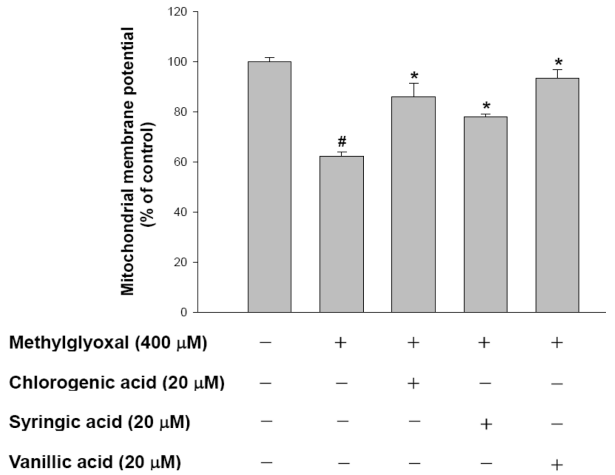


Figure 5. Effects of phenolic acids on mitochondrial membrane potential ($\Delta\Psi m$) in methylglyoxal-treated Neuro-2A cells. Results are expressed as percentages of mitochondria membrane potential. Values are mean ± SD of three independent experiments. #, $p < 0.05$ versus vehicle control. *, $p < 0.05$ versus methylglyoxal treatment only.

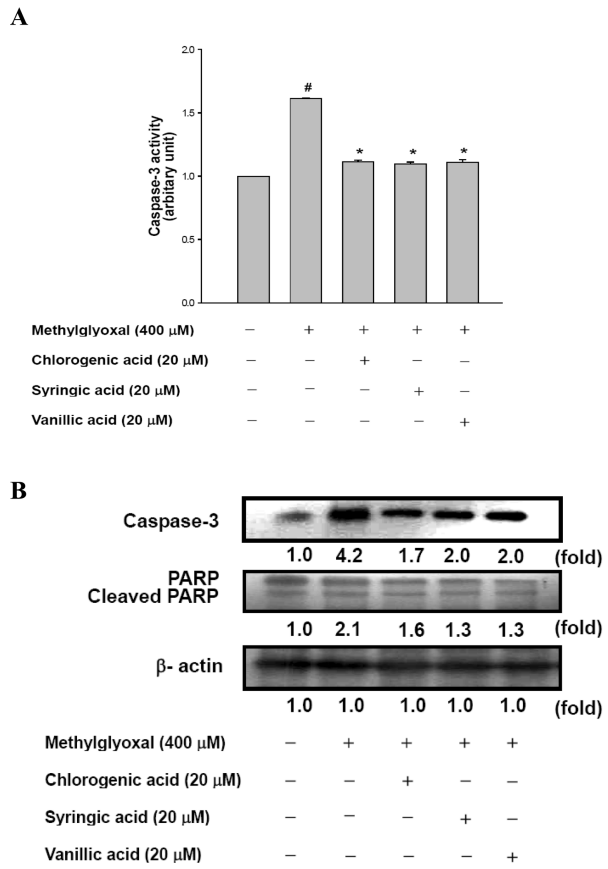


Figure 6. Effects of phenolic acids on activation of caspase-3 and cleavage of PARP in methylglyoxal-treated Neuro-2A cells. Cytosolic fraction of cells was analyzed for (A) ceaspase-3 activity by proteolytic fluorogenic substrates. Ac-DEVD-pNA was used as the substrate for caspase-3 activity of control cells was set to 100%, and the relative changes in the activity were shown. Values are mean ± SD of three independent experiments. #, $p < 0.05$ versus vehicle control. *, $p < 0.05$ versus methylglyoxal treatment only. (B) Cells were treated with methylglyoxal and phenolic acids for 36 h incubation. Total lysates extracted were analyzed for the proteolytic cleavage of PARP and caspase-3 by Western blot.

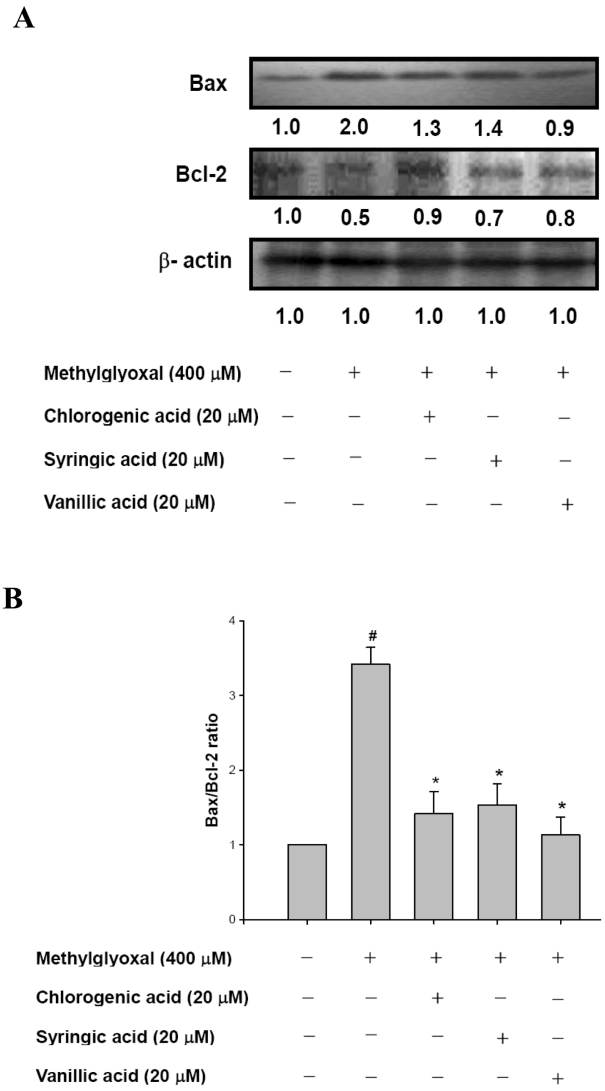


Figure 7. (A) Effects of phenolic acids on Bcl-2 and Bax protein expression in methylglyoxal-treated Neuro-2A cells. Total cell lysates of Neuro-2A cells were prepared after treating with methylglyoxal and phenolic acids at 36 h. Proteins separated by SDS-PAGE electrophoresis were immunoblotted and probed with antibodies of Bcl-2, Bax and β-actin. (B) Phenolic acids effect with methylglyoxal-treated Neuro-2A cells on Bax/Bcl-2 ratio. Data are mean ± SD of three independent experiments. #, $p < 0.05$ versus vehicle control. *, $p < 0.05$ versus methylglyoxal treatment only.

3.5 Effects of phenolic acids on ΔΨm in methylglyoxal-treated Neuro-2A cells

Mitochondria play an essential role in cell death signal transduction such as the permeability of transition pore opening and the collapse of ΔΨm resulting in a rapid release of caspase activators. Therefore, the effect of phenolic acids (chlorogenic, syringic and vanillic acid) on ΔΨm in methylglyoxal-treated Neuro-2A cells was investigated. As shown in Fig. 5, 400 μM of methylglyoxal induced serious mitochondrial disruption in Neuro-2A cells after 36-h incubation ($p < 0.05$). Phenolic acid treatment of Neuro-2A cells reduced the disruption of ΔΨm ($p < 0.05$).

3.6 Effects of phenolic acids on methylglyoxal-induced caspase-3 activation in Neuro-2A cells

In most of the apoptotic process, caspase-3 has been shown to play a pivotal role in the terminal, execution phase of apoptosis induced by diverse stimuli. The effect of phenolic acids (chlorogenic, syringic and vanillic acid) on caspase-3 activity in methylglyoxal-treated Neuro-2A cells was examined. As shown in Fig. 6A, the data indicated that treat-

ment with 400 μM of methylglyoxal of Neuro-2A cells for 48 h markedly increased the activity of caspase-3 ($p < 0.05$). However, a significant reduction of caspase-3 activity was observed with a treatment of 20 μM of the indicated phenolic acids ($p < 0.05$). Activation of caspase-3 leads to a cleavage of a number of proteins, including poly (ADP-ribose) polymerase (PARP). Furthermore, the effects of phenolic acids (chlorogenic, syringic and vanillic acid) on methylglyoxal-induced apoptotic pathways in Neuro-2A cells, transcription factor caspase-3 and PARP protein expression were evaluated by Western blot assay. As shown in Fig. 6B, treatment of methylglyoxal significantly stimulated caspase-3 protein expression, and induced cleavage of PARP protein with a significant effect in Neuro-2A cells as compared to control ($p < 0.05$). Treatment with phenolic acids of methylglyoxal-induced Neuro-2A cells indicated that phenolic acids markedly inhibited the expression of caspase-3 protein and decreased the cleavage of PARP protein ($p < 0.05$).

3.7 Regulatory effect of phenolic acids on Bcl-2 family proteins during methylglyoxal-induced Neuro-2A cell apoptosis

Since Bax and Bcl-2 play a crucial role in apoptosis, effect of phenolic acids on the expression of Bcl-2 family of pro- and anti-apoptotic protein in methylglyoxal-induced Neuro-2A cells was further analyzed. The Western blot analysis exhibited a significant increase of Bax protein, also a significant decrease of Bcl-2 protein in methylglyoxal-induced Neuro-2A cells for 36 h ($p < 0.05$). Treatment with phenolic acids (chlorogenic, syringic and vanillic acid) significantly decreased the expression of Bax protein, while increased the expression of Bcl-2 protein (Fig. 7A). Figure 7B shows that methylglyoxal-induced elevation of Bax/Bcl-2 ratio was significantly reduced by treatment with phenolic acids ($p < 0.05$). Our findings demonstrate an anti-apoptotic effect of phenolic acids on methylglyoxal-induced Neuro-2A cells.

3.8 Effects of phenolic acids on MAPK signaling pathways in methylglyoxal-induced apoptotic Neuro-2A cells

It is generally recognized that MAPK signaling pathways is involved in the growth and death of cell. The activation state of MAPK cascades was also determined in this study by immunoblotting technique. As shown in Fig. 8, cells were exposed to 400 μM of methylglyoxal and the protein phosphorylation levels of JNK and p38 were significantly increased after 24-h incubation. In contrast, no apparent change in the activation state of ERK pathway has been observed, suggesting that methylglyoxal stimulated the activation of JNK and p38 MAPK pathways in the process of Neuro-2A apoptosis. Furthermore, mehylglyoxal-induced activation of JNK and p38 MAPK pathways was markedly inhibited by the indicated phenolic acids (chlorogenic, syringic and vanillic acid). The results suggest that phenolic acids directly regulate the MAPK signal pathways (JNK and p38) to inhibit methylglyoxal-induced Neuro-2A cell apoptosis.

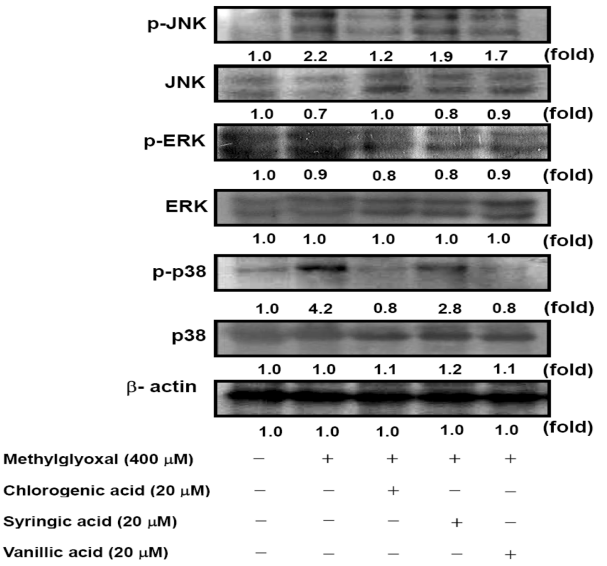


Figure 8. Effects of phenolic acids on the protein expression of total and phosphorylated forms of MAPK. Total cell lysates of Neuro-2A cells were prepared after treating with methylglyoxal and phenolic acids at 24 h. Proteins separated by SDS-PAGE were immunoblotted and probed to total and phosphorylated forms of MAPK and β-actin.

4 Discussion

Methylglyoxal is one of a series of glycation intermediates (dicarbonyls), which includes such reactive intermediates as glucosone, 3-deoxyglucosone and glyoxal that have been identified during Amadori rearrangement in the Maillard reaction [1]. Methylglyoxal is also formed from non-oxidative mechanisms [20, 21] by amine-catalyzed sugar fragmentation as well as spontaneous decomposition of triose phosphate intermediates in glycolysis, in which such glycation intermediates have been derived predominantly by non-oxidative means and might paradoxically induce oxidative stress and cell apoptosis [22].

Methylglyoxal is commonly formed via glycolytic metabolism. Therefore, many foodstuffs and beverages containing sugars represent exogenous sources of methylglyoxal. In general, methylglyoxal concentration in human blood is approximately 1 μM for the population, whereas diabetes mellitus patients perform three to six times of that amount and the levels in diabetic patients can remain elevated for several months and even years [23]. Methyl-

glyoxal is considered a toxic compound and has been correlated with diabetic complications, as it is a potent precursor of AGEs formation by the modification of proteins or lipids [24]. Such glycolytic intermediates-mediated glycation of protein has been implicated in the development of diseases, particularly chronic complications associated with diabetes such as macrovascular disease [25], retinopathy [26], neuropathy [27], nephropathy [28], Alzheimer's disease, and aging [4, 29].

Importantly, studies of methylglyoxal reported that the cytotoxicity of methylglyoxal to tissue or cells is mediated through an induction of apoptosis. The reactive methylglyoxal is capable of inducing apoptosis in Jurkat leukemia T cells by activating c-Jun N-terminal kinases [11]. *In vitro* study showed that methylglyoxal induced apoptosis on human leukemia 60 cells by alternation of DNA G1 growth arrest [30]. Diabetes neuropathy such as Alzheimer's disease is a neurodegenerative disease. Such neuropathy involved several mechanisms, including mitochondrial dysfunction, abnormal protein aggregation, and inflammation [6]. Recent study has reported that methylglyoxal induced peripheral nerve-derived Schwann cell apoptosis via the activation of p38 MAPK pathway, suggesting that glucose-derived methylglyoxal led to the development of diabetic neuropathy by damaging nerve system through cell apoptosis in hyperglycemia milieu [14].

Phenolic acids are widely distributed in plants and are present in considerable amount in human diet. Moreover, phenolic acids are potent antioxidants showing great biological activities in some *in vitro* and *in vivo* studies. In our preliminary experiments, we tested 14 types of phenolic acids, finding that chlorogenic acid, syringic acid, and vanillic acid showed the most inhibitory effects on methylglyoxal-induced Neuro-2A cell death (Fig. 2). A quantitative analysis of LDH activity can be used to determine the percentage of dead cells. LDH is a stable cytoplasmic enzyme, which is present in most cells. Therefore, we further examined the effects of phenolic acids on methylglyoxal-induced cytotoxicity. Our data indicated that significant inhibition of LDH leakage was observed after the treatment with 20 μM of the phenolic acids (Fig. 3). The results also suggested that chlorogenic acid, syringic acid, and vanillic acid might possess cytoprotective abilities against methylglyoxal-induced Neuro-2A cell injury.

Apoptosis is a type of physiological cell death accompanied by a special cellular mechanism and some distinctive morphological changes. During apoptosis, cells cycle stops and apoptosis is initiated prior to entry into S-phase [31]. Therefore, we confirmed the degree of Neuro-2A cell apoptosis, and the amount of sub-G_1 DNA was analyzed by flow cytometry technique. Figure 4 indicated that methylglyoxal treatment led to a significant accumulation on the percentage of apoptotic cells at sub-G1 phase for 72 h, whereas the phenolic acids decreased the accumulation of the percentage of cells at sub-G1 phase. Annexin V-FITC binds to phosphatidylserine and can be used to detect the early stages of apoptosis, and PI cannot enter cells with intact membranes and is used to differentiate between the normal, early apoptotic, late apoptotic and necrotic cells [25]. Thus, we further examined the cell populations of methylglyoxal-induced Neuro-2A cells. The numbers of apoptotic cells including early and late apoptotic cells increased after the treatment with 400 μM of methylglyoxal. Treatment with phenolic acids reduced methylglyoxal-induced Neuro-2A cell apoptosis (early and late apoptotic cells). Mitochondria play an essential role in death transduction such that the permeability of transition pore opening and the collapse of $\Delta\Psi m$ resulted in a rapid release of caspase activator [32]. As shown in Fig. 5, 400 μM of methylglyoxal induced serious mitochondrial disruption in Neuro-2A cells after 36-h incubation. Phenolic acids treatment of Neuro-2A cells decreased the damage to mitochondria membrane potential. These data demonstrate that methylglyoxal induced Neuro-2A cell apoptosis and treatment of phenolic acids effectively reduced the apoptotic cell death of Neuro-2A cells.

To clarify the effects of phenolic acids on molecular mechanisms of methylglyoxal-induced Neuro-2A cell apoptosis, we evaluated the protein levels of various key apoptosis-linked gene products, including caspase-3, PARP, Bcl-2, and Bax protein (Figs. 6 and 7). Many evidences suggest that activation of caspase cascade triggers apoptotic process in various cells. Caspase-3, a member of the caspase family, has been shown to play a key role in apoptosis induced by a variety of stimuli [33, 34]. Our results indicated that treatment of methylglyoxal significantly stimulated caspase-3 protein expression in Neuro-2A cells, and phenolic acids markedly inhibited the expression of caspase-3 protein. In addition, activation of caspase-3 leads to the cleavage of a number of proteins, one of which is PARP. This cleavage leads to its inactivation, thus preventing the futile DNA-repair cycle. Similar to the data for caspase-3, proteolytic cleavage of PARP was observed in methylglyoxal-treated Neuro-2A cells, whereas treatment of phenolic acids protected the cleavage of PARP (Fig. 6).

Some members of Bcl-2 family, such as Bcl-2 are apoptotic regulators suppressing cell death, while other homologues including Bax and Bak exhibit powerful death promoting abilities. The Bcl-2 family proteins play a central role in the process of cell apoptosis by interfering with the caspases. It is well established that the ratio between Bcl-2 and Bax is an important factor in the regulation of apoptosis rather than the level of each protein separately. Bax is a 21-kDa protein promoting mitochondrial membrane permeability, which has been demonstrated to accelerate apoptotic cell death. The ratio of Bcl-2 to Bax, rather than the levels of the individual proteins, is considered critical in the determination of survival/death of cells [35]. In this study, Fig. 7 showed that methylglyoxal-induced elevation of Bax/Bcl-2 ratio was significantly reduced by treatment with phenolic acids.

MAPK-signaling cascades are stimulated by many extracellular stimuli, such as cytokines, growth factor, and various stresses, and serve as a common signal transduction pathway shared by signals involved in cell proliferation, differentiation, functional activation, and stress responses [36]. Some previous studies have indicated that MAPK-signaling cascades are involved in the process of apoptosis [37, 38]. In this study, the activation state of MAPK-signaling cascades was determined by immunoblotting technique. As shown in Fig. 7, cells were exposed to 400 μM of methylglyoxal and the protein phosphorylation levels of JNK and p38 were significantly increased after 24-h incubation, suggesting that the activation of JNK and p38 pathway was associated with methylglyoxal-induced apoptosis. However, mehylglyoxal-induced activation of JNK and p38 MAPK pathways was markedly inhibited by the treatment with the indicated phenolic acids (Fig. 8).

In conclusion, the last-decade research has demonstrated that the neuropathy is a neurodegenerative disease. Increasing evidence suggests that neuronal cell-cycle regulatory failure followed by apoptosis is an important mechanism in the development of diabetic neuropathy complication. The data presented above showed that the glycation intermediate methylglyoxal was cytotoxic for neuronal cells in the progression of diabetic neuropathy. Methylglyoxal induced neuronal cell apoptosis via alternation of mitochondrial membrane potential and Bax/Bcl-2 ratio, activation of caspase-3, and cleavage of PARP. Taken together, we also demonstrated that activation of MAPK-signalling pathways (JNK and p38) participated in the methylglyoxal-induced neuronal cell apoptosis process. Naturally occurring antioxidants, especially phenolic acids have been recommended as the major bioactive compounds to prevent chronic diseases and promote health benefits. In this study, naturally occurring phenolics (chlorogenic, syringic and vanillic acid) showed cytoprotective effects against methyglyoxal-induced neuronal cell apoptosis, suggesting that phenolic acids might be a potential strategy in the prevention of diabetic neuropathy complications.

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5 References

[1] Singh, R., Barden, A., Mori, T., Beilin, L., Advanced glycation end-products: a review, *Diabetologia* 2001, *44*, 129 – 146.

[2] Chellan, P., Nagaraj, R. H., Protein crosslinking by the Maillard reaction: dicarbonyl-derived imidazolium crosslinks in aging and diabetes, *Arch. Biochem. Biophys*. 1999, *368*, 98 – 104.

[3] Uchida, K., Khor, O. T., Oya, T., Osawa, T., *et al*., Protein modification by a Maillard reaction intermediate methylglyoxal. Immunochemical detection of fluorescent 5-methylimidazolone derivatives *in vivo*, *FEBS Lett*. 1997, *410*, 313 – 318.

[4] Brownlee, M., Biochemistry and molecular cell biology of diabetic complications, *Nature* 2001, *414*, 813 – 820.

[5] Wong, A., Luth, H. J., Arendt, T., Munch, G., Advanced glycation endproducts co-localize with inducible nitric oxide synthase in Alzheimer's disease, *Brain Res*. 2001, *920*, 32 – 40.

[6] Picklo Sr, M. J., Montine, T. J., Amarnath, V., *et al*., Carbonyl toxicology and alzheimer's disease, *Toxicol. Appl. Pharmacol*. 2002, *184*, 187 – 197.

[7] Hail, G. M., Jr., Lo, T. W., Thornalley, P. J., Methylglyoxal concentration and glyoxalase activities in the human lens, *Exp. Eye Res*. 1994, *59*, 497 – 500.

[8] Abordo, E. A., Minhas, H. S., Thornalley, P. J., Accumulation of alpha-oxoaldehydes during oxidative stress: a role in cytotoxicity. Biochem. Pharmacol. *Biochem. Pharmacol*. 1999, *58*, 641 – 648.

[9] Thornally, P. J., Jahan, I., Ng, R., Suppression of the accumulation of triosephosphates and increased formation of methylglyoxal in human red blood cells during hyperglycaemia by thiamine *in vitro*, *J. Biochem*. 2001, *129*, 543-549.

[10] Thornalley, P. J., The glyoxalase system in health and disease, *Mol. Aspects Med*. 1993, *419*, 287 – 371.

[11] Du, J., Suzuki, H., Nagase, F. A., Akhand, A., *et al*., Methylglyoxal induces apoptosis in Jurkat leukemia cells by activating c-Jun N-terminal kinase, *Cell Biochem*. 2000, *77*, 333 – 44.

[12] Sima, A. A., Sugimoto, K., Experimental diabetic neuropathy: an update, *Diabetologia* 1999, *42*, 773 – 788.

[13] de Arriba, S. G., Krugel, U., Ralf, R., Vissiennon, Z., *et al*., Carbonyl stress and NMDA receptor activation contribute to methylglyoxal neurotoxicity, *Free Radic. Biol. Med.* 2006, *40*, 779 – 790.

[14] Fukunaga, M., Miyata, S., Higo, S., Hamada, Y., *et al*., Methylglyoxal induces apoptosis through oxidative stress-mediated activation of p38 mitogen-activated protein kinase in rat Schwann cells, *Ann. NY Acad. Sci.* 2005, *1043*, 151 – 157.

[15] Nardini, M., Pisu, P., Gentili, V., Natella, F., Di, M., *et al*., Effect of caffeic acid on tert-butyl hydroperocide-induced ocidative stress in U937, *Free Radical. Biol. Med.* 1998, *25*, 1098 – 1105.

[16] Lodovici, M., Guglielmi, F., Meoni, M., Dolara, P., Effect of natural phenolic acids on DNA oxidation *in vitro*, *Food Chem. Toxicol*. 2001, *39*, 1205 – 1210.

[17] Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem*. 1976, *72*, 248 – 254.

[18] Hsieh, C. L., Yen, G. C., Chen, H. Y., Antioxidant activities of phenolic acids on ultraviolet radiation-induced erythrocyte and low density lipoprotein oxidation, *J. Agric. Food Chem*. 2005, *53*, 6151 – 6155.

[19] Yeh, C. T., Yen, G. C., Induction of hepatic antioxidant enzymes by phenolic acids in rats is accompanied by increased levels of multidrug resistance-associated protein 3 mRNA expression, *J. Nutr*. 2006, *136*, 11 – 15.

[20] Thornalley, P. J., Westwood, M., Lo, T. W., McLellan, A. C., Formation of methylglyoxal-modified proteins in vitro and in vivo and their involvement in AGE-related processes, *Contrib. Nephrol.* 1995, *112*, 24 – 31.

[21] Thornalley, P. J., Pharmacology of methylglyoxal, *Gen. Pharmacol*. 1996, *27*, 565 – 573.

[22] Thornalley, P. J., Langborg, A., Minhas, H. S., Formation of glyoxal, methylglyoxal and 3-DG in the glycation of protein, *Biochem. J.* 1999, *344*, 109 – 116.

[23] McLellan, A. C., Thornalley, P. J., Benn, J., Sonksen, P. H., Glyoxlase system in clinical diabetes mellitus and correlation with diabetic complications, *Clin. Sci*. 1994, *87*, 21 – 29.

[24] Frye, E. B., Degenhardt, T. P., Thrope, S. R., Role of the Maillard reaction in aging of tissue proteins, *J. Biol. Chem*. 1998, *273*, 18714 – 18719.

[25] Vermes, I., Haanen, C., Steffens-Nakken, H., Reutelingsperger, C., A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V, *J. Immunol. Methods* 1995, *184*, 39 – 51.

[26] Yamagishi, S., Nakamura, K., Matsui, T., Advanced glycation end products (AGEs) and their receptor (RAGE) system in diabetic retinopathy, *Curr. Drug Discov. Technol.* 2006, *6*, 83 – 88.

[27] Yagihashi, S., Diabetic neuropathy, *Nippon Rinsho*. 2006, *3*, 155 – 160.

[28] Kurowski, R., Mantitus, J., Advanced glycation end products (AGEs) and renal failure, *Przeql. Lek*. 2006, *63*, 203 – 208.

[29] Munch, G., Thome, J., AGE's in aging and Alzheimer's disease, *Brain Res. Rev.* 1997, *23*, 134 – 143.

[30] Kang, Y., Edwards, L. G., Thornalley, P. J., Effect of methylglyoxal on human leukaemia 60 cell growth: modification of DNA G1 growth arrest and induction of apoptosis, *Leuk. Res*. 1996, *20*, 397 – 405.

[31] Kajstura, J., Bolli, R., Sonnenblick, E. H., Anversa, P., *et al*., Cause of death: suicide, *J. Mol. Cell. Cardio.* 2006, *40*, 425 – 437.

[32] Ly, J. D., Grubb, D. R., Lawen, A., The mitochondrial membrane potential (deltapsi(m)) in apoptosis; an update. Apoptosis: An International Journal on Programmed Cell Death, *Apoptosis* 2003, *8*, 115 – 128.

[33] Li, Y. H., Gong, P. L., Neuroprotective effects of dauricine against apoptosis induced by transient focal cerebral ischaemia in rats via a mitochondrial pathway, *Clin. Exp. Pharmacol. Physiol.* 2007, *34*, 177 – 184.

[34] Luan, J., Zhou, B., Qi, Z., Tolerance of mice to lipopolysaccharide is correlated with inhibition of caspase-3-mediated apoptosis in mouse liver cells, *Acta Biochem. Biophys. Sin.* 2007, *39*, 96 – 100.

[35] Scorrano, L., Korsmeyer, S. J., Mechanisms of cytochrome c release by proapoptotic BCL-2 family members, *Biochem. Biophys. Res. Commun.* 2003, *304*, 437 – 444.

[36] Seger, R., Krebs, E. G., The MAPK signaling cascade, *FASEB J*. 1995, *9*, 726 – 735.

[37] Cross, T. G., Scheel-Toellner, D., Henriquez, N. V., Deacon, E. *et al*., Serine/threonine protein kinases and apoptosis, *Exp. Cell Res*. 2000, *256*, 34 – 41.

[38] Franklin, R. A., McCubrey, J. A., Kinase: positive and negative regulators of apoptosis. Leukemia, *Leukemia*. 2002, *14*, 2019 – 2034.