Mechanism for ginkgolic acid (15 : 1)-induced MDCK cell necrosis: Mitochondria and lysosomes damages and cell cycle arrest

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[ABSTRACT] Ginkgolic acids (GAs), primarily found in the leaves, nuts, and testa of ginkgo biloba, have been identified with suspected allergenic, genotoxic and cytotoxic properties. However, little information is available about GAs toxicity in kidneys and the underlying mechanism has not been thoroughly elucidated so far. Instead of GAs extract, the renal cytotoxicity of GA (15 : 1), which was isolated from the testa of Ginkgo biloba, was assessed in vitro by using MDCK cells. The action of GA (15 : 1) on cell viability was evaluated by the MTT and neutral red uptake assays. Compared with the control, the cytotoxicity of GA (15 : 1) on MDCK cells displayed a time- and dose-dependent manner, suggesting the cells mitochondria and lysosomes were damaged. It was confirmed that GA (15 : 1) resulted in the loss of cells mitochondrial trans-membrane potential (ΔΨm). In propidium iodide (PI) staining analysis, GA (15 : 1) induced cell cycle arrest at the G0/G1 and G2/M phases, influencing on the DNA synthesis and cell mitosis. Characteristics of necrotic cell death were observed in MDCK cells at the experimental conditions, as a result of DNA agarose gel electrophoresis and morphological observation of MDCK cells. In conclusion, these findings might provide useful information for a better understanding of the GA (15 : 1) induced renal toxicity.

[KEY WORDS] Ginkgolic acids (15 : 1); Cytotoxicity; Mechanism; Necrosis

[Introduction] Ginkgo biloba L., a kind of living fossil, has the longest history than any other living tree on the earth [1]. As an important traditional herbal medicine, Ginkgo biloba is widely used for the treatment of cerebrovascular disorders, cardiovascular diseases, as well as Alzheimer’s diseases [2-4]. EGb761, a standardized formulation of the extracts, which is consisted of ginkgolides, bilobalide, and flavonoids, is an alternative medicine available in European countries [5]. However, a group of alkylphenols (anacardic or ginkgolic acids, cardanols and cardols) in crude Ginkgo extracts has been identified to process strong allergenic, mutagenic, and carcinogenic properties [6]. Ginkgolic acids (GAs) are defined as salicylic acid derivatives with extensive pharmacological and toxic activities, mainly existing in leaves, fruits, and testa of Ginkgoaceae. Actually, some therapeutically desired effects of GAs have been reported (e.g., anti-tumor and antiviral activities). The anti-tumor action of GA monomer (C22H34O3; molecular weight: 374.6; purity > 99.9%) has been investigated in tumorigenic Hep-2 (human epithelial larynx cancer cell line), Tac8113 (human tongue squamous carcinoma cell line) cell lines due to inhibit the proliferation by retarding the progress of cell cycle, making GA as a new anti-tumor drug candidate [7]. And another research has shown that GA (15 : 1), GA (17 : 1), and GA (17 : 2) are capable of inhibiting FAS activity, providing a new insight into the mechanism underlying the anticancer potential [8]. Lü et al. have found that, compared with the control, GA (31.2 µg·mL–1) could inhibit HIV protease activity by 60% and the effect is concentration-dependent [9]. However, there are also strong indications of cytotoxic, allergic contact dermatitis, as well as possible mutagenic and neurotoxicity potential of these constituents [10-13]. Besides, the structures of some GAs metabolites have been determined [14-15].
the toxicities, GAs must be limited to less than 5 \(^{12}\).

Several reports have been published to study the nephrotoxic potential of GAs. Hecker et al. have evaluated the influence of GA mixture on the rhesus monkey tubular cell line (LLC-MK2), which displays an enhanced sensitivity to the toxicity of GA mixture with a low IC\(_{50}\) value of 4.6 mg·L\(^{-1}\) in neutral red uptake assay \(^{16}\). In parallel, GAs also cause a concentration-dependent release of LDH in this cell line. Morphological examination by electron microscopy revealed that the cytotoxicity of GAs might be mediated by transforming mitochondria to a longitudinally orientated type, as a result of uncoupled oxidative phosphorylation. Madin-Darby canine kidney (MDCK) cell line has been applied to evaluate drug developed nephrotoxicity before \(^{17-18}\). However, the possible toxic mechanism of GAs has not been fully explored.

In order to clarify the potential mechanism, the present study was designed to determine the toxicity of GA (15 : 1) (Fig. 1), which accounts for almost 45% of the total GAs in Ginkgo biloba, in MDCK cells \(^{19}\).

**Materials and Methods**

**Chemicals and reagents**

GA (15 : 1) was isolated from the testa of ginkgo biloba and its purity ( > 99.0%) was determined by UPLC-PDA-MS analysis in our laboratory \(^{20}\). The chemical structure was identified by \(^1\)H NMR and \(^{13}\)C NMR, consistent with the reference data \(^{21}\). Neutral red, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT), rhodamine 123 (Rho 123), propidium iodide (PI), trypsin blue, and ethidium bromide were purchased from Sigma-Aldrich (St. Louis, MO, USA). High glucose dulbecco’s modified eagle medium (DMEM) were purchased from Invitrogen (Carlsbad, CA, USA). High glucose dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco, Waltham, MA, USA), 1% nonessential amino acid, 1% penicillin, and 1% streptomycin, in a humidified atmosphere at 37 °C in 5% CO\(_2\). The medium was refreshed every 2 days, and the cells were trypsinized by 0.25% Trypsin-(1 mmol·L\(^{-1}\)) EDTA solution and then passaged, when the cell growth reached 80% confluence.

**Cell culture**

Madin-Darby canine kidney cell line (MDCK) was obtained from Peking Union Medical College (Beijing, China). The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco, Waltham, MA, USA), 1% nonessential amino acid, 1% penicillin, and 1% streptomycin, in a humidified atmosphere at 37 °C in 5% CO\(_2\). The medium was refreshed every 2 days, and the cells were trypsinized by 0.25% Trypsin-1 mmol·L\(^{-1}\) EDTA and then passaged, when the cell growth reached 80% confluence.

**Neutral red uptake assay**

The cytotoxic effects of GA (15 : 1) on MDCK cells were determined by the Neutral red uptake assay according to the method described by Borenfreund and Puerner \(^{22}\). In brief, the MDCK cells were seeded in 96-well plates at a density of 1.0×10\(^5\)–2.0×10\(^5\) cells/mL for 24 h. Then the medium was removed and replaced with serum-free medium containing GA (15 : 1) and the cells were cultured for 2, 6, and 24 h. After incubation, the medium was removed and the cells were exposed to fresh medium involving neutral red dye reagent (100 μg·mL\(^{-1}\)). After 2 h of incubation, the cells were washed and 150 μL of EtOH/HAC (50%/1%) were added to each well. Then the plates were oscillated gently for 10 min to achieve dissolution. Absorption of neutral red was measured at 540 nm using SpectraMax M2 microplate reader (Molecular devices, Sunnyvale, CA, USA).

**MTT assay**

MTT assay was conducted on the basis of the previous report \(^{23}\). The procedure was the same with Neutral red uptake assay. Following exposure to GA (15 : 1), the cells were incubated with MTT reagent (5 mg·mL\(^{-1}\)) for 4 h. At the end, the medium was removed and formazan was dissolved by adding 150 μL of DMSO. The detection wavelength was set at 570 nm.

**Detection of DNA content**

As with the method of the measurement of mitochondrial transmembrane potential described above, the treated cells were harvested by centrifugation and re-suspended in pre-cooling 70% (V/V) ethanol and then fixed at 4°C for 24 h. The cell concentration was adjusted to 1.0 × 10\(^5\) cells/mL with 1 mL PBS containing RNase A (20 μg·mL\(^{-1}\)), and the cells were incubated at 37 °C for 30 min. Propidium iodide (50 μg·mL\(^{-1}\) in PBS) was applied to stain the cells for 30 min. The fluorescence of cells was analyzed on flow cytometry (Becton Dickinson, Lincoln Park, NJ, USA) with an excitation wavelength of 480 nm \(^{24-25}\).

**Electrophoretic analysis of DNA Fragmentation**

After exposed to GA (15 : 1) at various concentrations for 2 h, the MDCK cells (1.0×10\(^5\)–2.0×10\(^5\)) were first lysed in appropriate volume of lysis buffer (0.1 mol·L\(^{-1}\) of NaCl; 10 mmol·L\(^{-1}\) of Tris-HCl, pH 8.0; 10 mmol·L\(^{-1}\) of EDTA; 0.5% sodium lauryl sulfate; and 100 μg·mL\(^{-1}\) of proteinase K) at 37 °C for 12 h and then incubated with 200 μg·mL\(^{-1}\) of RNase A for addi-
DNA was extracted from the lysate by equal volume of phenol/chloroform/isoamyl alcohol mixture (25 : 24 : 1) and centrifuged at 10 000 r·min⁻¹, 4 °C for 10 min. DNA in water phase was extracted by equal volume of phenol/chloroform (1 : 1) and centrifuged again. Finally, DNA was precipitated by two volumes of ethanol with 1/10 volumes of 0.3 mol·L⁻¹ of sodium acetate at –20 °C overnight. After centrifugation at 12 000 r·min⁻¹ at 4 °C for 60 min, the DNA pellets were washed with 70% ethanol, centrifuged for 30 min, and air-dried. The DNA pellets were re-suspended in TE buffer (10.0 mmol·L⁻¹ of Tris–HCl and 1.0 mol·L⁻¹ of EDTA, pH 8.0) and separated on 1.8% agarose gels containing 0.5 mg·mL⁻¹ of ethidium bromide by electrophoresis (EC250-90, EC Apparatus Corporation, Pittsburgh, PA, USA). The results were photographed by Bio-Rad GD2000 (Universal Hood, Bio-Rad, Opa Locka, FL, USA).

**Data analysis**

The data were analyzed using DAS 2.0 Software and GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA). Values were expressed as means ± SD.

**Results**

**Cytotoxicity of GA (15 : 1) in MDCK cells**

MTT and neutral red uptake assays were employed to

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Fig. 2  The time and dose-dependent cytotoxic effects of GA (15 : 1) on cell viability of MDCK cells determined by the MTT and neutral red uptake assay. The cells were exposed to GA (15 : 1) (5–80 μmol·L⁻¹) for 2 h, 6 h, and 24 h, respectively. The results are presented as means ± SD from three independent experiments.
evaluate the cytotoxic effects of GA (15 : 1) on cell viability of MDCK cells, which were exposed to GA (15 : 1) ranging from 0 to 80 μmol·L⁻¹ for 2, 6, and 24 h, respectively (Fig. 2). For 2-h exposure to GA (15 : 1) within 10 μmol·L⁻¹, no apparent cytotoxicity was observed in the neutral red uptake and MTT assays. When the concentration of GA (15 : 1) increased to 80 μmol·L⁻¹, cell viability in MDCK cells was decreased to 20% (Fig. 2). In MTT assay, cell viability declined rapidly in MDCK cells treated with greater than 20 μmol·L⁻¹ of GA (15 : 1) for 6 h (Fig. 2). However, with the increasing concentration of GA (15 : 1) at the same conditions, the loss of cell viability in MDCK cells was rather gentle in neutral red uptake assay. The cell viability of cells exposed to GA (15 : 1) at 60 μmol·L⁻¹ for 6 h was more than 40% (Fig. 2). It was noted that the cytotoxicity of GA (15 : 1) enhanced significantly after a 24-h exposure. When the concentration of GA (15 : 1) exceeded 20 μmol·L⁻¹, there were no cells survived (Fig. 2). The results revealed that GA (15 : 1) caused cytotoxic effects on MDCK cells in a time- and dose-dependent manner.

Reduction in mitochondrial transmembrane potential

To investigate whether dysfunction of mitochondria occurred during GA (15 : 1) treatment, mitochondrial transmembrane potential (ΔΨm) was monitored by fluorescence of Rho 123, a potential-sensitive dye, using flow cytometry. Compared with the 5 μmol·L⁻¹ GA (15 : 1) treatment group for 8 or 24 h, the cells in control group showed a high mitochondrial transmembrane potential (Fig. 3A). After incubation with GA (15 : 1) for 8 h, Rho 123-stained cells were divided into two parts according to fluorescent strength, and 29.83% of cells exerted a reduction of the ΔΨm (Fig. 3B). The

![Fig. 3](image-url)  The mitochondrial membrane potential of MDCK cells treated with 5 μmol·L⁻¹ GA (15 : 1) for 8 h and 24 h. Cells were stained with Rho 123 and ΔΨm was monitored by flow cytometry. A: Control; B: 8 h; C: 24 h
percentage of ΔΨm loss was more obvious when the cells were exposed to GA (15 : 1) for 24 h, accounting for 36.14% decrease (Fig. 3C). The results revealed that GA (15 : 1) induced a reduction in ΔΨm, which reflected the serious damaging state of mitochondrial function.

**Effect of GA (15:1) on cell cycle distributions**

Evident changes in cell cycle distributions were observed after treatment with high concentrations of GA (15 : 1) (Fig. 4) and the proportions of cells at different cell cycle phases are shown in Table 1. The percent of G0/G1 phase cells increased from 29.04% to 35.22% and 42.22%, in GA (15 : 1) treated cells at 20 and 40 μmol·L−1, respectively. With the increase of the concentration, the ratio of G2/M phase cells also presented an escalating trend, simultaneously, and the cells in S phase decreased to 27% from 58.59%. Besides, studies concerning time-course effects of GA (15 : 1) on cell cycle distributions were conducted (Figs. 5 and 6). The cells remained in S phase declined, along with an increase of G0/G1 and G2/M fractions, occurring after incubation with GA (15 : 1) at 1 and 5 μmol·L−1 for 8 and 24 h (Tables 2 and 3). After exposure to GA (15 : 1) at high concentration for short period or at low concentration for long time, the numbers of cells in G1 and G2 phase were increased, but that in S phase decreased, indicating that GA (15 : 1) inhibited cell growth through influencing cell mitosis.

**Cells Morphology**

Morphologic features of the control group were characteristic fusiform shape. The cells were treated with 30, 40, and 60 μmol·L−1 of GA (15 : 1) for 2 h. As shown in arrow “a”, the cells that survived from exposure to high concentration of GA (15 : 1) presented cellular swelling. Meanwhile, most cell membrane broke down (Fig. 7, arrow “b”). Trypan blue entered into the MDCK cells treated with 30 and 40 μmol·L−1 of GA (15 : 1) and conjugated with the cell nucleus closely (Figs. 7-3 and 7-4). These findings suggested MDCK cell morphology was affected by GA (15 : 1), resulting in apoptosis or necrosis.

Fig. 6  Cell cycle distributions of MDCK cells treated with 1 μmol·L⁻¹ GA (15 : 1) for 8 h and 24 h. Cells were stained with PI and determined by flow cytometry. (A): Control, (B): 8 h, (C): 24 h. Histograms are representative of three independent experiments.

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<th>Table 1</th>
<th>Cell cycle distributions during treatment with 0, 20, 40 μmol·L⁻¹ GA (15 : 1)</th>
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<tr>
<td>Cell cycle phase (%)</td>
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<td>G0/G1</td>
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DNA fragmentation in MDCK cells
Electrophoresis was employed to detect DNA fragmentation in MDCK cells treated with GA (15 : 1) (Fig. 8). When the concentration of GA (15 : 1) was below 20 μmol·L⁻¹, the majority of DNA stayed at the sample loading site, without migration. As shown in Fig. 8, electrophoretic analysis of DNA showed that exposure of MDCK cells to GA (15 : 1) (20–60 μmol·L⁻¹) for 24 h did not result in a characteristic DNA laddering pattern, a biochemical hallmark of apoptosis. Instead, fuzzy successive bands were observed, suggesting that cell death was caused in a necrotic manner. Most DNA fragmentation focused on 100 bp region, arising from GA (15 : 1) at 60 μmol·L⁻¹ for 2 h. The results further illustrated the GA (15 : 1) induced cell necrosis.

Fig. 7  Morphology of MDCK cells treated with GA (15 : 1). 1: Control; 2: 60 μmol·L⁻¹ GA (15 : 1) for 2 h; 3: treatment with trypan blue after 40 μmol·L⁻¹ GA (15 : 1) for 2 h; 4: treatment with trypan blue after 30 μmol·L⁻¹ GA (15 : 1) for 2 h

Fig. 8  Effect of GA (15 : 1) on DNA fragmentation in MDCK cells treated for 2 h and analyzed on 1.8% agarose gels. Results are representative of two independent experiments. lane 1, control cells; lane 2, 20 μmol·L⁻¹ GA (15 : 1), 2 h; lane 3, 30 μmol·L⁻¹ GA (15 : 1), 2 h; lane 4, 40 μmol·L⁻¹ GA (15 : 1), 2 h; lane 5, 60 μmol·L⁻¹ GA (15 : 1), 2 h; lane 6, marker

Discussion
Once toxic substances enter into the cells, the cells may inevitably get damaged. Several methods were developed to
detect cytotoxicity in vitro. Chemical dyes can be absorbed into the living cells, like gentian violet and trypan blue dyes, and hence, the number of survival cells can be measured. The release of lactate dehydrogenase (LDH) has been an indicator of enzyme in the supernatants cell culture, reflecting cytotoxicity. The MTT assay is commonly applied to drug-induced cytotoxicity for its validity in various cell lines. A sensitive and rapid technique, single cell gel electrophoresis (SCGE), also known as the comet assay, could be conducted to quantify the extent of DNA damage, especially in the area of cancer research. Flow cytometry allows simultaneous multi-parametric analysis of the physical and chemical characteristics of up to thousands of particles per second, including the information about cell size, the content of DNA or RNA, and so on. As such, this technique has been widely used in analysis of cytotoxicity.

In the present study, MTT and neutral red uptake assays were used to determine cell viability in vitro. Within a certain range, the amount of generated formazan in MTT assay is directly proportional to the number of living cells. Besides, living cells are able to take up neutral red dye reagent and accumulate them in the lysosomes, while dead cells can’t uptake the reagent for the damage of cell membrane or lysosome membrane. In our study, cytotoxicity of GA (15 : 1) was analyzed using MTT and neutral red uptake assays in a time- and dose-dependent manner, indicating that this compound has potential ability to destroy both mitochondria and lysosomes in MDCK cells and the mechanism of cytotoxicity caused by GA (15 : 1) may be complicated. In addition, we also found that the toxic effect of GA (15 : 1) on MDCK-MDR1 was weaker than that on MDCK cells (data not shown), in consistent with the prior published results. According to the previous research, GA (15 : 1) is a preferred substrate of P-gp. Thus, P-gp, which is localized to the apical (luminal) membrane of the renal epithelial cells, could help GA (15 : 1) excretion and reduce its toxicity.

Mitochondria are kinds of pivotal organelles in controlling cell life and death, where they produce adenosine triphosphate (ATP), and frequently the target of injury after controlling cell life and death, where they produce adenosine triphosphate (ATP), and frequently the target of injury after mitochondrial dysfunction, which is also closely interrelated with mitochondrial membrane permeability. As time went on, the reduction of ΔΨm was notable in MDCK cells exposed to 5 μmol·L⁻¹ of GA (15 : 1) for 8 and 24 h, respectively, suggesting a serious mitochondrial dysfunction aroused by GA (15 : 1). Consequently, the lack of ATP production would result in cells death by apoptosis or necrosis.

Some important physiological processes, for example, DNA replication, protein synthesis and cell mitosis, are involved in cell cycle progression, which includes the gap before DNA replication (G1), the DNA synthetic phase (S), the gap after DNA replication (G2), and the mitotic phase that culminates in cell division (M). It is reported that the appearance of sub-G1 peak was a suggestive character of apoptosis by other researchers. DNA degradation in regular necrotic cells is random, along with different sizes of DNA fragments. In that case, smaller peaks than sub-G1 should have appeared by flow cytometric detection. We tried to investigate whether GA (15 : 1) could induce MDCK cell apoptosis by PI staining. However, there were no sub-G1 peaks, and hence, further apoptosis research was not conducted. High concentration of GA (15 : 1) treatment might cause necrosis. But strangely, we failed to detect smaller peaks than G1 peak by flow cytometry. It is likely that the toxic effect of GA (15 : 1) on MDCK was relatively prominent, and DNA in necrotic cells was easily discharged during cell separation. If so, the fixed cells might only be living cells, while necrotic cells were omitted with flow cytometry. After short-term exposure to high concentration and long-term exposure to low concentration of GA (15 : 1), the numbers of MDCK cells in G1 and G2 phase were increased, while that in S phase decreased, indicating that MDCK cells were blocked both in G0/G1 and G2/M phases. We speculated that GA (15 : 1) might have influence on the DNA synthesis and cell mitosis, which were responsible for DNA damage and cell proliferation, leading to cell death eventually.

Two patterns of cell death, apoptosis and necrosis, were first discovered by Kerr et al. Apoptosis is an active and inherently programmed phenomenon, whereas necrosis, usually caused by internal or external factors, such as injury or toxins, is a passive process of cell death that differs from apoptosis, showing chromatin clumping, organelles swelling, membrane breakdown, and cell disintegration eventually. Trypan blue staining can be used to distinguish living cells from death cells, based on the cell membrane integrity. After treating with GA (15 : 1) at concentrations of greater than 20 μmol·L⁻¹ for 2 h, the majority of cells were positive for trypan blue, suggesting extensive membrane damage. Besides, detection of DNA ladder is regarded as one key feature of apoptosis, since DNA is damaged and broken into distinct fragments by endonuclease activity. As to necrosis, the DNA is preserved initially, and then disintegrated non-specifically later. The results of DNA fragmentation evaluated by agarose gel electrophoresis showed smeary bands (about 100 bp) of cells in the presence of high concentrations of GA (15 : 1), which represented necrotic cell death. Presumably, short-term treatment with high concentration of GA (15 : 1) primarily induced necrosis in MDCK cells.

In conclusion, the mechanism of GA (15 : 1) toxicity was multiple and complicated. GA (15 : 1) could result in several types of cellular damages, including necrosis via mitocho-
dria and lysosomes damage, cell membrane smash, cell swelling, DNA fragmentation and cell cycle arrest. These findings, to some extent, might provide a basis for understanding GA (15 : 1)-induced renal toxicity.

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