Total Flavonoids of Litsea Coreana Enhance the Cytotoxicity of Oxaliplatin by Increasing Gap Junction Intercellular Communication

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Oxaliplatin is widely used in the treatment of variety of cancers, including cancer of the testis and colorectum. Gap junctions (GJs) can amplify the cytotoxicity of antineoplastic drugs through the bystander effect in different cancer cells. In this study, we demonstrate that total flavonoids of litsea coreana (TFLC), one extract from the dried leaves of litsea coreana leve, increase the cytotoxicity of oxaliplatin in mouse testicular cancer I-10 cells. We found that cell survival was substantially decreased only when functional GJs formed in I-10 cells. TFLC increased oxaliplatin cytotoxicity (inducing cell death and apoptosis) by enhancing gap junction intercellular communication (GJIC) through elevated Cx43 protein expression. Furthermore, apoptosis-related protein (Bax, Bel-2, caspase-3/9) results showed that the Bax/Bel-2 ratio and activated caspase-3/9 increased when TFLC was used compared with treatment with oxaliplatin alone, which suggests that the mechanism of increased oxaliplatin-induced apoptosis was through the mitochondrial pathway. These results demonstrate that TFLC can enhance the cytotoxicity of oxaliplatin, and that these processes may be regulated in testicular tumor cells through GJ-mediated regulation of tumor cell apoptosis.

Key words litsea coreana; gap junction; apoptosis; oxaliplatin

Gap junctions (GJs) are intercellular channels that connect the cytoplasm of adjacent cells. They have vital implication in regulation of homeostasis, morphogenesis, cell differentiation, and growth control in multicellular organisms. GJs are consisted with core protein-connexin. A considerable number of studies have established that GJs are decreased in variety of tumors.1–4) Furthermore, enhancement of GJs increases cytotoxicity during cancer chemotherapy and radiotherapy.5,6)

It is well known that dietary intake of fruit or herbal health drinks containing sufficient flavanoids will reduce the occurrence risk of cancer (e.g. breast, lung and colon). Total flavonoids of litsea coreana (TFLC), one of the flavonoids, is the key pharmacologically active constituents extracted from litsea coreana leve which is a traditional Chinese medicine. TFLC primarily consists of five flavonoids, identified as quercetin-3-β-D-glucoside, quercetin-3-β-D-glucoside, kaempferol-3-β-D-glucoside, kaempferol-3-β-D-galactoside and catechin.7)

Many studies have shown that flavonoids can increase the gap junction intercellular communication (GJIC) via the alteration of connexin protein expression. Liu et al. reported berberine, a flavonoids drug, enhanced the function of GJ8) and amplify the radiotherapy induced apoptosis in cancer cells. Hong et al. demonstrated that up-regulation of GJ promoted the cytotoxicity of cisplatin in testicular cancer cells and down-regulation of GJ decreased cytotoxicity.9)

Oxaliplatin (OHP) [1,2-diaminocyclohexane (trans-1) oxolatoplatinum(II)], a related platinum based chemotherapeutic agent, reacting with GC-rich areas of DNA, forming both intrastrand and interstrand cross-links, leading to G2 arrest and to apoptosis or necrosis.10) It is commonly used in the treatment of testicular cancer. Wang et al. also demonstrated that the cytotoxicity of oxaliplatin was increased in the presence of GJ and increasing GJ function enhanced cytotoxicity of oxaliplatin in the cells with functional GJ.11) And a simple inference from these reports is that induction of apoptotic or necrotic processes in one cell can cause a molecular “death signal” to be transmitted to neighboring cells via GJs. At present, TFLC has been reported in the treatment of hyperlipidemia, immune regulation and diabetes.12–17) As a flavonoid agent, little is known about probable effect of TFLC on gap junction.

The aim of this study is to determine the effect of TFLC on the cytotoxicity of oxaliplatin and the relationship between this effect and the modulation of GJ function in testicular tumor cells.

MATERIALS AND METHODS

Drugs, Antibodies and Reagents Total Flavonoids of Litsea Coreana (TFLC) was a generous gift from Dr. Jun Li (School of Pharmacy, Anhui Medical University, Anhui, China). Calcein-AM (acetoxymethyl ester) and CM-DiI were from Invitrogen. Rabbit anti-Bax and rabbit anti-Bcl-2 antibodies were from Santa Cruz. Anti-caspase-9 polyclonal antibody was purchased from Abcam. Anti-Cx43 antibody was from Sigma. Secondary antibodies for Western blotting were from Millipore (Massachusetts, U.S.A.). Binding buffer, Annexin V-fluorescin isothiocyanate (FITC) and propidium iodide (PI) were from BD (New York, U.S.A.). Cell culture reagents were obtained from Gibco. All other reagents were from sigma unless stated otherwise.

Cell Lines and Cell Culture Mouse Leydig tumor cell line (I-10) was obtained from American Type Culture Collection (Manassas, VA, U.S.A.). I-10 cells were cultured in F-12 supplemented with 15% horse serum, 2.5% fetal bovine serum. The cells were grown at 37°C in an atmosphere of 5% CO2 in air.

Standard Colony-Forming Assay Oxaliplatin stock so--
lutions were prepared fresh at 10 mM in phosphate buffered saline (PBS). TFLC stock solutions were dissolved at 100 mg/mL in dimethyl sulfoxide (DMSO). Retinoic acid (RA), a GJ promoter, stock solutions were dissolved at 10 mM in DMSO. Cells exposed to oxaliplatin or TFLC were performed in the dark. When combined treating with oxaliplatin, TFLC was added to cells 24 h before oxaliplatin. Toxicity was assessed by standard colony forming assay adapted from the protocol of Wang et al.11 Cells were seeded at low density (300 cells/cm²) or high density (3000 cells/cm²). After 1 h exposure to oxaliplatin with or without TFLC, cells were washed with PBS, harvested by trypsinization, counted, and seeded into six-well plates at 600 cells/well. The cells were incubated for another 5–10 d, then fixed and stained with 1% crystal violet in ethanol. Colonies containing 50 or more cells were scored. Colony formation was normalized to the colony forming efficiency of non-drug-treated cells.

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\text{surviving fraction (\%)} = \frac{\text{drug-treated cell clones number}}{\text{non-drug-treated cell clones number}} \times 100
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**“Parachute“ Dye-Coupling Assay** Functional GJIC was examined as described by Tao and colleagues8–20. Cells were grown to confluence in 12-well plates. The donor cells were double-labeled for 30 min with 5 µM CM-DiI, a membrane dye that does not spread to coupled cells, and 5 µM calcein-AM, which is converted intracellularly into the gap junction-permeable dye calcein at 37°C. The donor cells were then trypsinized and seeded into the receiver cells at a 1:200 donor/receiver ratio. They were allowed to attach to the monolayer of receiver cells and form gap junctions for 4 h at 37°C and pH 7.4, and then examined with a fluorescence microscope. The average number of receiver cells containing dye per donor cell was considered as a measure of the degree of GJIC.

**Western Blotting** Cells were washed three times with cold PBS and then harvested using lysis buffer. Cells lysated were centrifuged at 12000 rpm for 30 min at 4°C. DC protein assay kit was used to determine protein concentration (Bio-Rad Co., Hercules, CA, U.S.A.). Fifty micrograms protein were stained with 50 ng/mL hoechst 33258 and incubated for 30 min at room temperature and washed twice with PBS. Fixed cells were stained with 50 ng/mL hoechst 33258 and incubated for 30 min at room temperature and washed twice with PBS. Late apoptotic cells were identified by condensation and fragmentation of nuclei examined by fluorescence microscopy. Typical apoptotic morphology (prolapses of cytoplasm, apoptotic bodies and shrunken cells) was easily distinguishable in I-10 cells treated with toxic doses of oxaliplatin. A minimum of 500 cells were counted for each treatment.

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\text{late apoptotic rate (\%)} = \frac{\text{late apoptosis cells number}}{\text{total cells number}} \times 100
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**Statistical Analysis** All of the experiments have a minimum of three determinations. The statistical analysis between groups was performed by unpaired Student’s t-test with spss16. Data were presented as mean±standard deviations (S.D.). Differences with \(p<0.05\) were considered significant.

**RESULTS**

Connexin/GJ and Cytotoxicity of Oxaliplatin Oxaliplatin toxicity was assessed by standard colony forming assay. Figure 1 provides the surviving fraction of I-10 cells exposed to oxaliplatin under conditions where GJ is not possible (low...
density: 300 cells/cm²; cells are isolated from others and have no opportunity to form GJ) or possible (high density: 30000 cells/cm²). Treated with oxaliplatin (2.5–40 µM) for 1 h reduced the surviving fraction of cells at both low density and high density. The toxic effect of oxaliplatin was substantially greater at high density than at low density, indicating that the cells were more sensitive to the toxic effects of oxaliplatin only when cells were at high density. The results demonstrate the gap junctions in the testicular cancer cells increase the cytotoxicity of oxaliplatin.

It has been demonstrated that gap junction function was also manipulated by enhancement of junctional channels by retinoic acid (RA). In the present study, treating the cells with RA, a widely used GJ promoter for 24 h, which was verified to increase the function of Cx43-composing GJ, markedly reduced the cell survival only in high-density cells. However, at low cell density, there was no effect of RA on oxaliplatin cytotoxicity (Fig. 2). RA had no effect on the cell survival when applied alone. This confirmed that enhancement of GJ can increase the cytotoxicity of oxaliplatin in testicular tumor cells I-10.

Effects of TFLC on Connexin/GJ To determine whether TFLC can affect the function of GJ in I-10 cells or not, the cells were treated with 5–20 µg/mL TFLC which had no effect on the I-10 cell survival (Supplemental Fig. 1). We examined the effect of TFLC on GJ function between cultured cells by the parachute assay. Figure 3A showed that TFLC markedly increased the dye spread from donor cells to receiver cells and the enhancement of TFLC on dye spread was most obviously in the 20 µg/mL concentration. It has been reported that the functional GJs can be mediated by the expression of Cx43 in testicular cells.

Therefore, Western blotting was used to identify the effect of TFLC on Cx43 in I-10 cells. Figure 3B illustrated that treatment with 5–20 µg/mL TFLC for 24 h increased the total level of Cx43 expression and the expression of Cx43 was the highest in the 20 µg/mL concentration. The concentration (20 µg/mL) of TFLC of increasing Cx43 expression is the same to that in the enhancement of GJ function.

Effects of TFLC on Cytotoxicity of Oxaliplatin and Oxaliplatin-Induced Apoptosis The effect of TFLC on oxaliplatin-induced cytotoxicity was examined in I-10 cells expressing Cx43. Cells seeded at high or low cell density were treated with 20 µg/mL TFLC for 24 h, followed by exposing to 10 µM oxaliplatin for 1 h. The effect of TFLC on cytotoxicity of oxaliplatin was detected by colony formation assay. Figure 4A showed that TFLC had no effect on oxaliplatin cytotoxicity in low-density cultures, but at high density, it increased a substantial portion of the oxaliplatin cytotoxicity, the cell surviving fraction was 75.99 ± 1.48% in oxaliplatin group, and it was 62.01 ± 3.28% in combination group. The increased cytotoxicity occurred only in the high-density, which has opportunity to form GJs.

Apoptosis is the critical physiological process in the cell development and homeostasis. Figure 4B illustrated the early stage apoptosis (Annexin V⁺/PI−, lower right quadrant) induced by 30 µM oxaliplatin for 8 h with pretreated with 20 µg/
Fig. 3. Effects of TFLC on Cx43 Expression and GJIC

(A) Fluorescence images show the degree of dye coupling by the parachute assay. The dye spread of cells treated with a range of TFLC (5–20 µg/mL) concentrations for 24h. (B) Western blotting analyses of TFLC on Cx43 expression in I-10 cells. Bar graphs are derived from densitometric scanning of the blots. Columns, mean from four independent experiments, bars, SD. **p<0.01 significantly different from control group. (C) Immunofluorescence assay was used to detect the expression of Cx43 on membrane of I-10 cells. The cells membrane were stained with anti-Cx43 antibody and with FITC coupled anti-mouse IgG (upper row). The nucleus were stained with DAPI (lower row).
mL TFLC for 24h at high density. The result showed that the early apoptosis rate was 6.70±0.82% in oxaliplatin group, and it was 12.93±0.98% in combination group. That is, pretreatment of cells with 20µg/mL TFLC increased the early apoptosis rate by approximately 92.99% compared with oxaliplatin group at high density. TFLC had no effect on the early apoptosis induced by oxaliplatin at low density (Supplemental Fig. 2).
Figure 4C showed that the late stage apoptosis induced by 20 µM oxaliplatin for 24 h with pretreated with 20 µg/mL TFLC for 24 h assessed by hoechst 33258 assay at high density. The result also showed that the late apoptosis rate was 15.13±0.36% in oxaliplatin group, and it was 25.92±0.34% in combination group. The late apoptosis rate was increased by approximately 71.32% when pretreated with TFLC. TFLC had no effect on the late apoptosis induced by oxaliplatin at low density (Supplemental Fig. 3).

Thus, these results suggest that TFLC increased apoptosis induced by oxaliplatin in I-10 cells through enhanced GJ.

Effects of TFLC on Oxaliplatin-Induced Changing of Bax/Bcl-2 and Activation of Caspase

Fig. 5. Effects of TFLC on Oxaliplatin-Induced Changing of Bax/Bcl-2 and Activation of Caspase-3, -9

(A) The expression of apoptosis-related protein was measured by Western blotting. (B) The ratio of Bax and Bcl-2 was determined using a densitometer. (C) Quantification of cleaved caspase-3 and cleaved caspase-9 protein from Western blotting using densitometry was analysed. Columns, mean from four independent experiments, bars, S.D. **p<0.01 significantly different from control group, ##p<0.01, significantly different from oxaliplatin group.
duction and amplification after antineoplastic agents exposure. Western blotting was performed after the cells treated by 10 µM oxaliplatin with or without 20 µg/mL TFLC (Fig. 5A). These data showed that TFLC obviously enhanced the expressions of Bax, which was used as a positive control for promoting oxaliplatin-induced apoptosis, decreased Bcl-2 (protected against apoptosis) expression and the Bax-to-Bcl-2 ratio (Fig. 5B) was significantly higher at the combination group than the oxaliplatin group (p < 0.01). Additionally, the results to detect the cleavage of caspase-9 and caspase-3 also showed that cleavage of caspase-9 and caspase-3 were greatly increasing when cells were pretreated with TFLC (Fig. 5C). TFLC had no effect on the expression of Bax, Bcl-2, caspase-9 and caspase-3 at low density (Supplemental Fig. 4).

DISCUSSION

The present study demonstrated that TFLC can enhance the cytotoxicity of oxaliplatin determined by cell survival and induction of apoptosis in I-10 cells. However, the increased toxicity of oxaliplatin was found only in high density (GJ formed) and TFLC had no effect on oxaliplatin cytotoxicity (no GJ formed). Therefore, the syner-gism influence of TFLC on oxaliplatin antineoplastic effect is through enhanced GJIC composed of Cx43.

Previous work showed that toxic effect of chemotherapy and radiotherapy is enhanced by the presence of functional gap junctions. In the present study, importantly, and in contrast to previous study, we show here for the first time that the TFLC enhanced the function of GJ and the expres-sion of Cx43 in testicular tumor cells I-10. The function of GJ is mainly regulated by GJ channel permeability and connexin structure, amount, distribution. Phosphorylation plays a significant role in GJ function, mediating almost all stages of the Cx43 life cycle. Recent study showed that flavonoid, liquiritin, increases the function of GJ via inhibiting the Cx43 phosphorylation level. Peterson-Roth et al. reported that gap junction may be increased by inhibiting the Src kinase. And Wang et al. showed that the augment of GJIC is due to the decrease in PKC-mediated Cx43 phosphorylation and thereby the increased membrane localization of Cx43 in murine Leydig tumor cell line (MLTC-1). So, we supposed that TFLC may inhibit Src kinase or PKC, the phosphorylation of connexins reduce, so that the function of GJ increased.

Furthermore, we show here, for the first time that TFLC increased the cytotoxicity of oxaliplatin. It has been reported that flavonoid, baicalein, induced cell cycle arrest and apoptosis in cancer cells, mitochondrial-dependent caspase activation pathway was involved in baicalein-induced apoptosis in human hepatoma J5 cells. Some studies reported that another flavonoid, berberine, exerted auxiliary anti-cancer property via induced apoptosis in human hepatoma cells and human promonocytic U937 cells. These reports indicated that TFLC may enhance cytotoxicity induced by oxaliplatin via accelerating apoptosis through enhancing GJ in I-10 cells. The results of flow cytometry and hoechst 33258 staining showed that pretreatment of cells with 20 µg/mL TFLC increased the early apoptosis rate by approximately 92.99% compared with oxaliplatin group, and the late apoptosis rate was increased by approximately 71.32% when pretreated with TFLC. These results demonstrated that TFLC elevated apoptosis induced by oxaliplatin in I-10 cells, however, only when GJ formed.

To explore the mechanism by which TFLC enhances the apoptosis induced by oxaliplatin, we examined the expression of apoptosis-related protein in I-10 cells by Western blotting. It has been reported that flavonoids drugs can induce apoptosis through a mitochondria/caspases pathway in human hepatoma cells, and in human promonocytic U937 cells via activation of caspase-9 and caspase-3. In addition, Kamertitsch et al. showed Cx43 and Cx40 but not Cx37 promote apoptosis via gap junctional transfer of pro-apoptotic signals between cells and previous work, revealed that, the process which cisplatin induced apoptosis was only the mitochondrial pathway, and not the death receptor pathway, that was modulated by GJIC. The caspase cascade system played an important role in the induction, transduction and amplification of intracellular apoptotic signals. There are two main pathways leading to caspase activation. One depends upon the participation of mitochondria and the other involves interaction of a death receptor with its ligand. In the mitochondrial pathway, release of cytochrome c from mitochondria activates caspase-9. Activation caspase-9 can continue to activate caspase-3, resulting in the morphological and biochemical changes associated with apoptosis. Therefore we detected the expression of Bax, Bcl-2, caspase-3 and caspase-9. Consistent with previous reports, the results of the present study show that TFLC could enhance the expressions of Bax and decrease Bcl-2, at the same time, cleavage of caspase-9 and caspase-3 were greatly increasing when cells were pretreated with TFLC only in high density. So, this result suggested that effect of TFLC on GJIC modulated the mitochondrial apoptosis pathway.

In summary, the data collected here indicated TFLC potentiated connexin expression in I-10 cells during the period when TFLC enhances GJ function and then increased the cytotoxicity of oxaliplatin. Further studies are needed to detect the mechanism by which TFLC enhances the expression of Cx43 in I-10 cells. It has been suggested the possibility that TFLC may develop a non-toxic chemo-adjuvant used to increase the efficacy of anticancer chemotherapies by up-regulation or maintenance of GJ functionality.

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REFERENCES


