Extracts from guava fruit protect renal tubular endothelial cells against acetaminophen-induced cytotoxicity

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Abstract. Acetaminophen (APAP) is an analgesic and antipyretic agent primarily used in the clinical setting. However, high doses of APAP can cause oxidative stress. Guavas have been reported to provide anti-inflammatory, anti-microbial, anti-oxidative and anti-diarrheal functions. In addition, guavas have been reported to prevent renal damage due to progression of diabetes mellitus. Therefore, the aim of the present study was to investigate whether guavas can reduce APAP-induced renal cell damage. In the present study, extracts from guavas were obtained and added to APAP-treated renal tubular endothelial cells. The present results demonstrated that APAP induces cytotoxicity in renal tubular endothelial cells, while guava extracts inhibited this cytotoxicity. In addition, the study demonstrated that the protective effects of guava extracts against APAP-induced cytotoxicity may be associated with inhibition of oxidative stress and caspase-3 activation.

Introduction

Acetaminophen (APAP) is widely used clinically as an antipyretic and analgesic medicine (1-3). Generally, APAP is considered to be safe when used within the therapeutic dose range; however, APAP overdose can cause liver and renal damage (4-6). Because APAP is cheap and readily available, patients may overdose easily, leading to reports of self-poisoning in numerous countries (7-9). Many studies have shown that high doses of APAP can induce cell death through either the apoptotic or necrotic death pathways (10-12). Previous studies have demonstrated that APAP-induced cytotoxicity is related to increased oxidative stress and glutathione depletion (13-16). It is well-known that cellular glutathione can convert H_2O_2 to H_2O via a glutathione peroxidase reaction to attenuate cellular oxidative stress (17,18). Therefore, APAP-induced glutathione depletion may cause the H_2O_2 level to increase in APAP-treated cells (3,6,19). In addition, mitogen activated protein kinase (MAPK) and caspase signals can be activated in APAP-treated cells (3,20,21). Because APAP is generally used in a clinical setting, understanding how to reduce the threshold of APAP-induced cytotoxicity is important.

Guavas have many functional phytochemicals such as vitamins, tannins, phenolic compounds, flavonoids, and triterpenoid acids (22-24). Therefore, many studies indicated that guavas have anti-inflammatory, anti-microbial, anti-oxidative, anticancer, and anti-diarrheal functions (25-27). Many studies have demonstrated that guavas can improve anti-oxidant functions such as glutathione levels and activities of superoxide dismutase, catalase, and glutathione peroxidase against oxidative stress-induced damage (28-31). Guavas can inhibit arsenic-induced (29), streptozotocin-induced (30,31), and alloxan-induced (28) oxidative stress. Furthermore, a recent study suggested that guavas can inhibit caspase activity to attenuate cell apoptosis in type II diabetic rats (32). Because APAP-induced cytotoxicity is related to oxidative stress and caspase activity (13-16,33,34), and because guavas can inhibit oxidative stress and caspase activity, the present study investigated whether guavas can attenuate APAP-induced renal cytotoxicity.

Guava fruit has antioxidant activities (35-38). Previous studies have demonstrated that guavas can protect renal cells

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against oxidative stress-induced damage (32,39,40). Therefore, we hypothesized that guava may be a potential diet to reduce the threshold of APAP-induced renal cytotoxicity. Although guavas have many antioxidant components including ascorbate, flavonoids, glutathione, superoxide dismutase, catalase, peroxidase, ascorbate peroxidase, and glutathione reductase, the types and quantity of these antioxidant components are different at different stages of maturation (38,41). Generally, the ripening stages of guavas are classified as immature green (IG), mature green (MG), turning fruits (T), ripe (R), and over-ripe (OR) (41). Many components and high levels of antioxidant phytochemicals are found at the MG and T stages (41). This study showed that antioxidant molecules including glutathione reductase, total glutathione, and GSH are found predominately at the MG stage, antioxidant molecules including catalase, POX, ascorbate, and ascorbate peroxidase are primarily found at the T stage, and the antioxidant molecule, SOD, is found at the R stage. However, short antioxidant molecules are found at the IG and OR stages. In addition, a recent study showed that different guava cultivars have different antioxidant components and activities (37). In order to find potential guava extracts to protect renal cells against APAP-induced renal damage, the extracts of pearl guava, imperial guava, and red pulp guava were investigated in this study.

Materials and methods

Materials. Pearl guavas, imperial guavas, and red pulp guavas were kindly provided from farmer Lin Chao Hsiung (A Fong guava farm, Tainan, R.O.C.). Luminol and lucigenin were bought from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). An MTT assay kit was bought from Bio Basic Canada Inc. (Markham, ON, Canada). Anti-tubulin bought polyclonal antibody from Bioworld (Louis Park, MN, USA). Anti-caspase-3 and anti-cleaved-caspase-3 rabbit polyclonal antibodies and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Western Lightning Chemiluminescence Reagent Plus was bought from PerkinElmer, Inc. (Waltham, MA, USA). Fetal bovine serum, DMEM, non-essential amino acids, L-glutamine, and penicillin/streptomycin were bought from Gibco-BRL (Invitrogen Life Technologies, Carlsbad, CA, USA).

Cell line and cell culture. Rat renal tubular epithelial cells (NRK-52E) and human renal tubular epithelial cells (HK-2 cells) were bought from the Bioresource Collection and Research Center (Shinchu, Taiwan). Cells were cultured and maintained in DMEM (containing 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin, and 0.1 mM non-essential amino acids) at 37˚C in a humidified atmosphere containing 5% CO_2.

Guava extract preparation. Guava extracts were obtained using a similar method to a previous study (32). The extracts from the MG and T stages of the pearl guavas, imperial guavas, and red pulp guavas were used in this study following cleaning. The fruits were cut and the seed sections were removed, before grinding the crude juice by treated with pure juice machine (National MJ-C85 N) without extra water. The crude juice was centrifuged at 4,000 x g (Allegra X-15®; Beckman Coulter, Inc., Brea, CA, USA) for 30 min, following which the supernatant was collected. The guava extracts were obtained after the supernatant was filtered with a 0.22 mM filter. The final concentration of guava extracts was ~2 g/ml (guava weight/final liquid volume). The guava extracts were stored at -80˚C. Guava extracts (1 and 20 ml) were used in the study because 1 and 20 ml guava extracts had antioxidant activity and did not cause cell cytotoxicity.

Cell viability assay. An MTT assay kit was used for cell viability assay (3,42). Cells were cultured used 96-well plates ($6x10^3$ cells/well). Cell viability was determined every 24 h. The control and experimental samples were added with an MTT assay kit and incubated for 3 h at 37˚C. The absorbance of the reactive product was measured at 570 nm (A570) by using a Multiskan™ FC Microplate Photometer (Molecular Devices, Sunnyvale, CA, USA). The cell viability (%) was calculated as (A570 experimental group)/(A570 control group) x 100.

H_2O_2 level determination. Intracellular H_2O_2 levels were determined by using the lucigenin-amplified chemiluminescence method (6,32). Each sample (200 μl containing 8,000 cells) was treated with luminol solution (100 μl; 0.2 mM/ml) and incubated for 5 min. The H_2O_2 levels of these samples were analyzed by using a chemiluminescence analyzing system (CLA-FSI; Tohoko Electronic Industrial Co., Ltd., Sendai, Japan).

SDS PAGE and western blotting. Primary anti-tubulin polyclonal antibody (1:1,000; cat. no. BS1699) were bought from Bioworld. Primary anti-caspase-3 (1:1,000; cat. no. 9965), anti-cleaved caspase-3 antibody (1:500; cat. no. 9662) and secondary horseradish peroxidase conjugated goat anti-rabbit IgG (1:2,000, cat. no. 7074) were bought from Cell Signaling Technology, Inc. (Beverly, MD, USA). These antibodies were used for western blotting. The control and experimental cells (~$3x10^7$ cells) were collected and lysed with RIPA buffer (cat. no. 20-188; EMD Millipore, Billerica, MA, USA). After centrifugation (16,000 x g; at 4˚C) for 20 min, cellular proteins were obtained from the supernatant layer. The protein concentration was determined with a protein assay kit (cat. no. 23200; Thermo Fischer Scientific, Inc.). Equal quantities (40 μg) of protein were loaded onto a 13.3% SDS gel and separated by SDS electrophoresis, prior to being transferred onto polyvinylidene difluoride membranes (EMD Millipore). The membranes were blocked with 5% non-fat milk solution for 2 h at room temperature and washed with PBS three times (each time for 5 min). Next, the membranes were treated with primary antibodies for 2 h at room temperature and washed with PBS three times at room temperature. The membranes were subsequently treated with anti-rabbit HRP-conjugated secondary antibodies for 1 h at room temperature. Finally, the membranes were treated with Western Lightning® Chemiluminescence Plus reagent (PerkinElmer, Inc.) and immunolabeled proteins were observed using a Luminescence Image Analysis system (LAS-4000; FujiFilm Electronic Materials Taiwan, Co., Ltd., Tainan, Taiwan).

Statistical analysis. Data were collected and calculated from four independent experiments and presented as the mean ± SE.

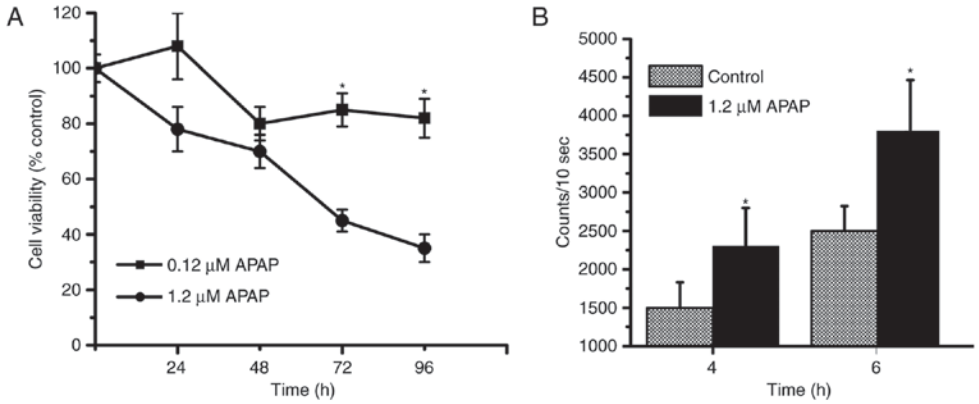

Figure 1. Cell viability and H_2O_2 levels. (A) NRK-52E cells were incubated with 0.12 and 1.2 μM APAP for 96 h. Cell viability was determined by conducting an MTT assay. *P<0.05, compared to the 1.2 μM APAP group. (B) H_2O_2 levels were determined in the control group and APAP-treated group. *P<0.05, compared to the control group. Data were analyzed from four independent experiments and presented as mean ± SD. APAP, acetaminophen.

Means were calculated with the Student's t-test method by using Microsoft Excel (http://microsoft-excel-2010.updatestar.com/zh-tw) and Bonferroni correction (SPSS 20.0 statistical software; IBM SPSS, Armonk, NY, USA). P-value <0.05 was considered to indicate a statistically significant difference.

Results

High-doses of APAP induces cell cytotoxicity and increases H_2O_2 levels. Based on our previous studies (4,6,19), a therapeutic dose of APAP (0.12 mM) is considered safe, whereas a high dose of APAP (1.2 mM) is toxic to cells and induces increases in H_2O_2 levels. The concentrations of APAP that induce cytotoxicity were determined in this study. The cell viability was above 80% in the 0.12 mM APAP-treated group at 24 and 96 h; however, the cell viability was below 40 and 30% in the 1.2 mM APAP treated-group at 72 and 96 h, respectively (Fig. 1A). Our data indicated that the high dose of APAP is cytotoxic to rat renal tubular epithelial cells (NRK-52E cells). Next, we determined whether high-dose APAP could induce H_2O_2 level increases. Our results showed that H_2O_2 levels significantly increased in the APAP-treated group at 4 and 6 h (Fig. 1B).

Guava extracts decrease H_2O_2 levels and are not toxic to NRK-52E cells. A previous study showed that guavas may be classified as IG, MG, T, R, and OR according to their ripeness (41). In this study, the extracts from pearl guava, imperial guava, and red pulp guavas in the MG and T stages were used. As different guava cultivars present different antioxidant activities (37), we first determined whether the extracts from three guava cultivars could decrease H_2O_2 levels. Our data suggested that all extracts from the three cultivars could decrease H_2O_2 levels, especially at the MG stage (Fig. 2). We further determined the cytotoxicity in NRK-52E cells after treatment with the three guava cultivar extracts. As shown in Fig. 3, cell viability rates were approximately 100% in pearl guava and imperial guava-treated groups (Fig. 3A and B), and cell viability was ~80% in the red pulp guava-treated group (Fig. 3C). Our data suggested that extracts of the three guava cultivars were not cytotoxic to NRK-52E cells.

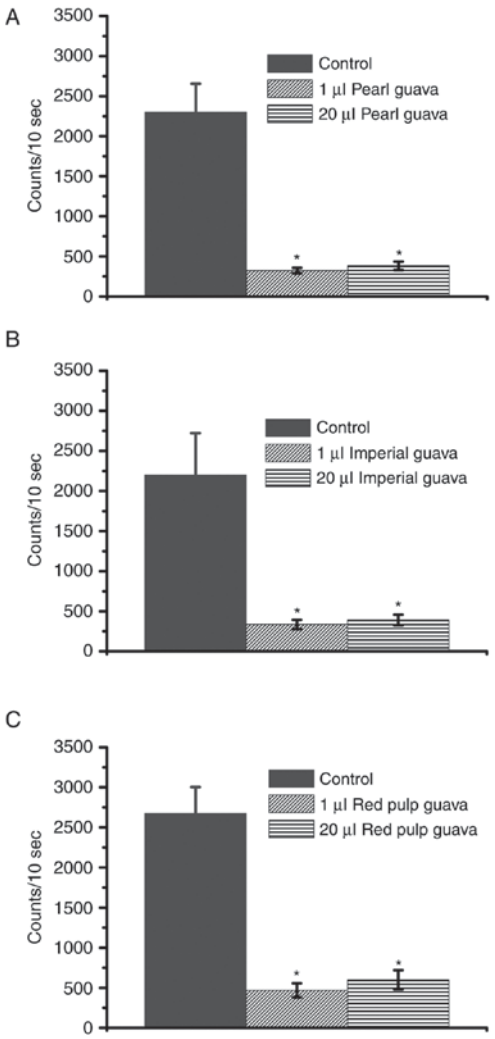

Figure 2. H_2O_2 levels. (A) H_2O_2 levels were determined in control, 1 and 20 μl pearl guava-treated groups. (B) H_2O_2 levels were determined in control, 1 and 20 μl imperial guava-treated groups. (C) H_2O_2 levels were determined in control, 1 and 20 μl red pulp guava-treated groups. Data were analyzed from four independent experiments and presented as mean ± SD. *P<0.05, compared to the control group.

Guava extracts inhibit APAP-induced H2O2 level increases. High-dose APAP caused H_2O_2 levels to increase in NRK-52E

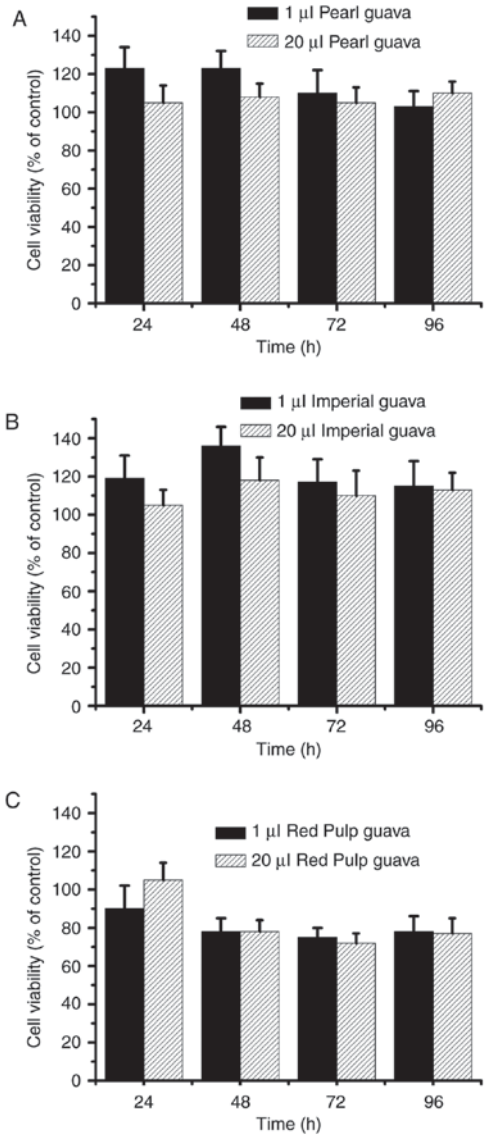

Figure 3. Cell viability. (A) NRK-52E cells were incubated with 1 and 20 μl pearl guava for 96 h. (B) NRK-52E cells were incubated with 1 and 20 μl imperial guava for 96 h. (C) NRK-52E cells were incubated with 1 and 20 μl red pulp guava for 96 h. Data were analyzed from four independent experiments and presented as mean ± SD.

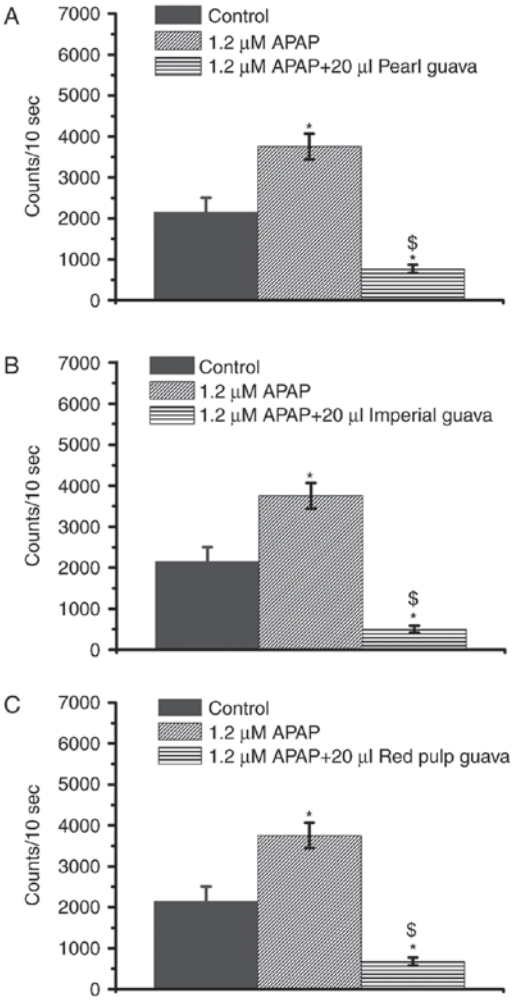

Figure 4. H_2O_2 levels. (A) H_2O_2 levels were determined in control, 1.2 μM APAP-treated, and 1.2 μM APAP plus 20 μl pearl guava-treated groups. (B) H_2O_2 levels were determined in control, 1.2 μM APAP-treated, and 1.2 μM APAP plus 20 μl imperial guava-treated groups. (C) H_2O_2 levels were determined in control, 1.2 μM APAP-treated, and 1.2 μM APAP plus 20 μl red pulp guava-treated groups. Data were analyzed from four independent experiments and presented as mean ± SD. *P<0.05, compared to the control group; $^{\$}$P<0.05, compared to the 1.2 μM APAP group. APAP, acetaminophen.

cells (Fig. 1B) while the guava extracts were indicated to decrease cellular H_2O_2 levels (Fig. 2). Therefore, we decided to further investigate whether guava could inhibit the increased H_2O_2 levels in APAP-treated NRK-52E cells. Our data showed that all extracts from three guava cultivars (pearl guava, imperial guava, and red pulp guava) effectively attenuated the APAP-induced increases in H_2O_2 level in the 1.2 mM group (Fig. 4). Previous studies indicated that increased H_2O_2 level is an important factor that results in APAP cytotoxicity (3,6,19). As shown in Fig. 4, guava extracts inhibited APAP-induced H_2O_2 levels, guava extracts were supposed to prevent APAP-induced cytotoxicity.

Pearl and imperial guava extracts inhibit APAP-induced cytotoxicity. Extracts from the three guava cultivars (pearl guava, imperial guava, and red pulp guava) could attenuate APAP-arose H_2O_2 levels (Fig. 4). We further determined whether the three guava cultivars extracts can inhibit APAP-induced cytotoxicity. Guava extracts were added to APAP-treated NRK-52E cells and the cell viability was determined. Following determination for 96 h, the cell viability was below 50% in the APAP-treated group at 48 and 96 h, however the cell viability was ~80% in APAP plus guava extract (pearl or imperial guava)-treated groups (Fig. 5A and B). Therefore, pearl and imperial guava extracts could inhibit APAP-induced-cytotoxicity in NRK-52E cells. However, the cell viabilities of the APAP-treated-group and the APAP plus red pulp guava extract-treated-group were similar (Fig. 5C). Taken together, our data indicated that pearl and imperial guava extracts inhibited APAP-induced-cytotoxicity effectively, while red pulp guava extracts did not.

Guava extracts inhibit APAP-activated caspase-3 activity. Previous studies showed that APAP causes cell cytotoxicity via the caspase-3 signaling death pathway (3,33). In this study,

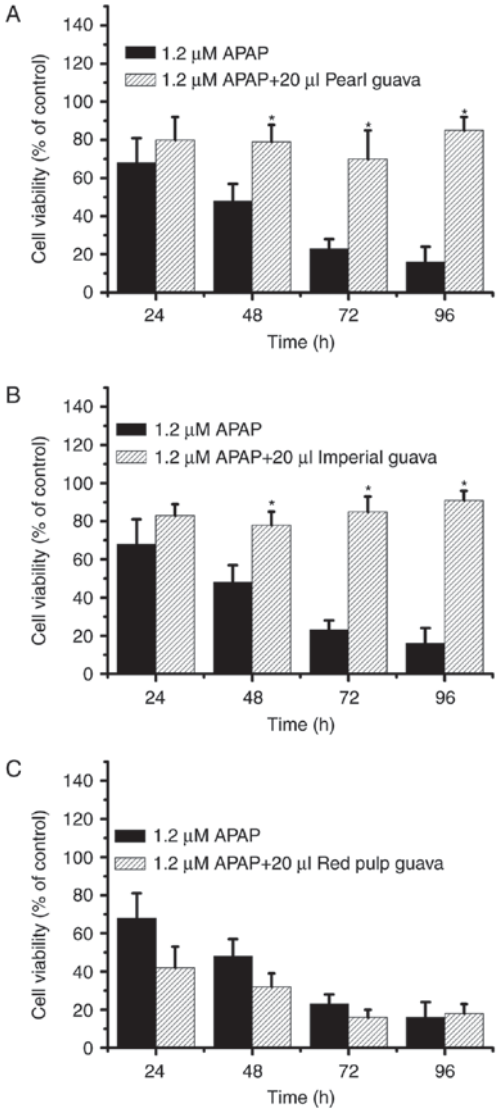

Figure 5. Cell viability. (A) NRK-52E cells were incubated with 1.2 μM APAP and 1.2 μM APAP plus 20 μl pearl guava for 96 h. (B) NRK-52E cells were incubated with 1.2 μM APAP and 1.2 μM APAP plus 20 μl imperial guava for 96 h. (C) NRK-52E cells were incubated with 1.2 μM APAP and 1.2 μM APAP plus 20 μl red pulp guava for 96 h. Data were analyzed from four independent experiments and presented as mean ± SD. *P<0.05, compared to the 1.2 μM APAP group. APAP, acetaminophen.

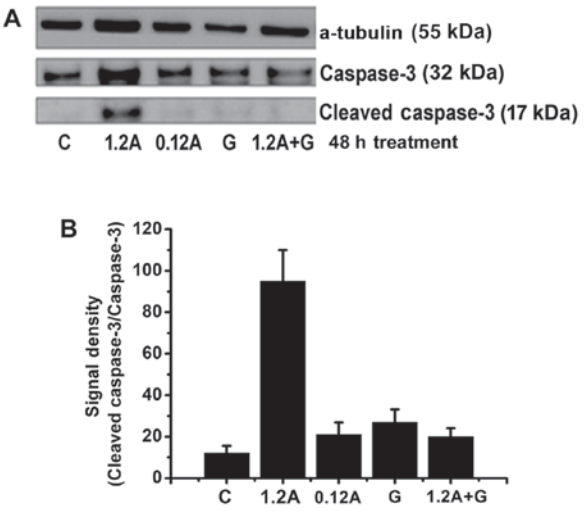

Figure 6. Caspase-3 activation. (A) Caspase-3 activity was analyzed by western blot analysis. Tubulin, caspase-3, and cleaved caspase-3 were observed on (C) control, (1.2A) 1.2 μM APAP, (0.12A) 0.12 μM APAP, (G) guava, and (1.2A+G) 1.2 μM APAP plus guava groups at 48 h. (B) Densitometric analysis of caspase-3 activity was performed and the results show the cleaved caspase-3/caspase-3 ratio. APAP, acetaminophen.

different concentrations of APAP were used to treat NRK-52 cells. The caspase-3 activation was observed easily with APAP-treated for 48 h, therefore, the 48-h incubation time were showed in Fig. 6. The results showed that the levels of cleaved caspase-3 levels obviously increased in the 1.2 mM APAP-treated group, but not in the 0.12 mM APAP-treated group (Fig. 6). Therefore, high-dose APAP induced the caspase-3 signaling pathway while low-dose APAP did not. In addition, compared with the 1.2 mM APAP-treated group, cleaved caspase-3 levels obviously decreased in the 1 2 mM APAP plus guava extract-treated group (Fig. 6). The data suggested that guava extracts could inhibit high-dose APAP-activated caspase-3 signals.

Guava extracts inhibit APAP-induced cytotoxicity in HK-2 cells. NRK-52E cells are rat renal tubular epithelial cells. As shown in Fig. 5, guava extracts were able to inhibit APAP-induced cytotoxicity in NRK-52E cells. HK-2 cells are human renal tubular epithelial cells. In order to determine whether guava extracts had similar anti-APAP effects on HK2 cells, both 48- and 96-h points were used to determine the cell viability of HK-2 cells. As shown in Fig. 7, the cell viability rate was below 50% at 96 h in the APAP-treated group, while the cell viability rates were approximately 100% in the APAP plus pearl guava-treated group (Fig. 7A) and the APAP plus imperial guava-treated group (Fig. 7B). Furthermore, similar to the data presented Fig. 5C, our study showed that the pulp guava extracts did not effectively inhibit APAP-induced-cytotoxicity in HK-2 cells (data not shown). Taken together, these data (Figs. 5 and 7) indicated that pearl and imperial guava extracts inhibited APAP-induced cytotoxicity effectively in NRK-52 and HK-2 renal tubular cells.

Discussion

Previous studies showed that high-dose APAP causes kidney and liver damage related to ROS increases, especially increases in H_2O_2 levels (3,6,19). Similar to these studies, our data demonstrated that high-dose APAP could decrease the cell viability rate in renal tubular cells and arise H_2O_2 increased level. In addition, our data showed that increased H_2O_2 levels were found at 4 and 6 h after high-dose APAP treatment, while cell viability rates obviously decreased after high-dose APAP treatment for 72 h. Cleaved caspase-3 was observed following high-dose APAP treatment for 48 h. These data suggested that the increased H_2O_2 level was upstream of APAP-induced cytotoxicity in renal tubular cells, and caspase-3 activation was downstream of APAP-induced cytotoxicity in renal tubular cells.

Previous studies have demonstrated that cytochrome P450 enzymes can regulate cell proliferation (43,44). APAP

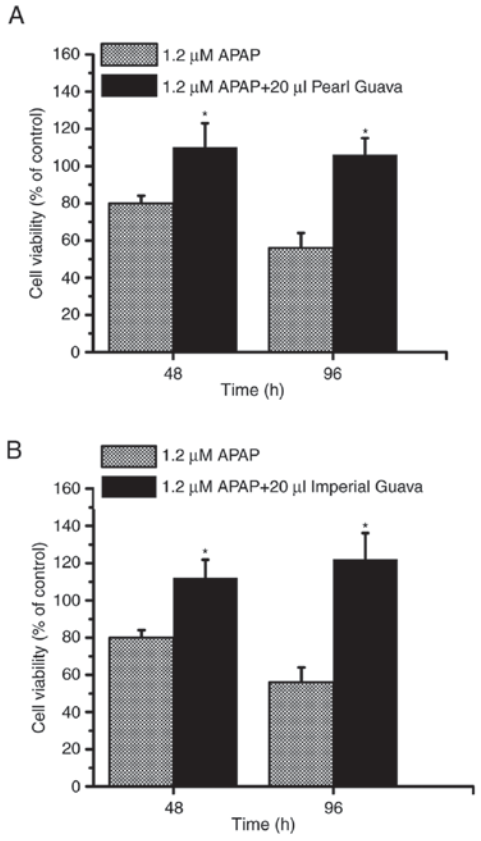

Figure 7. Cell viability. (A) HK-2 cells were incubated with 1.2 μM APAP and 1.2 μM APAP plus 20 μl pearl guava. (B) HK-2 cells were incubated with 1.2 μM APAP and 1.2 μM APAP plus 20 μl imperial guava. Cell viability was recorded at 48 and 96 h. Data were analyzed from four independent experiments and presented as mean ± SD. *P<0.05, compared to the 1.2 μM APAP group. APAP, acetaminophen.

interacts with the active sites of cytochrome P450 enzymes related to APAP-induced cytotoxicity (45,46). Many studies have shown that APAP-induced cytotoxicity requires bioactivation by cytochrome P450 enzymes (47,48), and APAP can induce the apoptotic death pathway by increasing cytochrome P450 activity (34). On the other hand, a study has shown that guava juice can inhibit cytochrome P450 enzyme activities (49). The results of the present study demonstrated that guava extracts can attenuate APAP-induced cytotoxicity. These studies indicated that the protective effects of guava extracts against APAP-induced cytotoxicity may be via cytochrome P450 signals. However, a recent study showed that APAP-induced cytotoxicity may occur either in a cytochrome P450-dependent manner or independent manner (47). Whether cytochrome P450-independent signals are involved in the effects of guava extracts against APAP-induced cytotoxicity remain to be investigated.

There are many different guava cultivars planted worldwide. Different guava cultivars have different components and quantity of phytochemicals; therefore, antioxidant activities may differ in different guava cultivars (37). In this study, pearl guava, imperial guava, and red pulp guava were investigated. Although their phytochemical components and antioxidant activities have not previously been elucidated, our study indicated that the three guava cultivars had a similar activity against H_2O_2 levels. Guavas are either classified as immature green (IG), mature green (MG), turning fruits (T), ripe (R), and over-ripe (OR). Generally, most antioxidant phytochemicals exist in the MG and T stages; however, short antioxidant molecules were found in the IG, R, and OR stages (41). Therefore, guava extracts from the MG and T stages were choice against APAP-induced cytotoxicity and exhibited anti-H_2O_2 activities in this study. Guavas in the IG stage are immature and bitter-tasting, deeming their extracts unsuitable for drinking. Therefore, guava extracts from the IG stage were not used in our study. Guava extracts from T, R, and OR stages had been pre-tested and it was shown that these extracts did not exhibit any anti-H_2O_2 activities. This study suggested that guava extracts exhibited anti-H_2O_2 activities relating to each different guava stage, as different stages have different levels of antioxidant molecules.

In our primary studies, we also found that the extracts from the MG stage guava had a better activity against H_2O_2 levels than the T stage guava extract (data not shown). A previous study showed that high glutathione levels and high glutathione reductase activity were found at the MG stage, while high ascorbate and catalase activities were found at the T stage (41). These studies indicated that distinct antioxidant molecules exhibited different anti-H_2O_2 activities. In addition, glutathione is a major cellular factor to convert H_2O_2 to H_2O (17,18). We considered high GSH levels and glutathione reductase activity to be important factors related to MG stage-expressed anti-H_2O activity. In addition, *N*-acetylcysteine (NAC), a precursor of glutathione, is common clinical drug to treat acute APAP-induced intoxication (50-52). Therefore, we determined that the MG stage of guava fruits can decrease APAP-induced cytotoxicity.

As shown in Figs. 4, 5 and 7, both extracts obtained from pearl guava and imperial guava could attenuate APAP-induced increases to H_2O_2 levels and inhibit APAP-induced cytotoxicity in renal tubular cells effectively. However, extracts obtained from red pulp guava also attenuated APAP-induced increases to H_2O_2 levels but did not inhibit APAP-induced cytotoxicity. The results indicated that H_2O_2 level increases was one of the possible factors resulting in APAP-induced cytotoxicity; other factors leading to APAP-induced cytotoxicity should be investigated in future studies. In addition, different phytochemicals may be present in different guava cultivars (37). Whether different phytochemicals exist in pearl guava, imperial guava, and red pulp guava influence APAP-induced cytotoxicity remains unclear.

Proliferating cell nuclear antigen (PCNA) is a proliferating maker found abundantly in proliferating cells such as tumor cells, stem cells and regenerating liver (53,54). However, PCNA levels were not abundant in renal epithelial cells (55). Up to now, whether APAP or guava can alter PCNA expression in renal tubular cells remained unclear. Today our primary date indicated PCNA levels were very few in NRK-52E cells and were not obvious difference in control, APAP-treated and APAP plus pearl guava-treated group (data not show). Our studies suggested APAP-induced cytotoxicity in NRK-52 cells was not related to PCNA and guava extracts inhibited-APAP-induced cytotoxicity was also not associated with PCNA. In conclusion, this study suggests that extracts of pearl guava and imperial guava could inhibit APAP-induced cytotoxicity in renal tubular cells.

Acknowledgements

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