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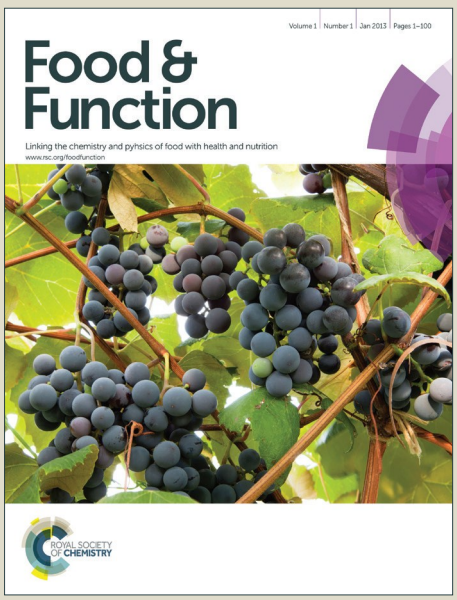

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Genistein promotes the metabolic transformation of acetaminophen to glucuronic acid in human L-O2, HepG2 and Hep3b cells via the Nrf2/Keap1 pathway

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Abstract： This study aimed to explore the effects of genistein on regulating the activation of UGTs via the Nrf2/Keap1 pathway and to elucidate the underlying mechanisms of detoxification and hepatic protection. Experiments monitoring genistein-induced protection against acetaminophen-induced cell damage were performed in L-02, HepG2 and Hep3b cells. The results of the MTT, AST, ALT, LDH, GSH and GSSG assays showed that genistein evidently protected the cells from acetaminophen-induced injury in a dose-dependent manner. The control cells were treated with 10 mM acetaminophen without genistein to compare with the effects of

the combination of acetaminophen and genistein on the expression of UGT1A1, 1A6 and 1A9, Nrf2 and Keap1 mRNAs, as well as the expression of Nrf2 and Keap1 proteins, which were tested by western blotting. The results showed that the expression of the Nrf2 mRNA and protein increased; in contrast, the expression levels of the Keap1 mRNA and protein were obviously reduced by genistein in a dose-dependent manner. Meanwhile, the expression of the UGT mRNA was increased, and UGT1A9 exhibited the highest expression among the three UGTs. Accordingly, the residual acetaminophen content was obviously reduced and acetaminophen glucuronidation increased after 24 hours of treatment with genistein in a dose-dependent effect.

Keywords： genistein, Uridine Diphosphate glucuronosyltransferases, Nrf2/Keap1, acetaminophen, glucuronidation

Abbreviations:

UGTs: uridine diphosphate glucuronosyltransferase;

UDPGA: uridine diphosphate-glucuronic acid;

APAP: acetaminophen, paracetamol;

ALT: alanine aminotransferase;

AST: aspartate aminotransferase;

LDH: lactate dehydrogenase；

GSH: glutathione;

GSSG : glutathione disulfide;

GR: Glutathione reductase

Nrf2 : nuclear factor erythroid 2-related factor 2;

Keap1: Kelch like-ECH-associated protein 1;

Gen: genistein;

APAP: acetaminophen, paracetamol;

Nrf2: nuclear factor erythroid 2-related factor 2;

Keap1: Kelch-like ECH-associated protein 1;

1. Introduction

Soybean isoflavones (genistein and daidzein) possess many biological functions; besides functioning as antioxidant and anthelmintic, many isoflavones have been shown to interact with animal and human estrogen receptors, causing effects in the body similar to those caused by the hormone estrogen. Isoflavones also produce non-hormonal and redox-active effects. Isoflavones may also interact with specific intracellular signalling proteins and with nucleic acids [1].

The activation of phase II detoxifying enzymes, such as UDP-glucuronyltransferase (UGTs), glutathione S-transferase (GST), and NAD(P)H: quinone oxidoreductases (NQR), by isoflavones results in the detoxification of carcinogens and represents one of their anticarcinogenic mechanisms [2]. Recently, it was documented that genistein prevents and protects against acetaminophen (also call paracetamol, APAP)-induced liver toxicity by inhibiting APAP biotransformation and the resistance to oxidative stress via modulation of the activities of the antioxidant and phase I/II metabolic enzymes [3].

It was shown that the expression of UGTs was up-regulated and APAP glucuronidation was enhanced, while genistein protected against APAP-induced hepatotoxicity in our normal animal experiments; however, APAP glucuronidation was reduced in nuclear factor erythroid 2-related factor 2 (Nrf2) null mice [4]. Nrf2-deficient mice were more susceptible to APAP toxicity [5]. Based on studies in animal models and cultured cells, the transcription factor Nrf2 is rapidly being

recognized as a critical regulator of the cellular stress response [6], and the Nrf2 antioxidant response pathway is the primary cellular defence mechanism against the cytotoxic effects of oxidative stress. Nrf2 is retained in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1). Under normal or quiescent conditions, Nrf2 is anchored in the cytoplasm through its interaction with Keap1. However, under oxidative stress, Nrf2 is not degraded, but instead travels to the nucleus, where it binds to a DNA promoter and initiates the transcription of genes encoding antioxidative and phase II metabolic enzymes.

Genistein may act as direct antioxidant, similar to many other isoflavones, and thus may alleviate damaging effects of free radicals in tissues, human cells turn on beneficial, detoxifying Nrf2 factor in response to genistein insult. This pathway may be responsible for observed health maintaining properities of small doses of genistein [7]. Although some reports have shown that isoflavones (genistein and daidzein) can activate the Nrf2/Keap1 signalling pathway and up-regulate the detoxifying and antioxidant defence genes to prevent cardiovascular disease[8], cancer[9], and catecholaminergic neurotoxicity [10], antioxidative enzymes, that are described in these literatures, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR), and glutamate cysteine ligase (GCL) are rarely involved in the activation of detoxification enzymes，such as UGTs and GST, the precise mechanisms of activation of detoxifying enzymes by which polyphenols (e.g., flavonols, flavones, isoflavones,

and anthocyanidins) promote the beneficial effects of Nrf2 remain to be elucidated [11].

In the present study, we showed that genistein activates UGTs and protects against APAP-induced cytotoxicity. In addition, it regulates the expression and dissociation of Nrf2 and the Keap1 signal pathway in human L-O2, HepG2 and Hep3b cells. We have attempted to focus this study on the more well-known and well-studied roles of genistein that are associated with the Nrf2/Keap1 pathway, the activation of UGTs and the level of APAP glucuronidation.

2. Materials and methods

2.1 Chemicals and reagents

Genistein from soybean (Gen, 4,5,7-trihydroxyisoflavone, PCode: 1001537637, purity: 98%, Sigma-Aldrich Chemie GmbH, Riedstr. China, Beijing) was dissolved in dimethylsulfoxide (DMSO, Sigma Co., Ltd., U.S.A) and diluted to its final concentrations in each culture medium. Acetaminophen (Paracetamol, APAP; $C_8H_9NO_2$, CAS: 103-90-2) was obtained from Aladdin Reagents Industrial Inc., (China, Shanghai) and was dissolved in DMSO to its final concentrations. MTT (3-4,5-dimethylthiahiazo-2-y1-2,5-diphenyl tetrazolium bromide) was purchased from Amresco, U.S.A; the *ρ*-acetamidophenyl β-D-glucuronide sodium salt

(APAP-Glu, PCode: 100157797) was purchased from Sigma-Aldrich Chemie GmbH, Riedstr. (U.S.A).

2.2 Cell lines and cell culture

The normal, immortalized human hepatic cell line L-O2 (HL-7702) and the human hepatocellular carcinoma cell lines HepG2 and Hep3b were all purchased from The Cell Bank of the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The L-O2 cells were cultured in Roswell Park Memorial Institute (RPMI, Mediatech Inc., Manassas, USA) medium with L-glutamine supplemented with 20% foetal bovine serum (FBS). The HepG2 and Hep3b cells were cultured in Dulbecco's modified Eagle medium (DMEM, Mediatech Inc., Manassas, USA) with 4.5 g L^{-1} D-glucose and L-glutamine and supplemented with 10% FBS. The cells were maintained at 37℃ in a humidified atmosphere of 5% CO_2 and 95% air in an incubator; and their respective media were changed every 48 hours.

2.3 Assaying the proliferation and viability of cells treated with APAP and Genistein

The effects of genistein and APAP on cell proliferation were measured with a modified MTT assay, based on the ability of live cells to cleave the tetrazolium ring in active mitochondria to a produce a molecule that absorbs at a wavelength of 570

nm. The cells were plated in 96-well microtiter plates at an initial density of 2×10^5 cells per well.

2.3.1 Genistein treatments

The L-O2, Hep3b and HepG2 cells were treated with genistein (0, 15, 30, 60, and 120 μM in medium) for 48 hours and then washed in PBS. The supernatant was removed, and 180 μl of fresh RPMI (L-O2) or DMEM (Hep3b and HepG2) and 20 μl of MTT solution (MTT was dissolved in PBS at 5 mg ml^{-1}) were added to each well and incubated for 4 hours to detect cell viability. The blank was treated with a uniform concentration of DMSO as the control.

2.3.2 APAP treatments

The L-O2, Hep3b and HepG2 cells were treated with APAP (0, 5 mM, 10 mM, or 20 mM in medium) and incubated for 48 hours. Then, the same MTT method as used for the genistein treatments was used to detected cell viability, with the same concentration of DMSO as the control.

2.3.3 Combined Genistein and APAP treatments

The L-O2, Hep3b and HepG2 cells were treated with 0, 15 μM, 30 μM, 60 μM, or 120 μM genistein in medium. Subsequently, 0, 5 mM, 10 mM, or 20 mM APAP was added to the medium, respectively, and incubated for 48 hours. Then, the MTT solution was added to the cells and incubated for 4 hours to detect cell viability.

2.4 Tests for AST, ALT, LHD, GSH and GSSG

The cells were treated with APAP (10 mM) plus genistein (15, 30, 60, and 120 μM in medium separately) and incubated for 48 hours. Then, the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were measured to determine whether the liver cells were damaged. The levels of ALT, AST and LDH were tested using specific assay kits (the ALT, AST and LDH kits were purchased from Jiancheng Bioengineering Institute., Nanjing, China), according to the manufacturer's instructions; the sample from each concentration was repeated in triplicate.

The levels of glutathione (GSH) and glutathione disulfide (GSSG) in the experimental cells were determined using an assay kit (GSH and GSSG Assay Kit，purchased from the Beyotime Institute of Biotechnology, Shanghai, China) after the cells were treated with APAP and genistein, as described above. After treatment, each group of cells was lysed at intervals of 0.5, 1, 2, 4, 8, 24 and 48 hours to analyse the GSH and GSSG levels. According to the manufacturer's instructions, the total glutathione levels can be determined colourimetrically by reacting GSH with DTNB (Ellman's reagent) in the presence of glutathione reductase. Glutathione reductase reduces GSSH to GSH, which then reacts with DTNB to produce a yellow coloured 5-thio-2-nitrobenzoic acid (TNB), which absorbs at 412 nm.

131 **2.5 Tests for APAP and APAP-Glu**

132

133 The experimental cells were treated with APAP (10 mM) plus genistein (15, 30,
134 60, and 120 μM in medium) and incubated for 24 hours. To generate standard curves,
135 APAP-Glu (0.125, 0.25, 0.5, 2, and 3 μM) and APAP (0.5, 1, 2.5, 5, and 10 mM)
136 were dissolved in methanol. APAP-Glu and APAP were detected by HPLC using a
137 Waters RP-18 column (4.6 mm×150 mm, 5 μm) and a mobile phase composed of a
138 phosphate buffer solution (pH 4.5) and acetonitrile (90∶10) at a flow rate of 1.0 ml
139 min^{-1}. The detection wavelength was 250 nm. The APAP-Glu and APAP
140 concentrations were calculated based on the prepared standard curves.

141

142 **2.6 Analysis of the expression of the UGT1A1, 1A6, 1A9, Nrf2 and Keap1**
143 **mRNAs**

144 The expression levels of the UGT1A1, 1A6, 1A9, Nrf2 and Keap1 mRNAs were
145 measured to determine their catalytic effects on the glucuronidation of APAP and
146 genistein. The L-O2, Hep3b and HepG2 cells were treated with different
147 concentrations of genistein (0, 15, 30, 60, and 120 μM) and APAP (10 mM) and
148 incubated for 24 hours. The cells were sampled and tested separately. To determine
149 the expression of the UGT1A1, 1A6, and 1A9 mRNAs in the L-O2, Hep3b and
150 HepG2 cells by qRT-PCR (quantitative real-time PCR) and RT-PCR analysis. The
151 total RNA was isolated using TRIZOL Reagent (Beyotime Institute of Biotechnology.,
152 China, Shanghai) according to the manufacturer's protocol. RT-PCR was performed

153 using a Revert Aid^{TM} First Strand cDNA Synthesis Kit (Thermo Scientific Fermentas,
154 vicegerent Jiancheng Bioengineering Institute., China, Nanjing). The primers used in
155 this study were based on the GenBank primer sequences (KeyGen Biotech, Sangon
156 Biotech Co. Ltd., China, Shanghai). The primer sequences were as follows:
157 UGT1A1 (F): 5- AACAAGGAGCTCATGGCCTCC-3,
158 (R): 5- GTTCGCAAGATTCGATGGTCG-3;
159 UGT1A6 (F): 5- CTTCCTGCAGGGTTTCTCTTCC-3,
160 (R): 5- CAACGATGCCATGCTCCCC-3;
161 UGT1A9 (F): 5- GAACATTTATTATGCCACCG -3,
162 (R): 5-ATTGATCCCAAAGAGAAAACCAC -3;
163 Nrf2 (F): 5- TGCCCCTGGAAGTGTCAAACA -3,
164 (R): 5- CAACAGGGAGGTTAATGATTT-3;
165 Keap1 (F): 5- CATCCACCCTAAGGTCATGGA-3,
166 (R): 5- GACAGGTTGAAGAACTCCTCC-3;
167 The primers for the human β-actin sequence were: (F): 5-
168 GGGAAATCGTGCGTGACAT-3,
169 (R): 5- CTGGAAGGTGGACAGCGAG -3.
170

171 **2.7 Western blot analysis of the Nrf2 and Keap1 proteins**

172

173 The L-O2, HepG2 and Hep3b cells were pre-incubated with five different
174 concentrations of genistein (0, 15, 30, 60, and 120 μM) for 48 hours, then fresh

medium was added, and each sample was treated with 10 mM APAP for 24 hours. After being washed in PBS and removal of the supernatant, the cells were lysed, and the total protein concentrations were determined using the Bradford protein assay. The cytoplasmic and nuclear fractions extracted from L-O2, HepG2 and Hep3b cells were subjected to immunoblot experiments of Keap1, Nrf2 and cellular proteins were isolated using whole cell protein extraction kit according to the manufacturer's instruction. Cytosolic and nuclear proteins were isolated as described in NE-PERs nuclear and cytoplasmic extraction kit. Protein concentration was detected by BCA Kits, and all the samples in the same experiment were normalized to the equal protein concentration. The extracted proteins (20 μg/well) were separated with an SDS-PAGE Gel Preparation Kit (Beyotime Institute of Biotechnology, China, Shanghai). The fractionated proteins were transferred onto a 0.45-μm PVDF membrane (Millipore, Germany) using a Trans-Blot Cell System (Bio-Rad, USA). Cruz Marker Molecular Weight Standards were purchased from Santa Cruz Biotechnology; the Nrf2 and the Keap1 proteins were detected using goat polyclonal IgG antibodies (sc-772 and sc-33569, respectively, Santa Cruz Biotechnology, USA).

2.8 Statistical analysis

All statistical analyses were performed using SPSS, Version 19.0 software. The data are expressed as the means±standard deviation from at least three separate

replicates. The differences in response to the genistein and APAP treatments were analysed by one-way analysis of variance (ANOVA) in combination with Tukey's t-test. Significant differences were designated in instances where p was less than 0.05.

3. Results

3.1 Genistein and APAP influence cell viability, as determined by the MTT method

Cell damage was determined by measuring the MTT reduction ability of the L-O2, HepG2 and Hep3b cells treated with APAP or Gen alone for 48 h, The cells were seeded at 2×10^5 cells ml^{-1} and then treated with APAP (5, 10 and 20 mM) or with genistein (15, 30, 60 and 120μM) for 48 hours. The number of cells and the cell viability were determined using the metabolic dye-based MTT assay (Fig.1.a and b).The cells that were not treated with APAP or genistein but were treated with DMSO alone were used as controls (100%). APAP inhibited cell viabilities in a dose-dependent manner and damaged the normal L-O2 cells (88.36% ± 0.05, 77.32% ± 0.04 and 55.69% ± 0.02 and all was $p < 0.05$, corresponding to APAP concentration 5mM, 10mM, 20mM, respectively), and the inhibition of cells viabilities of HepG2 and Hep3b respectively were 43.58% ± 0.04 ($p < 0.05$) and 43.34% ± 0.02 ($p < 0.05$) at 20mM of APAP (Fig. 1.a). Genistein (120 μM) significantly decreased cancer cell viability of Hep3b (66.07% ± 5.19, $p < 0.05$) and HepG2 (68.68% ± 1.94, $p < 0.05$), However, in the normal L-O2 cells, genistein (120 μM) does not impacte on cell

growth compared with DMSO (87.19% ± 7.18, $p < 0.05$) (Fig.1.b).

The interaction of APAP and genistein showed abnormal changes in the cells activities (Fig.2). Genistein (15, 30, 60 and 120μM) enhance resistance to APAP (5, 10 and 20mM) in a dose-dependent manner; the cells viabilities at concentrations of 120μm genistein and 20mM APAP respectively were 84.65% ± 4.18 ($p < 0.05$, L-O2，Fig.2.a), 85.64% ± 1.88 ($p < 0.05$, HepG2, Fig.2.b) and 81.18% ± 4.72 ($p < 0.05$, Hep3b, Fig.2.c); the viabilities of L-O2 cells respectively were 58.51% ± 4.17, 69.8% ± 3.41 and 80.89% ± 3.42 corresponding concentration of genistein 15, 30 and 60μM respectively mixed with 20mM of APAP (Fig.2.a); for cancer cells of HepG2 and Hep3b, at lower concentration of genistein (15μM and 30μM) with 20mM APAP, and the viabilities of HepG2 were 51% ± 2.68 and 52.66% ± 3.42 (Fig.2.b), the viabilities of Hep3b were 43.24% ± 4.85 and 46.46% ± 2.9 (Fig.2.c).

APAP significantly decreased the number of live cells (Fig.1.a), indicating the cytotoxicity of APAP-induced cells necrosis (＜80% viability in 5, 10 and 20mM of APAP, checked by trypan blue staining). Genistein (15μM-120μM) non cytotoxic reaction was observed (>80% viability) for the normal human cells of L-O2, genistein at high concentration (120μM) resulted in the cells viability of HepG2 and Hep3b of cancer cells apoptotic death (Fig.1.b, 66%～68% viability); However, the capacity of genistein (120μM) induced cancer cells apoptosis attenuated in the presence of high concentration of APAP (20mM) (viability of HepG2, 85.64% ± 1.88, $p < 0.05$, Hep3b, 81.19 ±4.71, $p < 0.05$) (Fig 2. HepG2 & Hep3b). This result was consistent with the effect of caffeic acid prevention against the hepatotoxicity induced by APAP in cells

of L-02 and HepG2 [12].

3.2 Tests for ALT, AST, LDH

The ALT and AST levels were measured to determine if the cells were damaged or diseased because ALT and AST or their ratio (AST/ALT ratio) is commonly measured in the clinic as a part of a diagnostic evaluation of hepatocellular injury to determine liver health. LDH is released during tissue damage; it is a marker of common liver injuries and diseases [13]. Compared with control (LDH activity$<$37 U L^{-1}), the activity of LDH were 50.28 U L^{-1}±4.74, 46.14 U L^{-1}±3.47 and 52.04 U L^{-1}±2.2 respectively corresponding L-O2, HepG2 and Hep3b treated with APAP (10mM) alone; in the treatment of APAP (10mM) added with genistein (120 μM), LDH activity were 30.25 U L^{-1} ± 2.23 (L-O2, Fig.3.a), 32.06 U L^{-1} ± 2.45 (HepG2, Fig.3.b) and 35.26 U L^{-1} ± 1.36 (Hep3b, Fig.3.c). The release levels of AST, ALT and LDH were in agreement with the experimental results of MTT test.

3.3 Detection of GSH and GSSG

APAP is mostly converted to pharmacologically inactive glucuronide and sulfate conjugates, with a minor fraction being oxidized to a reactive metabolite NAPQI (5-10%). Detoxification of NAPQI occurs through its binding to the sulfhydryl

group of glutathione (GSH) to form APAP-GSH, which is ultimately excreted in the urine as cysteine and mercapturic acid conjugates [14]. The ratio of GSSG to GSH is a dynamic indicator of the oxidative stress of an organism. In healthy cells and tissue, more than 90% of the total glutathione pool is in the reduced form (GSH), and less than 10% exists in the disulfide form (GSSG).

The experimental results showed that the GSH levels were at the lowest concentration at 1 hour (Fig.4.a, b and c which were corresponding the cells of L-O2, HepG2 and Hep3b), which represents the concentration of the compound that caused approximately 50% GSH depletion after 30 min to 1 hour [15]. Then, the concentration of GSH increased for 8 hours. The decrease in the GSH levels at 24 hours may be related to the contabescence of some of the cells. GSSG and GSH showed a dynamic relationship, which indicated the relationship between growth and decreased activity of the oxidation system. The GSH and GSSG levels did not change significantly in the control groups. The GSH level and the GSH/GSSG ratio were significantly decreased in the APAP group, while the GSSG level was acutely increased within 1 hour. Compared with the control group and the APAP group, the short-term depletion of GSH was recovered in the genistein-treated cells in a dose-dependent manner. The data suggest that genistein may restore the normal redox balance [16].

3.4 Measuring APAP and APAP-G

Glucuronidation pathway of APAP metabolism (APAP-glu, 52-57% of urinary metabolites) is catalyzed by UGTs, which make APAP molecule more water-soluble by transferring the glucuronosyl group from UDP-glucuronic acid [17]. APAP and its glucuronide conjugate (APAP-Glu) were detected in the experimental L-O2, HepG2 and Hep3b cells using HPLC chromatograms in the present study. The concentrations of APAP and APAP-Glu were zero in the untreated experimental cells and the control groups treated with APAP (10 mM) alone compared with the groups treated APAP plus Genistein (15 μM-120 μM) for 24 hours. The results showed that the residual APAP levels were reduced (Fig.5.a), for example, after 24 hours APAP surviving were 7.76 mmol L^{-1} ± 0.21, 7.87 mmol L^{-1} ± 0.13 and 8.08 mmol L^{-1} ± 0.15 respectively corresponding L-O2, HepG2 and Hep3b at 120μM of genistein with 10mM of APAP, comparated with control, the remaining percentage of APAP respectively were 85.27%, 81.81% and 84.87%. On the other hand, APAP-glucuronide (APAP-Glu) transformation was increased (Fig. 5 b) in the cells that were treated with different concentrations of genistein, the percent conversion of APAP-Glu increased by 184.84%, 184.21% and 174.77% respectively corresponding L-O2, HepG2 and Hep3b at 120μM of genistein with 10mM of APAP.

3.5 The expression levels of related the UGT mRNAs following genistein treatment

306 It has been well documented that genistein decreases the residual free APAP
307 levels and enhances the transformation of APAP-glucuronide in an organism's liver
308 because it activates UGTs. UGT1a1, 1a6 and 1a9 are involved in conjugating APAP
309 and the metabolites of phenolic compounds [18] and are highly expressed in hepatic
310 tissue. The results of qRT-PCR analysis revealed that the expression levels the
311 UGT1a1, 1a6 and 1a9 mRNA were down-regulated by APAP treatment alone,
312 whereas, they were up-regulated by genistein + APAP treatment; for instance, the
313 expression levels of UGT1a1, 1a6 and 1a9 mRNA of L-O2 cells (Fig.6.a), the
314 following is expressed in the median and the active units of mRNA are represented
315 by arbilitrary units, respectively were 1.29,1.61 and 1.39 corresponding untreated as
316 a control group, 1.12, 1.23 and 1.12 by APAP (10mM) treatment alone, 6.81, 9.45
317 and 13.93 treated by genistein 120μM + 10mM APAP; for HepG2 cells (Fig.6.b),
318 their expression levels of mRNA respectively were 1.34, 1.49 and 1.32 in the control
319 group, 1.1, 1.09 and 1.12 by APAP (10mM) alone, 6.58, 8.98 and 5.51 by genistein
320 120μM + 10mM APAP treatment; for Hep3b cells(Fig.6.c), their expression levels
321 of mRNA respectively were 1.32, 1.74 and 1.4 in the control group, 1.1, 1.09 and
322 1.12 by APAP (10mM) alone, 6.53, 6.67 and 7.3 by genistein 120μM + 10mM
323 APAP treatment.
324
325 **3.6 Analysis of Nrf2/Keap1 by PCR and western blotting**
326
327 The Nrf2/Keap1 system is now recognized as one of the major cellular defence

mechanisms against oxidative and xenobiotic stresses [19]. In this article, we focused on determining whether the Nrf2/Keap1 mRNA and protein levels were regulated by genistein using qRT-PCR (Fig.7) and western blotting (Fig.8), respectively, to study genistein's potential impact on the expression levels and activation of the UGTs through the Nrf2/Keap1 pathways [20].

The expression level of Nrf2 mRNA was up-regulated and Keap1 was down-regulated by APAP with genistein treatment, this is consistent with the expression of UGTs activities affected by APAP with genistein treatment. Compared with control, APAP alone treatment down-regulated the expression level of Nrf2 (86.82%, 82.09% and 84.85% corresponding L-O2, HepG2 and Hep3b) and Keap1 (76.39%, 84.84% and 63.16%, same as above cells), APAP treatment in presence of genistein enhanced Nrf2 (238.39%, 252.73% and 152.57%, L-O2, HepG2 and Hep3b, APAP 10mM added genistein 120μM vs APAP 10mM alone) and attenuated Keap1 (48.34%, 37.96% and 63.26%, same as above treatment) mRNA expression with dose dependent (Fig,7. below).

Normally, Nrf2 binds to Keap1 and is sequestered in the cytoplasm, when cells are exposed to oxidative or xenobiotic stress, Nrf2 escapes Keap1-mediated repression and translocates into the nucleus to activate targets genes. However, Nrf2 accumulation did not accompany an increase in Nrf2 mRNA in cytoplasm, this is because Keap1 directly promotes degradation of Nrf2 in cytoplasm and Nrf2 translocates into the nucleus [21]. Therefore, the Nrf2 protein in the nuclear fraction and of the Keap1 protein in the cytoplasmic fraction were detected and compared by

Western blotting (Fig.8.a, b and c), the result showed that the nuclear Nrf2 protein were increased and the cytoplasmic Keap1 protein were reduced with dose-dependent manner while genistein protection against APAP-induce cytotoxicity. The results are in agreement with the literatures of fenofibrate, coffee acid and so on [12, 21].

4. Discussion

Our previous animal studies have shown that genistein regulates the expression of antioxidant enzymes in the liver, and it also regulates phase Ⅱ metabolic enzymes to promote the transformation of xenobiotics [3]. This regulatory pathway may require Nrf2/Keap1 signalling. In this study, we used three types of human liver cells to further confirm the detoxification mechanism through this pathway.

Nrf2 is a key transcriptional regulator of antioxidant defence and detoxification through regulating a number of antioxidant signalling and detoxification genes. In higher organisms, a more sophisticated means of tightly regulating Nrf2 activity was introduced via Keap1, which modulates Nrf2 activity [22]. Nrf2 activation results in the induction of many cytoprotective proteins. These include, but are not limited to, the glutathione S-transferase family (GSTs), Glutathione reductase (GR) and the UDP-glucuronosyltransferase family (UGTs). GSTs catalyse the conjugation of GSH with endogenous and xenobiotic electrophiles, are induced by Nrf2 activation and represent an important route of detoxification [23]. UGTs catalyse the conjugation of a

372 glucuronic acid moiety to a variety of endogenous and exogenous substances, making
373 them more water soluble and enabling them to be readily excreted, important
374 substrates for glucuronidation include bilirubin and acetaminophen. Nrf2 has been
375 shown to induce UGT1A1 and UGT1A6 expression [24]. In addition, Multidrug
376 resistance-associated proteins (Mrps) have been shown to be upregulated by Nrf2, and
377 alterations in their expression levels can dramatically alter the pharmacokinetics and
378 toxicity of compounds [25,4]. Nrf2 activation may promote the de novo development of
379 cancerous tumours [26], as well as the development of atherosclerosis by increasing the
380 plasma cholesterol levels and cholesterol content in the liver [27], which may
381 overshadow the potential antioxidant effects of Nrf2 activation [28]. Nrf2 is
382 ubiquitously expressed, with the highest concentrations in the kidney, muscle, lung,
383 heart, liver, and brain [29].

384 The human diet provides a wide variety of bioactive nutrients that possess
385 beneficial effects on health and are able to activate the Nrf2 signalling pathway;
386 isothiocyanates, organosulfur compounds, polyphenols, and isoflavones have been
387 characterized as potent Nrf2 activators [30]. Many chemopreventive chemicals can
388 activate the Nrf2 promoter [31]. Genistein protected the liver cells from damage
389 induced by oxidative stress, which was accompanied by decreases in the intracellular
390 glutathione levels that could be explained by the generation of glutathionyl conjugates
391 of the oxidized genistein metabolite. Genistein increased the expression of the
392 γ-glutamylcysteine synthetase-heavy subunit (γ-GCS-HS) and Glutathione reductase
393 (GR) [32] protein and increased the cytosolic accumulation and nuclear translocation of

Nrf2. Genistein also increased the cytosolic accumulation and nuclear translocation of Nrf1 and increased the expression and activity of glutathione peroxidase (GSH-Px). The genistein-induced protective effects primarily depend on the activation of GSH-Px by Nrf1, and not Nrf2 or increased glutathione synthesis [33]. Accordingly, the genistein-induced recovery of the depleted GSH from γ-GCS-HS, GR will require further in-depth studies in the future. Genistein appears to mediate an increase of the phosphorylation and activation of the cell signalling pathway, with subsequent activation of the antioxidant and detoxification Nrf2/Keap1 transcription system, with a corresponding nuclear accumulation and enhanced DNA binding activity of Nrf2. Genistein also increased the levels of the antioxidant proteins that are downstream of Nrf2 [34].

Genistein produce non-hormonal effects of activation cell signaling and transcriptional factor, which trigger the activation of the genes related to the enzymes of antioxidant and detoxification. In the present study demonstrated that genistein protects from APAP induced-injure liver cells through the promotion of antioxidant and metabolic pathways and is associated with the activation of Nrf2/Keap1 signalling. Cellular GSH is critical for the detoxification of APAP when conjugating with its metabolic product NAPQI. Thus, the accumulation of NAPQI will deplete cellular GSH, so generating oxidative stress-induced liver injury [12]. The study results show that genistein induced the intracellular GSH level increased in a dose and time-dependent manner while APAP challenged, cellular GSH rapidly decreased within 1 hour after APAP treatment, and then the GSH level was

recovered slowly. The relationship between the changes of the intracellular GSH and GSSG was related to the growth and decline. The results indicate that genistein activate the enzymes related to GSH synthesis and GSSG reduction, which were γ-GCS-HS, GR and GSH-Px, via impaction on the Nrf2/Keap1 transcription factor pathway. The increase of APAP-glucuronidation and the decrease of the residual amount of APAP, as a specific substrate for UGT1a1, 1A6, and 1A9, were directly related to the activation of UGTs activity derived from regulatory pathway by genistein rather than the antioxidant properties of genistein itself, the hypothesis was confirmed by the synchronous enhancement of the UGTs activation, the expression mRNA level of UGTs and Nrf2/Keap1, as well as the expression of Nrf2 protein.

Genistein has multiple biological effects, such as inducing apoptosis of cancer cells, however, the impaction of APAP and/or genistein on cells activity have different biological effects. At low concentration (15, 30μM), genistein and APAP synergistically injured cells in a dose-dependent, including cancer cells of HepG2 and Hep3b; at high concentration (60, 120μM), genistein protected cells from APAP damage and made cancer cell lost the characteristic of apoptosis. The part results of flow cytometry method showed that the apoptotic cells by treatment with genistein (60μM) mixed APAP (10mM) were less than those by genistein or APAP alone, suggesting that the biological effect of genistein protection against xenobiotic have a strong dosage biological effect for cancer cells. The detailed reason is not clear and need to be further tested and explored.

In summary, the data presented in our study showed that genistein had a very

important effect on the antioxidant activity and detoxification via the Nrf2/Keap1 transcription system, with a corresponding increase in the activation of UGT1, UGT6 and UGT9, as well as replenished intracellular GSH levels in the antioxidant and detoxification system to balance the ratio of GSH/GSSG. Based on the hepatoprotection effects of genistein, we conclude that these findings provide important insights into the molecular mechanisms by which genistein detoxifies or metabolizes xenobiotics or carcinogens to promote health benefits.

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Figure Legends

Fig. 1. APAP and genistein inhibit the growth and viability of L-O2, HepG2 and Hep3b cells. The cells were seeded at 2×10^5 cells/ml and then treated with 5mM, 10mM, 20 mM APAP (Fig.1.a) or 15μM, 30μM, 60μM, 120μM genistein (Fig.1.b) for 48 hours. The number of cells and the cell viability were determined using the metabolic dye-based MTT assay. Each point represents the mean ± SD of three independent experiments. The significance was determined using ANOVA (*$p < 0.05$ vs. the untreated control).

Fig. 2. The L-O2 (Fig.2.a), HepG2 (Fig.2.b), and Hep3b (Fig.2.c) cells were treated with 10 mM APAP in the presence or absence (control) of genistein (15, 30, 60 and 120 μM) for 48 hours, and cell viability was determined using the MTT assay. The significance was determined using ANOVA (*$p < 0.05$ vs. the cells treated with 10 mM APAP without genistein).

Fig. 3. The cells were untreated (control) or were treated with 10 mM APAP in the presence or absence of genistein (15, 30, 60 and 120 μM) for 48 hours, and then the LDH, AST and ALT levels were determined. The AST and ALT units were reported as IU/L (25℃), LDH was reported as U/L (37℃). (*$p < 0.05$ vs. the untreated control, # $p < 0.05$ vs. the cells that were treated with 10 mM APAP without genistein).

Fig. 4. The cells were treated with 10 mM APAP and different concentrations of genistein (15, 30, 60 and 120 μM), as described above. After treatment, each group of cells were lysed at intervals of 0.5, 1, 2, 4, 8, 24 and 48 hours to analyse the GSH and GSSG levels. The untreated cells served as a control.

Fig. 5. The L-02, HepG2 and Hep3b cells were treated with APAP (10 mM) in the presence or absence of genistein and separately incubated for 24 hours. The cells that were treated with 10 mM APAP in the absence of genistein were used as a control. The cells that were treated with 10 mM APAP and genistein (15, 30, 60 and 120 μM) exhibited reduced levels of residual APAP (Fig.5.a) and increased APAP-glucuronide (APAP-Glu) transformation (Fig.5.b) (*$p < 0.05$ vs. the cells that were treated with 10 mM APAP without genistein).

Fig. 6. The L-O2 (Fig.6.a), HepG2 (Fig.6.b), and Hepb3 (Fig.6.c) cells were treated with APAP (10 mM) in the presence or absence of genistein treatment (15, 30, 60 and 120 μM) for 24 hours. The expression levels of the UGT1A1, 1A6, and 1A9 mRNAs were determined by qRT-PCR analysis and untreated cells were as control. (*$p < 0.05$ vs. control, # $p < 0.05$ vs. treatment with 10 mM APAP without genistein).

Fig. 7. The L-O2, HepG2, and Hep3b cells were treated with APAP (10 mM) in the presence or absence of genistein treatment (15, 30, 60 and 120 μM) for 24 hours. The expression levels of the Nrf2 and Keap1 mRNAs were determined by qRT-PCR analysis. The cells that were untreated with neither APAP nor genistein served as a control. (*$p < 0.05$ vs. control, # $p < 0.05$ vs. treatment with 10 mM APAP without genistein).

Fig. 8. Western blot analysis of the Nrf2 (nucleus) and Keap1 (cytoplasm) proteins. The L-O2 (Fig.8.a), HepG2 (Fig.8.b) and Hep3b cells (Fig.8.c) were treated with APAP (10 mM) in the presence or absence of genistein for 24 hours. (*$p < 0.05$ vs. control, #$p < 0.05$ vs. treatment with 10 mM APAP without genistein).

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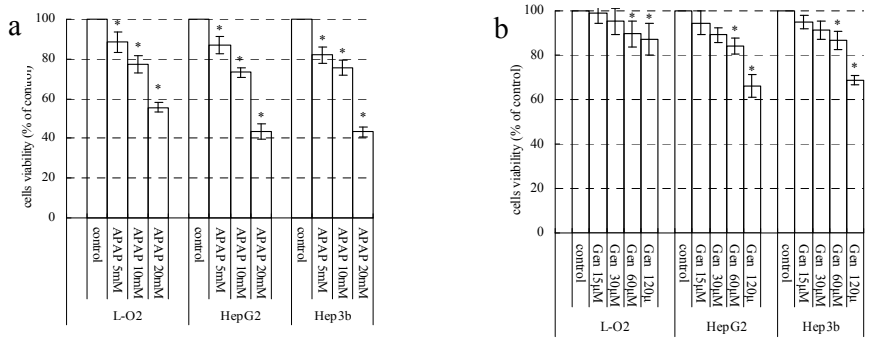
Fig.1



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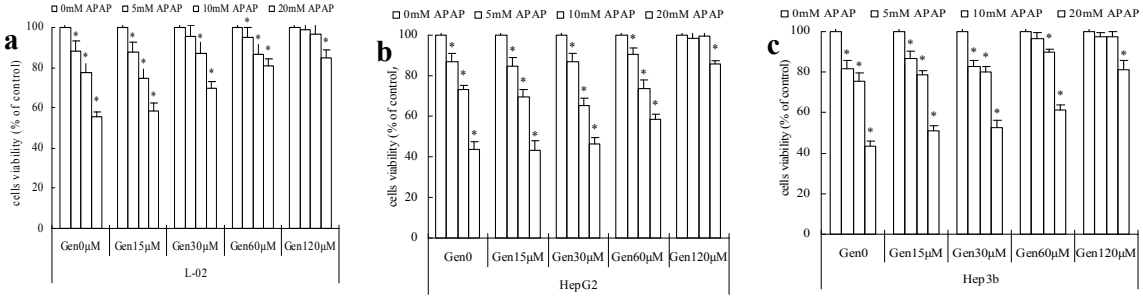

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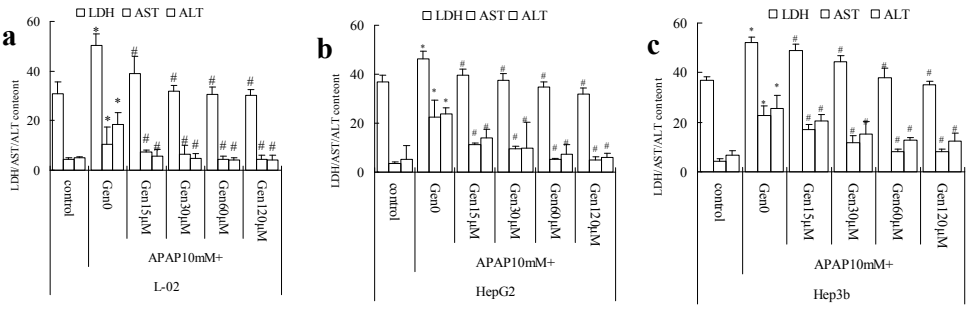

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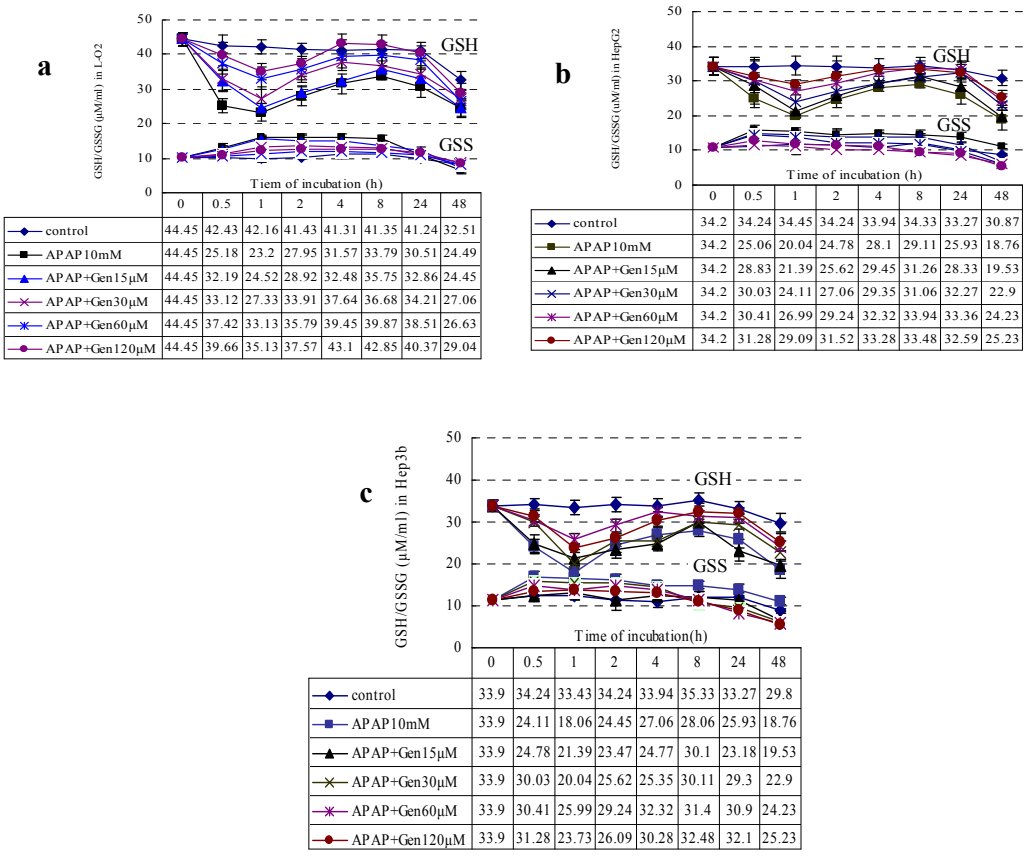


	0	0.5	1	2	4	8	24	48
control	44.45	42.43	42.16	41.43	41.31	41.35	41.24	32.51
APAP10mM	44.45	25.18	23.2	27.95	31.57	33.79	30.51	24.49
APAP+Gen15μM	44.45	32.19	24.52	28.92	32.48	35.75	32.86	24.45
APAP+Gen30μM	44.45	33.12	27.33	33.91	37.64	36.68	34.21	27.06
APAP+Gen60μM	44.45	37.42	33.13	35.79	39.45	39.87	38.51	26.63
APAP+Gen120μM	44.45	39.66	35.13	37.57	43.1	42.85	40.37	29.04

	0	0.5	1	2	4	8	24	48
control	34.2	34.24	34.45	34.24	33.94	34.33	33.27	30.87
APAP10mM	34.2	25.06	20.04	24.78	28.1	29.11	25.93	18.76
APAP+Gen15μM	34.2	28.83	21.39	25.62	29.45	31.26	28.33	19.53
APAP+Gen30μM	34.2	30.03	24.11	27.06	29.35	31.06	32.27	22.9
APAP+Gen60μM	34.2	30.41	26.99	29.24	32.32	33.94	33.36	24.23
APAP+Gen120μM	34.2	31.28	29.09	31.52	33.28	33.48	32.59	25.23

	0	0.5	1	2	4	8	24	48
control	33.9	34.24	33.43	34.24	33.94	35.33	33.27	29.8
APAP10mM	33.9	24.11	18.06	24.45	27.06	28.06	25.93	18.76
APAP+Gen15μM	33.9	24.78	21.39	23.47	24.77	30.1	23.18	19.53
APAP+Gen30μM	33.9	30.03	20.04	25.62	25.35	30.11	29.3	22.9
APAP+Gen60μM	33.9	30.41	25.99	29.24	32.32	31.4	30.9	24.23
APAP+Gen120μM	33.9	31.28	23.73	26.09	30.28	32.48	32.1	25.23

Fig. 5.

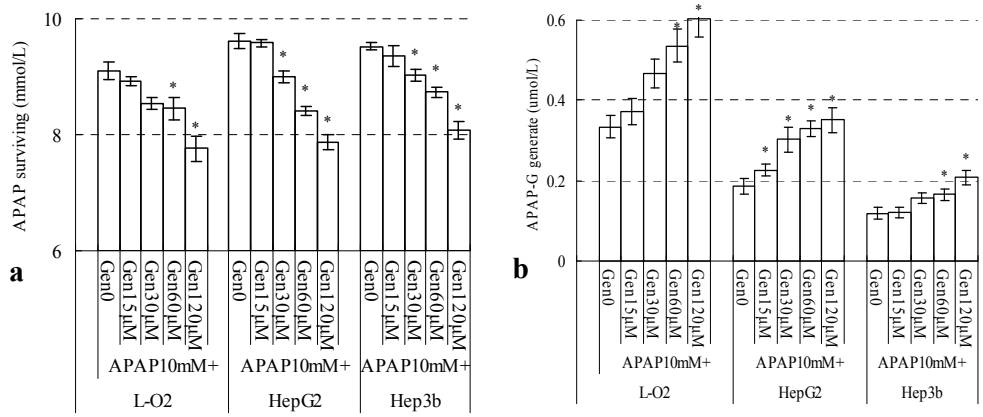


Fig. 6.

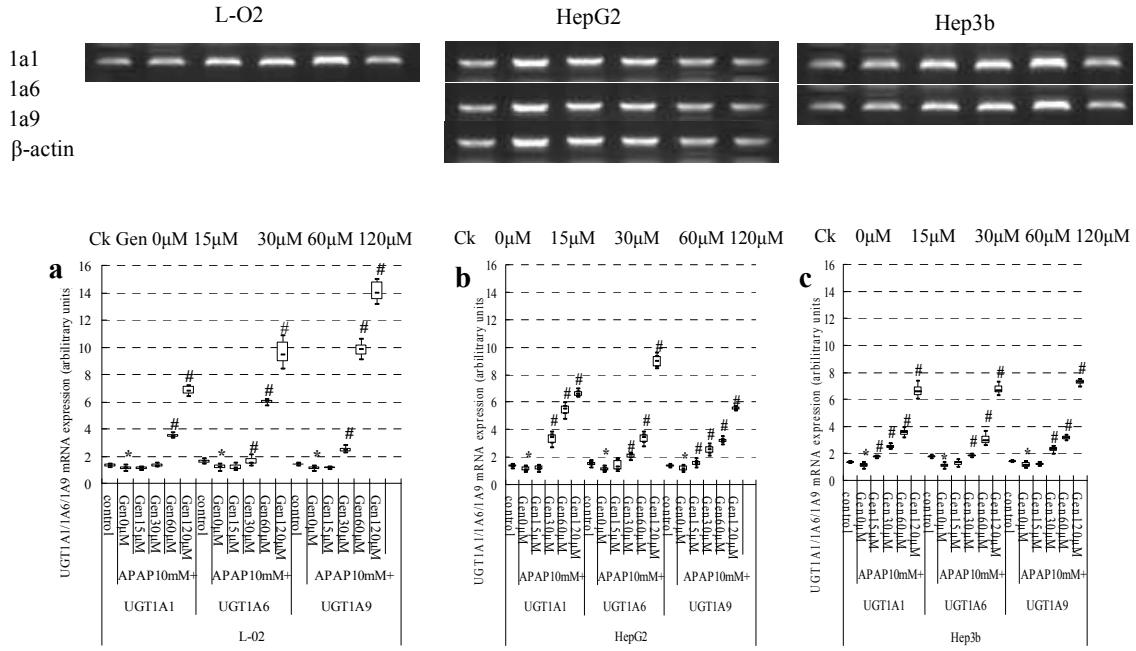


Fig. 7.

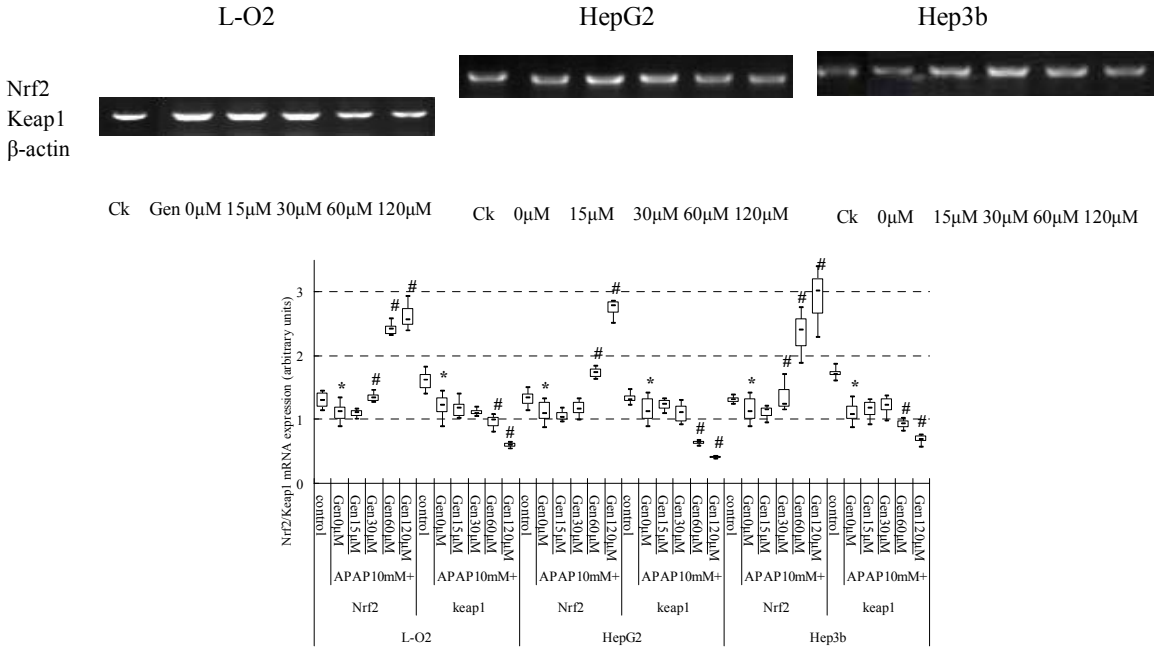
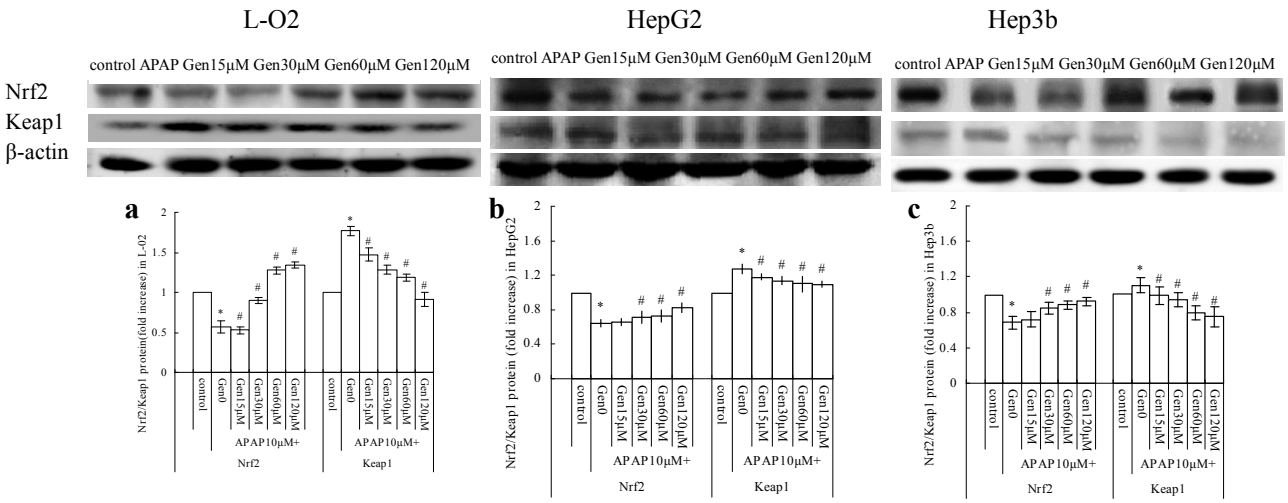


Fig. 8.



Graphical Abstract

Genistein can prevent and protect against APAP-induced liver toxicity due to the inhibition of APAP biotransformation and the resistance to oxidative stress via the Nrf2/Keap1 Cell signaling pathway to modulate the activities of UDP-glucuronosyltransferase and the antioxidant enzyme.