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Synergistic toxicity of some food additives used in non-alcoholic beverages on renal tubular epithelial cells

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ABSTRACT

Evidences supported many food additives (FAs) possess toxicity to human health due to chronic excessive exposure. Global hygienic standards strictly limit the dosage of each FA and mixture of the same functional FAs. However, the synergetic effects caused by the combination of FAs with different functions require careful evaluation. In the present study, the content of each FA in beverages was determined by HPLC-UV-Vis detection. The cytotoxic effects of selected typical FAs alone or their combination were evaluated in human renal tubular epithelial cells. Mathematical Modeling and bioinformatics methods were employed to evaluate the toxicity of FAs and to predict the key target proteins of FAs on renal tubular cell toxicity, which were verified by western blot. The results indicated above 5 FAs were used in each surveyed beverage. The content of each FA and the respective ratios of the same functional FAs in each beverage did not exceed the maximum permitted level. But it was intensively shown that the significant synergistic cytotoxicity for the combination of FAs with lower concentration. The intercellular signaling transduction pathways including JNK/STAT, PI3P/AKT, and MAPK pathways, which could also be activated by PDGF signaling, were predicted to be involved in Fas-induced cytotoxicity. The increased expression of p-STAT3, p-JNK and p-AKT was associated with renal tubular injury. The current study implied the synergistic cytotoxic effect caused by multiple FAs at no toxic dosages via activated cellular transduction pathways regulating cell survival and apoptosis function, which warning of the synergistic toxic effects of different types of FAs.

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Introduction

Evidences supported that long-term consumption of sugar-sweetened beverages is not only related to hypertension, diabetes and cardiovascular diseases (1-4) but also increases the incidence rate of chronic kidney disease (CKD) (5), which proved to be one of the important diseases threatening human health (6). Consumption of 2 or more glasses of cola per day or more than 4 cups of sugary beverages a week, was associated with more than 2-fold risk of CKD (7,8). Even long-term consumption of sugar-free beverages with low calories is also associated with an increased incidence of end-stage renal disease (ESRD) (9). In addition, if patients with CKD do not control the amount of beverage consumption, it will accelerate the progression of CKD to ESRD and increase all-cause mortality (10). Therefore, long-term excessive consumption of beverages can increase health risks including kidney injury, which deserves great attention from large consumers.

Previous studies have suggested that the kidney injury caused by beverages may be not only related to hyperuricemia, oxidative stress and renal calculus (11-13), but also associated with the food additives (FAs) used in the beverages (14). Thousands of FAs, including preservatives, antioxidants, sweeteners, coloring matters, flavor enhancers, thickeners, and emulsifiers, which mainly pertain to synthetic chemicals, are added to the beverages in order to improve the quality of products. The toxicity of FAs is generally negligible, but the health concern of FAs is mainly due to chronic excessive exposure. It has been reported that some commonly used FAs have toxicity to the kidney and other organs (15-17). Thus, many countries and international organizations have issued hygienic standards for the safe use of FAs (18), which prescribe a dosage limit for a single FA, or precautions for the mixed-use of the same functional FAs. However, there are a variety of FAs regularly used in each bottle of beverage except packaged drinking water, the synergetic toxic effects caused by the combination of different kinds of FAs would require careful evaluation. It is more important, especially for those who have long been severely addicted to beverages instead of drinking water for hydration. Thus, in order to illustrate the effects of the combination of multiple FAs, this study firstly investigated the common commercial beverages in the markets and further analyzed the contents of FAs in beverages to evaluate whether the usage of FAs in the beverages complied with the regulations of National Food Safety Standard - Standards for Uses of Food Additives of the people's Republic of China (GB 2760-2014) (19). Then the effects of selected FAs alone and their combination on cultured renal tubular epithelial cells were tentatively evaluated to clarify whether the synergistic side

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effects may occur due to the combination of multiple FAs with different functions, so as to promote rational and appropriate beverage consumption.

Materials and Methods

Survey of FAs in the beverages available for purchase in the local market

The shops and supermarkets located in the Xicheng District of Beijing listed in the Beijing Business Directory were selected as the investigation sites with the random sampling method. The samples of beverages in each investigation site were randomly selected according to their position on the shelves. The selected beverages were classified by the criteria of the General standard for beverages of the People's Republic of China (GB/T 10789-2015) (20). A detailed product specification sheet for each beverage was provided by the manufacturer. The FAs declared in the product specification of each beverage were documented for later analysis.

Quantitative analysis of FAs in the beverages

The analytical standards of 14 commonly used FAs were employed in the study. Sodium benzoate (purity > 98.8%), Potassium sorbate (purity > 99.0%), New red (purity > 98.0%), Caffeine (purity > 99.5%), Sodium saccharin (purity > 98.0%), Acesulfame K (purity > 98.0%), and Aspartame (purity > 99.7%) were purchased from Dr. Ehrenstorfer GmbH. Tartrazine (0.5 mg/ml), Sunset yellow FCF (0.5 mg/ml), Erythrosine (0.5 mg/ml), Amaranth (0.5 mg/ml), Ponceau 4R (0.5 mg/ml), Brilliant blue FCF (103 μg/ml), and Allura red AC (1 mg/ml) were purchased from China Academy of metrology (Beijing, China). Ammonium acetate and acetonitrile (chromatographic grade) were obtained from Sigma-Aldrich LLC (St. Louis, MO, USA).

The content of each FA in beverages was determined by high-performance liquid chromatography with ultraviolet-visible spectroscopic detection (HPLC UV-VIS). Briefly, 5g (accurate to 0.01 g) of each beverage was weighed into a 15 ml centrifuge tube using an analytical Balance (XPE 105, METTLER TOLEDO). The sample was centrifuged at 5000 RPM for 5 minutes using a high-performance centrifuge (BioSafe Avanti® J-26S XP, Beckman Coulter, Inc., Franklin Lakes, NJ, USA). The 2.5 ml of supernatant was then transferred into a 5 ml volumetric flask, diluted to the mark with ultrapure water and mixed well before filtration through membrane filters with pore sizes of 0.22 μm for HPLC analysis. The conditions using HPLC-2695 (Waters Corporation) were as follows. Kromasil C18 column (250 mmol/L × 4.6 mmol/L, 5 μm) was used for separation. Mobile phase: 20 mmol/L ammonium acetate solution (A) - acetonitrile (B). The flow rate was 1.0 ml/min. The injection sample volume was 10 μL with a temperature of the column was 35°C. Gradient elution procedure of HPLC: 0~5 min, 98% A; 5~10 min, 98%~76% A; 10~13 min, 76%~36% A; 13~15 min, 36%~30% A; 15~17 min, 30%~98% A. The scanning wavelength range of the diode array detector is 210~600 nm.

Evaluation of the cytotoxic effects of selected FAs alone and their combination

Human immortalized epithelial HK-2 cells obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified eagle medium (DMEM) medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and incubated in 5% CO_2 incubator at 37°C. Cells were passaged when cultured to 80%~90% confluence. After obtaining a homogeneous cell suspension, cells were counted by hemocytometer and seeded into 96-well plates at a density of 5000 cells/well. Cells were cultured for 4 h until fully attached and identified by observation under an inverted microscope. 10 μL DMEM containing different concentrations of each FA or composition of FAs was added in each well and cultured in a CO_2 incubator at 37°C for 24 h. Each sample was run in triplicate and each experiment included three groups: only DMEM in the well (blank group), cells culture in DMEM (control group), and cells culture with FA(s) (stimulus group). Pilot experiments were performed to determine the optimal incubation time to be 24 h, and then cell viability was determined using the Cell Counting Kit-8 assay (CCK-8, Dojindo Molecular Technologies, Inc., Kumamoto, Japan). After adding 10 μL of CCK-8 reagent into each well, the plate was incubated in 5% CO_2 at 37°C for 1 h. The absorbance of wells at 450 nm (reference wavelength, 650 nm) was measured with an iMark™ microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) Percent cell viability was calculated with a formula: ((absorbance from stimulus well) − (absorbance from the blank well)) / ((absorbance from the control well) − (absorbance from the blank well)) × 100. The cytotoxicity of FAs and their composition on HK-2 cells were evaluated based on the percentage of cell viability.

Mathematical Modeling for Evaluating the Toxicity of FAs Mixture

The combined effect of chemicals eventuates into three types of joint action an additive, synergistic and antagonistic effect. In this study, the action of each FA was determined by Toxicity Unit (TU). The half-maximal inhibitory concentration (IC_{50}) of the individual FAs (IC_{50i}) were determined respectively. The joint action of FAs in the mixture was calculated by equations 1 and 2, as described in the previous study (21).

$$TU_i = \frac{C_i}{IC_{50i}} \quad [1]$$

$$M_{TU} = \sum_{i=1}^{n} TU_i = \left(\frac{C_1}{IC_{50,1}} + \frac{C_2}{IC_{50,2}} + \cdots + \frac{C_n}{IC_{50,n}}\right) \quad [2]$$

In equation 1, Ci represented the concentration of each FA when the mixture was at its IC_{50}, TU_i was the toxic unit of each FA in the mixture. In equation 2, M_{TU} was the sum of TU_i. The effect of joint toxicity calculated by M_{TU} value is characterized in Table 1.

Screening of potential nephrotoxicity targets of FAs

After the canonical SMILES codes of six FAs inclu-

Table 1. The sum of TU values representing the interaction existing between FAs in the mixture.

M_{TU} (Sum of Toxic Unit)	Interaction
M < 0.8	Synergism
M between 0.8–1.2	Additive
M > 1.2	Antagonism

ding Acesulfame K, Potassium Sorbate, Sodium Benzoate, Tartrazine, Sunset yellow FCF and Amaranth were retrieved in the PubChem database (https://pubchem.ncbi.nlm.nih.gov/), the FAs related targets were predicted using SuperPred (https://prediction.charite.de/subpages/target_prediction.php) with a probability greater than 50% (22). Nephrotoxicity-related targets were retrieved using renal toxicity, kidney injury, renal fibrosis, and kidney disease as keywords from the GeneCards database (https://www.genecards.org) (23), the targets with a score ≥10.00 retrieved using each keyword were combined and regarded as the main targets of nephrotoxicity after deduplication.

The obtained FAs-related targets and the main targets of nephrotoxicity were normalized in the UniProt database (https://www.uniprot.org), and then intersection analysis was conducted to obtain the potential nephrotoxic targets of the above substances for subsequent research. The common targets between FAs and nephrotoxicity were obtained using the Online software Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/index.html) (24).

Construction of protein-protein interaction (PPI) network among potential nephrotoxic targets of FAs and prediction of key targets of nephrotoxicity

Import the screened potential nephrotoxicity targets of FAs into the STRING 11.5 database (https://string-db.org), set the biological type to "homo sapiens", set the active interaction source as an experiment and the medium confidence to 0.400, hide disconnected nodes, and the rest of the settings are as the default setting, a PPI network was constructed to analyze the interaction among the selected targets.

The PPI network was imported into Cytoscape 3.9.1 software to construct the network between FAs and targets. Topology analysis was conducted by calculating between-ness centrality, closeness centrality and degree centrality with the CytoNCA plugin to select the core targets of the network with the median value of centralities as cutoff value. The top 10 proteins ranked by the maximal clique centrality (MCC) method were considered as the hub proteins of the PPI network in the CytoHubba plugin. MCODE plugin was utilized to perform cluster analysis of the PPI network with the default settings to screen out the target protein sets involved in each functional module. The intersection of the results derived from the above three plugins of Cytoscape was identified as the key target proteins of FAs synergies to exert nephrotoxicity.

Enrichment analysis of the functions and pathways related to potential nephrotoxicity targets of FAs

GO gene ontology classification and KEGG pathway enrichment analysis of key targets associated with FAs-induced nephrotoxicity were plotted by https://www.bioinformatics.com.cn (last accessed on 5 Jun 2023), an online platform for data analysis and visualization. GO enrichment data were classified according to the biological pathway (BP), cellular composition (CC), and molecular function (MF). KEGG enrichment analysis was performed to identify the possible biological pathways.

Cell biology verification of key targets related to potential nephrotoxicity of FAs.

HK-2 cells were plated on 6-well plates (5×10^5 per well) in DMEM medium supplemented with 10% FBS and incubated for 24 h before intervention administration. DMEM or DMEM containing FAs mixture at the lowest concentration of cytotoxic effects was added in each well. Cell lysates were collected after further cultivation for 24 hours to determine the levels of STAT3, phosphorylated STAT3, Akt and phosphorylated Akt, JNK and phosphorylated JNK using the western blot method. Cell lysates were loaded in the well of sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and separated, then electrophoretically transferred to the nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% Bovine serum albumin (BSA) in Tris-buffered saline–Tween-20 (TBS-T) for 1 h. The membrane was incubated with antibody diluents at 4°C overnight. Blots were washed for 45 min in TBS-T before incubation with horseradish peroxidase (HRP)–conjugated anti-rabbit or anti-mouse IgG for 2 h at room temperature with rotation. Proteins were detected on X-ray film using chemiluminescence reagent plus (Perkin-Elmer Life Sciences, Waltham, MA, USA). For a more accurate result, each sample was analyzed in technical triplicates.

Statistical analysis

The experimental results are shown as mean ± standard deviation (SD) or median (interquartile range). For multiple group comparisons, a one-way analysis of variance (ANOVA) with Dunnett's test was used. Comparisons of frequency were performed using the Chi-square test. GraphPad Prism version 8.3 software (La Jolla, CA, USA) was utilized for statistical analysis. The criterion of significance was set at $P<0.05$.

Results

Number of FAs labeled in the product specification of beverages

A total of 48 kinds of beverages produced by 24 manufacturers from 5 large shopping malls, 5 regular supermarkets and 10 convenience stores were randomly selected in the survey. The 48 kinds of beverages were classified into 8 distinct categories according to general standard (GB/T 10789-2015) (20), including 18 fruit/vegetable juices, 4 protein beverages, 9 carbonated beverages, 3 tea beverages, 2 coffee beverages, 3 botanical beverages, 3 beverages for special uses and 6 flavored beverages.

According to the product specification of each beverage, there was no information on FAs in 2 traditional Chinese medicine beverages and 1 concentrated fruit juice beverage, 39 FAs were present in the other 45 beverages in all. The widely-used FAs in the investigated beverages included Citric acid, Sodium citrate, Vitamin C, Sodium carboxymethyl cellulose, Sodium hexametaphosphate, D-isoascorbate, Carotene, Acesulfame K, Sodium bicarbonate, Sodium benzoate, Aspartame, Potassium sorbate, Caffeine, Tartrazine, Sunset yellow FCF, Ponceau 4R, Amaranth and Brilliant blue FCF. Except for the three beverages with no FA indication, the number of FAs in each beverage was shown in Table 1. The average number of FAs for all beverages in the study was 5.3, with no difference among the categories of beverages. Protein beverages seemly contained more FAs with an average of 7.3, and a certain carbonated beverage contained up to 11 FAs.

Table 2. The concentration of selected FAs in beverages (mg/kg).

FAs	No. of beverages	Mean±SD	Median (IQR)	Min/Max	MPL
Acesulfame K	12	63.23±42.77	54.20 (60.88)	24.68/155.60	300~500
Sodium benzoate	24	114.75±58.39	146.20 (79.24)	7.76/169.20	200~2000
Potassium sorbate	10	117.46±48.15	121.55 (96.86)	40.27/176.20	500~2000
Aspartame	2	177.15±16.19	177.15	165.7/188.60	600
Caffeine	10	96.62±24.28	100.05 (19.24)	37.89/127.40	150
Tartrazine	9	7.43±3.34	6.68 (6.35)	2.56/11.13	100
Sunset yellow FCF	7	14.95±1.10	14.73 (0.60)	14.18/17.36	50~100
Ponceau 4R	1	14.09	14.09	14.09/14.09	25~50
Amaranth	3	3.4±0.14	3.40	3.26/3.55	25~50
Brilliant blue FCF	1	1.38	1.38	1.38/1.38	25

Abbreviation: IQR. IQR: interquartile range; Min/Max: minimum/maximum; MPL: Maximum permitted level.

Table 3. The concentration of FAs in cellular experiments (mmol/L).

FAs	Maximum concentration in previous studies	Initial concentration in this study
Sodium benzoate	200 (Yilmaz and Karabay 2018)	200
Potassium sorbate	200 (Mohammadzadeh-Aghdash et al. 2018)	200
Acesulfame K	50 (van Eyk 2015)	50
Tartrazine	64 (Soares et al. 2015)	64
Sunset yellow FCF	2.21 (Yadav et al. 2013)	2
Amaranth	8 (Mpountoukas et al. 2010)	8

Determining the content of FAs in the beverages

Among 45 beverages containing FAs, 10 FAs were determined by HPLC UV-VIS. Erythrosine, Allura red AC, New red and Sodium saccharin were not detected in any beverage. Table 2 showed the concentration of 10 FAs, which did not exceed their respective national maximum permitted level. The sum of respective ratios of FAs with the same functional class to the maximum level did not exceed 1 when used together in one beverage (data not shown), consistent with national food safety standards for the use of FAs (GB2760-2015).

Evaluating the cytotoxicity of FAs by CCK-8 assay

In order to understand the toxic effect of FAs, renal tubular epithelial cells cultured in vitro were treated with six frequently used FAs in beverages, including preservatives (Sodium benzoate, Potassium sorbate), sweetener (Acesulfame K), colorant (Sunset yellow, Tartrazine, Amaranth). A gradient dilution method was established to determine the concentrations of FAs in the experiments of cytotoxicity. The initial concentrations of the six FAs were referenced against the published studies (Table 3) (25-30). Tartrazine and Amaranth were not shown cytotoxicity within the given range of concentration, but the significant cytotoxicity on HK-2 cells of Sodium benzoate, Potassium sorbate, Acesulfame K, Sunset yellow FCF was shown as Figure 1, compared with the control group ($P<0.05$). The minimum concentration of cytotoxicity of the above four FAs was 25 mmol/L, 25 mmol/L, 50 mmol/L and 64 mmol/L respectively.

In order to confirm the in vitro cytotoxic effect of FAs combination, the above six FAs were mixed in equal volume at the initial concentration using in the above experiments (Sodium benzoate 200 mmol/L, Potassium sorbate 200 mmol/L, Acesulfame K 50 mmol/L, Tartrazine 64 mmol/L, Sunset yellow FCF 2 mmol/L, Amaranth 8 mmol/L), and the dosage of FAs combination contained each FA at a concentration of 1/6 of the individual initial concentration (IC). After gradient dilution, a 2-fold serial dilution of FAs combination was obtained that represented each additive at IC/6, IC/12, IC/24, and IC/48 to evaluate the synergistic cytotoxic effects of food additive combinations against HK-2 cells. The results were shown in Figure 2. The combination of FAs with IC/48 had no significant toxic effect, whereas the groups of IC/6, IC/12, and IC/24 were shown significant synergistic toxicity against HK-2 cells (compared with the control group, $P<0.05$). The concentrations of Sodium benzoate, Potassium sorbate, Acesulfame K, Tartrazine, Sunset yellow FCF, and Amaranth in the combination of IC/24 were only 8.33 mmol/L, 8.33 mmol/L, 2.08 mmol/L, 2.67 mmol/L, 0.08 mmol/L and 0.33 mmol/L respectively. The concentrations of the above four FAs in combination with cytotoxicity were considerably lower than that of each alone with cytotoxic effect, indicating the synergistic toxicity of FAs when they were mixed together even at a lower concentration.

Combined toxicity of FAs in the mixtures

The toxicity of any compounds might change when

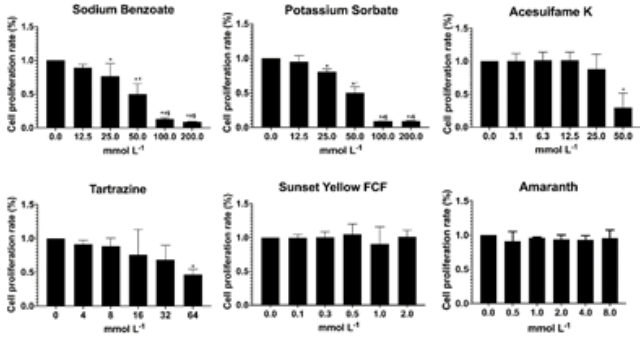

Figure 1. Effect of single FA on HK-2 cells. *P<0.05, compared with the control group. #P<0.05, compared with 25 mmol/L group. §P<0.05, compared with 50 mmol/L group.

they are mixed together especially for an extended period of time because they would interact with others in some way. Table 4 showed the IC50i of the FAs mixture or each FA, and Ci of each FA when the mixture was at its IC50. TUi represented the toxic unit of each FA in the mixture with IC50i. The IC50i of FAs Mixture, Acesulfame K, Potassium Sorbate, Sodium Benzoate, and Tartrazine was IC/15.09, 29.930 mM, 49.430 mM, 49.440 mM, 32.000 mM respectively. But Amaranth, Sunset Yellow FCF were not determined the toxicity in the current experiment. Thus the cellular toxicity of the mixture is mainly attributed to Acesulfame K, Potassium Sorbate, Sodium Benzoate, and Tartrazine. Their TUi was 0.111, 0.268, 0.268, 0.133 respectively, and the total MTU was 0.780. According to the evaluation approach, a synergistic effect arose when FAs were mixed together because the M_{TU} value was less than 0.8, indicating the cytotoxicity was amplified by the FAs mixture.

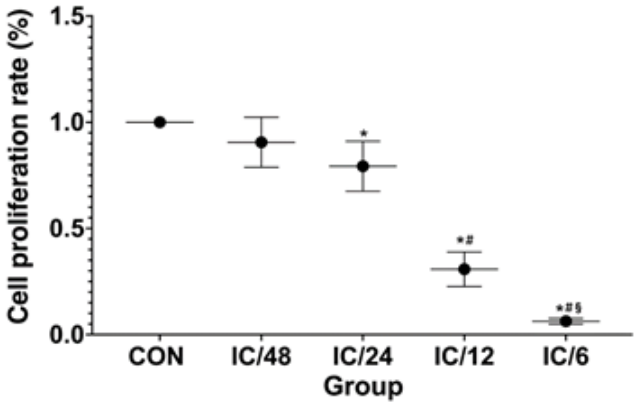

Figure 2. Effect of FAs combinations on HK-2 cells. *P < 0.05, compared with the control group. #P < 0.05, compared with the group of IC/24.

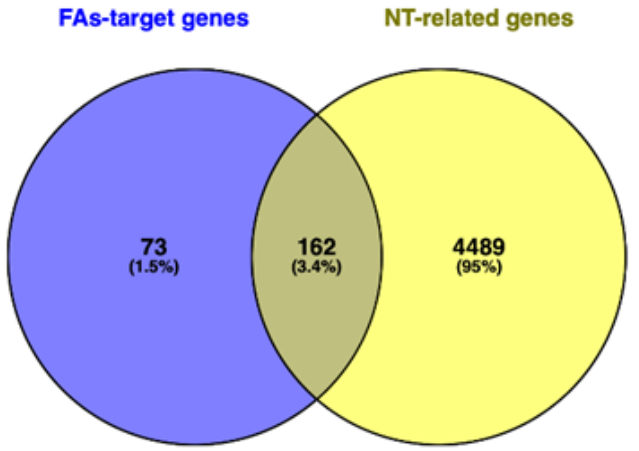

Figure 3. Numbers of potential target genes of FAs associated with nephrotoxicity. FAs: food additives, NT: nephrotoxicity. There were 235 potential targets for a mixture of FAs and 4651 nephrotoxicity-related targets. The 162 overlapped targets were identified as potential targets of FAs associated with nephrotoxicity.

Screening of potential nephrotoxicity targets of FAs

The FAs targets were predicted using SuperPred with a probability ≥50%. There 85, 94, 81, 67, 105 and 95 potential targets were retrieved for Acesulfame K, Amaranth, Potassium Sorbate, Sodium Benzoate, Sunset yellow FCF and Tartrazine respectively. A total of 235 potential targets for a mixture of the above 6 FAs were obtained after deduplication. By exploring the GeneCards database for the keywords "renal toxicity, kidney injury, renal fibrosis, kidney disease" and deleting the duplicates of the retrieved data for each keyword, the obtained 4651 genes were regarded as nephrotoxicity (NT)-related targets. There were 162 overlapped targets between NT-related targets and potential targets of FAs identified using Venny 2.1 (Figure 3), which were deemed as potential targets of FAs associated with nephrotoxicity.

Construction of protein-protein interaction (PPI) network among potential nephrotoxic targets of FAs and prediction of key targets of nephrotoxicity

The obtained 162 potential targets were analyzed using the STRING website according to the pre-determined criteria, and a PPI network with 70 intersecting targets was constructed (Figure 4A). The network between FAs and above 70 targets was constructed by Cyscape software including 77 nodes (1 node for a mixture of FAs, 6 FA nodes, and 70 target nodes) and 186 edges between FAs and targets, (Figure 4B). Different FAs could interact with the same target and different targets could interact with the same FA, except each FA interacted with special target(s), indicating the synergetic mechanisms of FAs for nephrotoxicity were based on the interaction between FAs and multitargets. The PPI network from the STRING website was then imported into Cyscape software and the potential key target proteins of FAs associated with nephrotoxicity, including Growth factor receptor-bound protein 2 (GRB2), Phosphatidylinositol 3-kinase regulatory subunit alpha (PIK3R1), Signal transducer and activator of transcription 1-alpha/beta (STAT1), Tyrosine-protein phosphatase non-receptor type 11 (PTPN11, a gene encoding the nonreceptor protein tyrosine phosphatase SHP2), Signal transducer and activator of transcription 3 (STAT3), Platelet-derived growth factor receptor beta (PDGFRB) and Platelet-derived growth factor receptor alpha (PDGFRA), were screen out using CytoNCA, cytoHubba and MCODE plugin. The PPI network of the 7 targets was shown in

Table 4. TU approach to determine the interaction effect of FAs in the mixture on cultural HK-2 cells.

FAs	IC50i (mM)	Ci (mM)	R2	TUi or MTU
Acesulfame K	29.930	3.314	0.783	0.111
Amaranth	unstable	0.530	0.015	
Potassium Sorbate	49.430	13.256	0.927	0.268
Sodium Benzoate	49.440	13.256	0.955	0.268
Sunset Yellow FCF	unstable	0.133	0.044	
Tartrazine	32.000	4.242	0.821	0.133
Mixture	IC/15.09		0.935	0.780

Figure 5.

Enrichment analysis of the functions and pathways related to potential nephrotoxicity targets of FAs

Gene ontology functional enrichment analysis yielded 351 GO entries (P<0.05), including 283 BP entries, 8 CC entries, and 60 MF entries, accounting for 80.6%, 2.3%, and 17.1%, respectively. The main biological processes involved in key targets of FAs-induced nephrotoxicity included positive regulation of protein kinase B signaling, regulation of protein kinase B signaling, protein kinase B signaling, cellular response to peptide hormone stimulus, cellular response to peptide, response to peptide hormone, interleukin-6-mediated signaling pathway, metanephric nephron development, cellular response to interleukin-6, cellular response to insulin stimulus. Cell components mainly included perinuclear endoplasmic reticulum, phosphatidylinositol 3-kinase complex, cell-cell junction, COP9 signalosome, cis-Golgi network, Schaffer collateral-CA1 synapse, microvillus, lysosomal lumen. Molecular functions mainly included insulin receptor substrate binding, protein phosphatase binding, phosphotyrosine residue binding, phosphatase binding, protein phosphorylated amino acid binding, cytokine receptor binding, phosphoprotein binding, platelet-derived growth factor binding, neurotrophin receptor binding, and platelet-derived growth factor receptor binding (Figure 6).

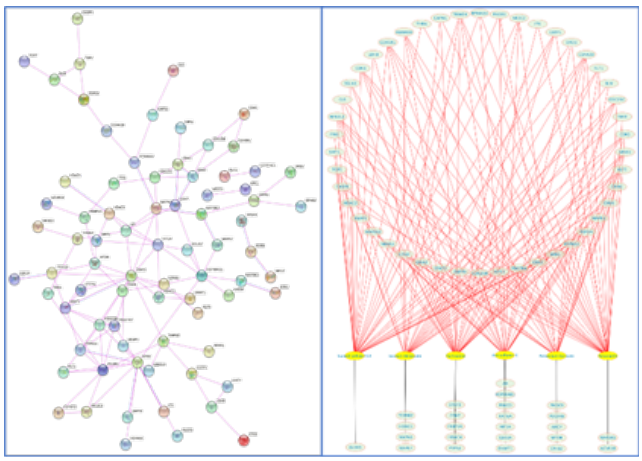

Figure 4. Potential targets of FAs associated with nephrotoxicity, indicating the synergetic mechanisms of FAs for nephrotoxicity were based on the interaction between FAs and multi-targets. A. PPI network with 70 intersecting targets constructed using the STRING website. B. The network between FAs and 70 targets was constructed by Cyscape software.

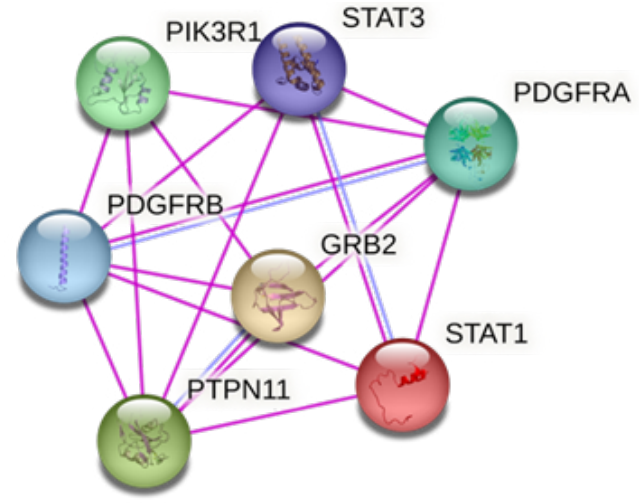

Figure 5. PPI network with 7 potential key targets of FAs associated with nephrotoxicity.

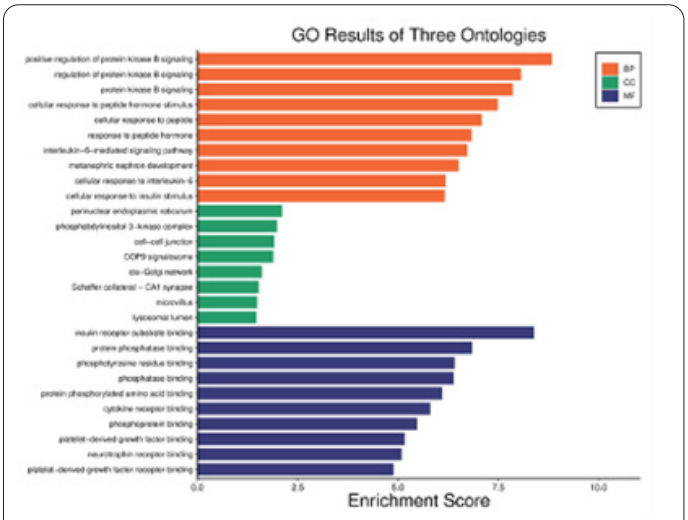

Figure 6. Top 10 significantly enriched GO (−log10 (p-value)) terms of the target genes.

The KEGG pathway enrichment and screening yielded 77 signaling pathways (P<0.05), which were shown in Figure 7. The key targets gene of FAs-induced nephrotoxicity communicates various signaling pathways, such as the JAK-STAT signaling pathway, EGFR tyrosine kinase inhibitor resistance, phospholipase D signaling pathway, prolactin signaling pathway, glioma, Ras signaling pathway, PD-L1 expression and PD-1 checkpoint pathway in cancer, prostate cancer, choline metabolism in cancer, MicroRNAs in cancer. Among them, the JAK-STAT signaling pathway, EGFR tyrosine kinase inhibitor resistance, phospholipase D signaling pathway, prolactin signaling pathway, and ras signaling pathway were associated with the pathological injury of renal tubular epithelial cells (Figure 7). It could be predicted that FAs could act on PDGFRB, PDGFRA, STAT1, STAT3, GRB2, SHP2, PIK3R1 and other genes to regulate apoptosis and other signaling pathways, to achieve nephrotoxic effect.

Cell biology verification of key targets related to potential cytotoxicity of FAs.

The above 7 target proteins associated with cellular signaling transduction passways, as well as each target-related FA(s), were depicted in Figure 8. PDGFR could activate JAK/STAT, PI3K/AKT, and MAPKs pathways, and each FA in the mixture could interact with several key proteins in the networks in which PDGFR participates in the regulation. The expression of p-STAT3, p-JNK and p-AKT were significantly increased in the FAs-treated HK-2 cells at the lowest concentration of cytotoxic effects (IC/24) as shown in Figure 9, indicating that the cytotoxic effects of FAs may be associated with multiple stimulation of passways involved in cell survival.

Discussion

There were a total of 39 FAs labeled in the product descriptions of 45 commercially available beverages investigated in the survey. Each beverage contained about 6 FAs on average and one of them incredibly contained up to 11

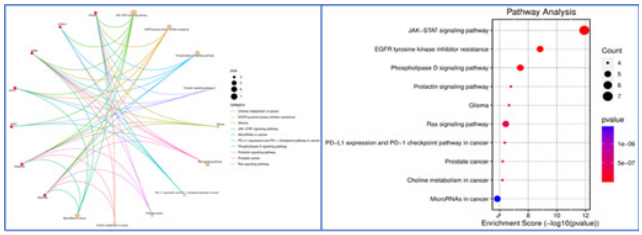

Figure 7. Top 10 significant KEGG pathways of the target genes associated with FAs-induced nephrotoxicity.

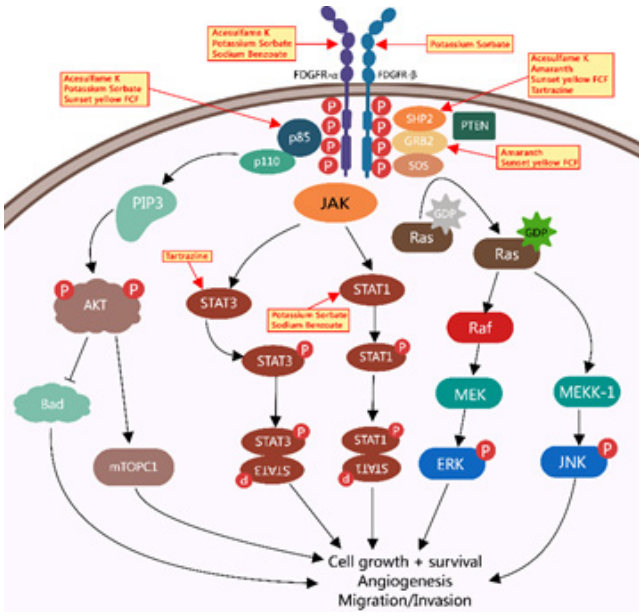

Figure 8. The predicted target proteins of FAs in the cellular signaling transduction pathways. JAK/STAT, PI3K/AKT, and MAPKs pathways might be activated by one FA or several FAs directly or indirectly via PDGFR. each FA in the mixture could interact with several key proteins in the networks which PDGFR participates in the regulation.

additives. Nowadays, FAs are widespread in the human diet including beverages, for all of them application and dosage are subject to strict regulations. Consumers are avoiding products with FAs because some of them were reported to pose undesired health effects (31,32). The abuse of FAs, including misuse or overuse of FAs, could cause food safety incidents (33). Thus, the standards for the safe use of FAs issued by countries and international organizations stipulated the dosage limit for a single FA, as well as the sum of the proportions of each additive less than 1 in the combined use of FAs with the same functional class (GB 2760-2014) (19). Delightfully, the respective dosage of each additive in the beverages was not exceed the maximum value allowed by the standards in this study.

But the safety of multiple FAs combined use in one beverage could really be ignored. In fact, a complex mixture of multiple additives was contained in most commonly consumed beverages in real life. For example, there was one beverage containing up to 11 additives with different functions in the study, which were classified into 6 categories: stabilizer, acidity regulator, preservative, colorant, antioxidant and sweetener. It is necessary to clearly state the safety of the combination of multiple FAs with different functions when they are mixed in one beverage, even if the concentration of individual additives was in accord with the standards. Regrettably, the previous risk assessment of additives was mostly focused on the analysis of the harmful effects caused by individual additives (34).

FAs, such as Sodium benzoate, Sunset yellow, Allura red, Amaranth and Tartrazine, could lead to multiple organ and system damage (16, 26, 28-30, 35, 36). The kidney is one of the important target organs of additives (37) because it plays a prominent role in mediating the toxicity of numerous substances as the important excretory organ for metabolic excretion in the body and participating in the transportation and metabolism of substances. Due to the numerous cell types organized into the nephron, any factors inducing injury of these cells, especially targeting renal tubular cells, can cause kidney damage and even renal failure. The toxic substances induced renal injury through pathophysiologic mechanisms involved in ATP depletion, oxidative stress, proximal tubule cell death and cell polarity (38). In our study, the toxic effect on renal tubular cells was observed out of expectation under the stimulation of the combination composed of FAs with IC/24, which was below the toxicity thresholds of the individual additives. The synergistic toxic effect of FAs combination was reinforced by the mathematical modeling method. It indicated that the combination of multiple FAs with different functions possesses synergistic toxic effects even if the dosage of individual additives did not exceed their respective limited range.

The development of bioinformatics provides powerful tools for studying the mechanisms of chemical substances. The 7 main target proteins were involved in PDGF receptor, and important intercellular signaling transduction pathways including JNK/STAT, PI3P/AKT, and MAPK pathways, which could also be activated by PDGF signaling. the 6 FAs were predicted to interact with PDGF receptors and bioactive molecules in the above pathways. These pathways are associated with biological effects such as cell survival, apoptosis, and proliferation. The interaction and integration of various cascade signaling molecules between these pathways ultimately determine the fate of the cells (39-41). In this study, the renal tubular epithelial cell toxicity induced by the combination of FAs was

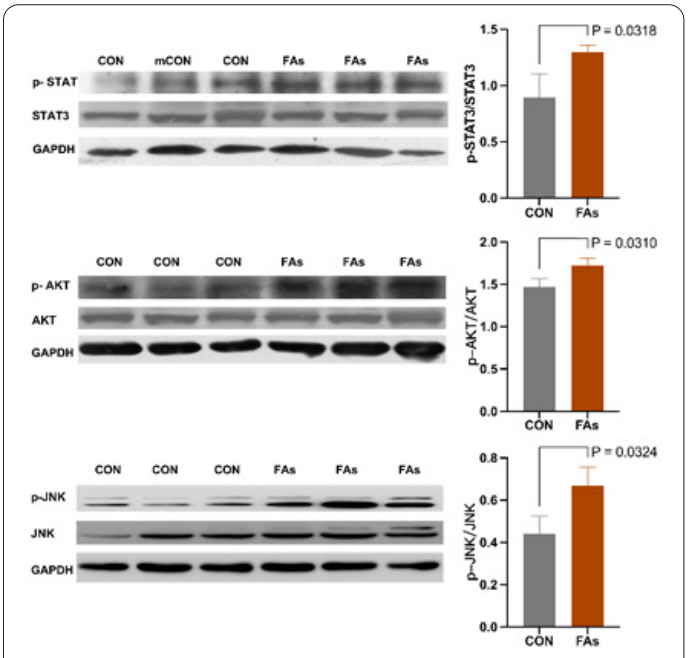

Figure 9. The phosphorylation of STAT3, JNK and AKT was significantly increased in the FAs-treated renal tubular cells.

confirmed, which may be related to individual effects or combined effects of these FAs on cell membrane receptors and multiple intracellular signaling cascade molecules. Although the study only investigated a partial mechanism of the toxic effects from the perspective of PDGFR-activated signaling pathways. the predictive tools suggest that these FAs may also induce cytotoxicity through other biological pathways, which help us to comprehensively understand the synergistic mechanisms of biological toxicity caused by different types of chemicals.

The previous safety assessment process for FAs mainly depended on the results from experimental studies. The small dosage of FAs determined by experiments was generally regarded as safe, but not all the effects on the human body could not be revealed in a short time. Based on the known side effects of individual additives, the dosage of a single additive or FAs with the same functional class in one beverage has been strictly limited according to the standards, whereas the effects of a mixture of dissimilar-acting FAs were greatly underestimated. The toxicological research on the complex mixture of FAs is gaining more attention from beverage manufacturers and regulatory authorities. It is especially important for children because increasing scientific evidence suggests potential adverse effects on children's health from food additives (42). The children who indulged in beverages intake regularly beverages larger than water (43), indicating their health is vulnerable to FAs. Today the consumption of beverages is popular among children in both rural and urban areas in China (44, 45). It will bring a serious health problem in society if most of the children become accustomed to consuming beverages because once the dietary habits have established in childhood, it would track into adulthood which is difficult to reverse without reinforcement intervention and may be induce later diseases in adult age (46-48). Long-term consumption of beverages, even containing very low-concentration multiple FAs, may induce the accumulation of FAs and synergistic reaction between FAs to enhance toxicity, or interaction with the substances in blood or organs to produce new toxic substances (49). It is conceivable that the toxic effect(s) on health by long-term consumption of beverages containing multiple FAs is a serious matter in urgent need of further study.

As we know, there are thousands of FAs have been commonly used in various kinds of food and beverages. To better ensure food safety to protect public health, the assessment of long-term toxicity effects of FAs should be taken seriously, not only based on the research results of a single FA, but also depend on a more comprehensive evaluation of the synergistic reactions induced by combined exposure of FAs. The disadvantage of this study is that the synergistic toxic effect of FAs was only derived from in vitro cellular culture system. It is better to conduct animal experiments to confirm the positive results, which may take several months even above one year. More full understanding of the toxic effects caused by the long-term combined use of artificial synthetic and natural FAs under different conditions will contribute to reducing the risk of kidney and other organ damage (50,51). But to determine the effects of long-term low-concentration multiple FAs exposure is a challenging task. It should urgently develop appropriate novel methodologies, including human stem cell cultures, 3-D cell cultures, organs-on-chips and so on, combining mathematical modelling to mimic human organs under different circumstances to comprehensively evaluate the advantages and adverse effects of FA combinations and identify the associated molecular mechanisms (52,53). A more detailed understanding of FAs will enhance the correct usage of FAs in the future.

Collectively, the study implied that the synergistic renal tubular cell toxicity may be caused by low dosages of multiple FAs, which partly depended on the activation of cellular transduction pathways regulating cell survival and apoptosis function. Further comprehensive studies are needed to understand the health consequences of long-term low dosages of multiple FAs in beverages and food, to protect human health more effectively.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Conflict of Interests

The authors declared no conflict of interest.

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