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Research article

Hexafluoropropylene oxide dimer acid (GenX) exposure induces apoptosis in HepG2 cells

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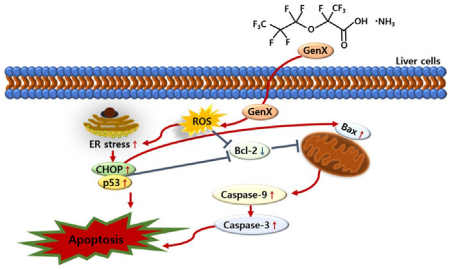
HIGHLIGHTS

- Hexafluoropropylene oxide dimer acid (GenX) can be harmful to the liver.
- Through the mediation of ROS, GenX causes apoptosis in HepG2 cells.
- GenX activates Bax, caspase 3/9, CHOP, and p53 via the apoptosis.
- The intrinsic pathway links GenX mode of action to apoptosis-related stimuli.

GRAPHICAL ABSTRACT



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ABSTRACT

Hexafluoropropylene oxide dimer acid, also known as GenX, is a poly- and perfluoroalkyl substance (PFAS). PFASs are nonvolatile synthetic substances that can be readily disseminated into the environment during processing and use, making them easy to implement in the soil, drinking water, and air. Compared to other PFASs, GenX has a comparatively short carbon chain length and is expected to have a lower tendency to accumulate in humans; therefore, GenX has recently been used as a substitute to other PFASs. However, the mechanisms underlying GenX action and intoxication in humans remains unclear. In this study, the apoptotic capacity of GenX in human liver cells was investigated. When representative human-derived liver cells (HepG2 cells) were treated with GenX for 12 h, cell viability was reduced, and apoptosis was greatly increased. In addition, GenX increased the generation of intracellular reactive oxygen species (ROS), indicating the induction of oxidative stress in a dose-dependent manner. GenX treatment increased the expression of major apoptosis-related genes relative to the untreated control group. This research indicates that GenX causes apoptosis through ROS mediation in HepG2 cells, which may expand our knowledge of the molecular and toxicological mechanisms of GenX.

1. Introduction

Perfluorinated alkyl substances (PFASs) are organic fluorine-based compounds in which fluorine-containing hydrophobic carbon chains are bound to different hydrophilic heads (Fromme et al., 2009). They contain strong covalent bonds between carbon and fluorine and are thus not easily degraded by acids or bases, and do not readily undergo oxidation, or reduction. Additionally, because of their heat resistance, they are commonly used in household and industrial products (Meegoda et al., 2020). Perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) are two PFASs that have been studied extensively (Trudel et al., 2008). Several studies have revealed that PFOA is organ-toxic

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(Jiang et al., 2012; Lv et al., 2019; Rashid et al., 2020; Shane et al., 2020) and harmful to developmental processes (Tucker et al., 2015). It also causes abnormalities in the immunological and endocrine systems (Corsini et al., 2014; Kang et al., 2016; Lee et al., 2017; Shane et al., 2020; Smits and Nain, 2013).

Because of the widespread toxicity of existing PFASs, several studies have explored safer replacements. A representative potential alternative is GenX, a hexafluoropropylene oxide dimer acid. Although research on GenX is limited and its toxicity is unclear, it is commonly used as a substitute and is believed to exhibit lower bioaccumulation when compared to that of PFOA (Gannon et al., 2016). GenX is considerably more hydrophilic than other PFASs and has a reduced molecular size, rendering separation difficult in water treatment facilities (Sun et al., 2016). Furthermore, in maize, shorter-chain PFASs displayed a higher uptake rate than longer-chain PFASs (Krippner et al., 2014). The authors suggested that the consumption of maize, a global agricultural crop, might particularly contribute to an increase in human exposure to short-chain PFASs. GenX has been widely distributed and dispersed in rivers worldwide, including South Korea, China, the United Kingdom, the Netherlands, Sweden, and the United States (Pan et al., 2018). In particular, in the Netherlands, GenX was found in drinking water as a local pollutant in the exposure route near a fluorochemical processing site, at concentrations ranging from 1.4 to 8.0 ng/L. High concentrations of GenX above national safety standards limits were also found in soil, surface water, groundwater, and vegetation samples (Brandsma et al., 2019; Gebbink and van Leeuwen, 2020). Although the half-life of GenX in human blood is unclear, the presence of GenX in blood samples from residents exposed to GenX through drinking water suggests that GenX may have a long half-life comparable to that of PFOA. However, another research suggests that the residence period of GenX in the body could be short (Thompson et al., 2019). A modeling research on male rats indicated that GenX is more likely to be harmful than PFOA, because it received a higher toxicity score in modeled serum and liver concentrations (Gomis et al., 2018). In addition, the binding ability of GenX to the human hepatic fatty acid-binding protein was greater than that of PFOA in the human liver HL-7702 cell line (Sheng et al., 2018). These findings suggest that, despite its expected short half-life in the body, GenX can be more toxic than PFOA. Furthermore, there was a significant increase in liver weight, hepatic histological abnormalities, and prevalence of placental anomalies following GenX exposure (Blake et al., 2020). Therefore, to address the rising concerns about the safety of GenX, a more detailed toxicity evaluation is needed.

The purpose of this study was to investigate the effects of GenX on the human liver, using the human cell line HepG2 as the experimental model, as well as rat primary hepatocytes in a separate experiment. Our results showed that GenX induces apoptosis of liver cells and triggers liver toxicity.

2. Materials and methods

2.1. Materials

GenX, also known as ammonium perfluoro (2-methyl-3-oxahexanoate) (Synquest Laboratories, Alachua, FL, USA), was used in this study. PFOA, N-acetylcysteine (NAC), dimethyl sulfoxide (DMSO), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Enhanced chemiluminescence (ECL) Western blot detection reagents were purchased from Abclone (Seoul, Republic of Korea). The bicinchoninic acid (BCA) protein assay kit and SuperSignal™ West Femto Maximum Sensitivity substrate were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Immunoblotting was performed using monoclonal or polyclonal antibodies against Bax, Bcl-2, Caspase-3, Caspase-9, p53, CHOP, and GAPDH. All antibodies used for Western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Information on the antibodies used is displayed in Supplementary Table 2. All stock solutions (GenX, PFOA) were prepared before the experiments and dissolved in phosphate-buffered saline (PBS; pH 7.4). For treatment, the stock solutions were diluted 100 times in the medium.

2.2. Cell culture

The human liver hepatocellular carcinoma cell line HepG2 was purchased from ATCC (Manassas, VA, USA) and maintained according to the ATCC guidelines. For the cell culture, low-glucose Dulbecco's modified Eagle's medium (Gibco Life Technologies, Grand Island, NY, USA) and fetal bovine serum (FBS; Hyclone, Logan, UT, USA) were used. The culture medium was supplemented with penicillin (100 U/mL) and 10% FBS. The medium was modified to contain Earle's balanced salt solution, 1mM sodium pyruvate, 2mM L-glutamine, 1.5 g/L sodium bicarbonate, and non-essential amino acids.

2.3. Cell morphology

HepG2 cells were exposed to 40, 100, 250, and 500 μM GenX for 12 h. As a positive control, cells were exposed to 40 μM PFOA. For dose selection, we referred to previous studies (Eriksen et al., 2010; Wen et al., 2020). All experiments were repeated in triplicate, and morphological alterations in the cells were observed using an inverted microscope (IX73P2F; Olympus Optical Co., Ltd., Tokyo, Japan). We evaluated the dynamics of cell attachment and cytoskeletal organization, and further compared the shapes of the cells.

2.4. Cell viability assay

The cell viability of HepG2 cells was measured by the MTT assay as previously described (Yang et al., 2019). HepG2 cells were co-incubated with 40 μM PFOA and different concentrations (40, 100, 250, and 500 μM) of GenX in 12-well plates. After the treatments, the supernatant was suctioned, MTT solutions were added, and incubated for 3 h. Subseuently, the same volume (200 μL) of DMSO was added to each well, and absorbance was detected using a Hidex sense multi-plate reader (Hidex, Turku, Finland).

2.5. Isolation and culture of primary rat hepatocytes

The collagenase perfusion method was performed as previously described, using 7-week-old male Wistar rats (Yang et al., 2019). The culture medium was replaced 4 h after plating, and cells were incubated for 12 h in an incubator under a humidified atmosphere of 95:5% O_2/CO_2 at 37 °C. All experimental protocols were approved by the Committee for the Care and Use of Laboratory Animals at Korea University (KUIACUC) (KUIACUC-20200057).

2.6. Intracellular reactive oxygen species (ROS) formation

ROS production was assessed as previously described (Yang et al., 2019). To assess the amount of ROS produced by HepG2 cells, cells were seeded in black 96-well plates at 5×10^5 cells/mL and cultured for 24 h. After 24 h, the supernatant was suctioned, and the cells were incubated with 100 μM DCFH-DA for 30 min. The supernatant was removed and the cells were washed twice with PBS. After washing, the cells were treated with 40 μM PFOA and various concentrations of GenX (40, 100, 250, and 500 μM) for 12 h and 48 h. The fluorescence intensity of DCFH-DA was detected using a Hidex sense multi-plate reader (Hidex, Turku, Finland; excitation, 485 nm; emission, 535 nm).

2.7. Flow cytometric analysis

To assess the apoptotic rate, eBioscience™ Annexin V-FITC Apoptosis Detection kit (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's guidelines. HepG2 cells were seeded in 6 well-plates and

exposed to 40 μM PFOA and GenX (40, 100, 250, and 500 μM) for 12 h. The apoptotic rate of the sample after treatment was measured using a BD Accuri C6 Plus Flow Cytometer Analyzer (BD Biosciences, San Jose, CA, USA).

2.8. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay

For the assessment of mRNA expression in HepG2 cells, qRT-PCR was performed as previously described (Yoo et al., 2019). Cells were exposed to 40 μM PFOA and various concentrations of GenX (40, 100, 250, and 500 μM) for 12 h. Total RNA extraction reagent (Takara, Shiga, Japan) was used to extract RNA. Legene Premium Express first-strand cDNA synthesis system (Legene Biosciences, San Diego, CA, USA) was used for cDNA synthesis. Apoptosis-related gene expression levels were assessed using SYBR Green real-time PCR master mix (Elpis, Daejeon, Republic of Korea) and iQ5 detection system (BioRad, Hercules, CA, USA). The results were calculated using the 2-ΔΔCt method (Livak and Schmittgen, 2001), and normalized against β-actin as a housekeeping gene. The sequences of primers used are shown in Supplementary Table 1.

2.9. Western blot analysis

Western blot analysis was performed as described previously (Shin et al., 2019). HepG2 cells were treated with 40 μM PFOA and GenX (40, 100, 250, and 500 μM) for 12 h. Cells in each well were lysed using radioimmunoprecipitation buffer (Elpis-Biotech, Daejeon, Korea). The lysis mixture was collected in a tube and centrifuged at 15,000xg for 20 min at 4 °C. Protein samples were prepared using the BCA assay and separated by SDS-PAGE. The separated protein sample was transferred to a polyvinylidene fluoride membrane, and 5% skim milk was used for membrane blocking. The membrane was incubated overnight in a solution containing the primary antibody and subsequently washed and incubated for 1 h with the secondary antibody at 4 °C. For the visualization of protein bands, SuperSignal ™ West Femto substrate and chemiluminescent ECL solution were used. Protein bands were quantified using the ImageJ image-analysis software developed and released from the National Institutes of Health, Bethesda, MD, USA.

2.10. Statistical analysis

Statistical analyses were carried out using one-way analysis of variance (ANOVA) according to Tukey's post hoc test with SAS version 9.4 (SAS Institute, Cary, NC, USA). The *p*-values were considered statistically significant at <0.05.

3. Results

3.1. Cell morphology induced by GenX

We treated HepG2 cells with 250 μM GenX for 12 and 48 h and observed changes in cell morphology (Figure 1). When cultured *in vitro*, HepG2 cells often take on an epithelial-like form (Singh et al., 2011), with healthy cells being spherical and closely connected. However, the cell morphology of the unit treated with PFOA or GenX demonstrated irregular shapes; cells were often observed to have separated from the clusters and shrunk, suggesting necrotic features.

3.2. Assessment of cell viability

The viability of HepG2 cells after exposure to 40 μM PFOA and GenX (40, 100, 250, and 500 μM) for 12 and 48 h is shown in Figure 2. The groups treated with 250 and 500 μM GenX for 12 and 48 h had a significant ($p < 0.05$) decrease in viability of 7.04% and 23.0% (for 12 h treatment) and 23.3% and 52.0% (for 48 h treatment), respectively. These results suggest that GenX is cytotoxic to HepG2 cells in a time-and dose-dependent manner. PFOA is a well-known hepatotoxic PFAS that decreases liver cell viability (Panaretakis et al., 2001). Cells treated with 40 μM PFOA for 12 and 48 h showed significantly ($p < 0.05$) decreased viability by 11.8% and 43.4%, respectively. The cell viability of the group exposed to 500 μM GenX was comparable to that of the positive control group treated with 40 μM PFOA.

3.3. Assessment of ROS

After HepG2 cells were exposed to GenX (40, 100, 250, and 500 μM), the quantity of cellular ROS generated was measured. As shown in Figure 3, in all groups treated with GenX, ROS production significantly increased in a dose-dependent manner. Cells exposed to 250 μM GenX or 40 μM PFOA, produced 1.35 and 1.14 fold higher ROS produced by the control groups, respectively. These findings indicated that GenX can cause oxidative stress and trigger the release of ROS.

3.4. Assessment of apoptotic rate by flow cytometric analysis

To assess the proportion of apoptotic HepG2 cells treated with GenX and distinguish between living, necrotic, and early and late apoptotic cells, flow cytometric analysis was performed. The third quadrant represents the distribution of living cells, the fourth quadrant represents early apoptosis, the second quadrant represents late apoptosis, and the first quadrant contains necrotic cells that can be identified as dead cells (Krysko et al., 2008). As seen in Figure 4, the apoptotic rate increased by approximately 1.44 times in the PFOA-treated cells than in the control, and in the 40, 100, and 250 M GenX-treated cells, the apoptotic rate increased by approximately 1.18, 1.28, and 1.77 times compared to the control, respectively.

3.5. Assessment of apoptosis-related gene expression by qRT-PCR

To assess apoptosis-related gene expression after GenX exposure in HepG2 cells, a qRT-PCR assay was performed. Interestingly, the mRNA expression level of Bax increased in a dose-dependent manner in all groups exposed to GenX (40, 100, and 250 M), and was even higher than

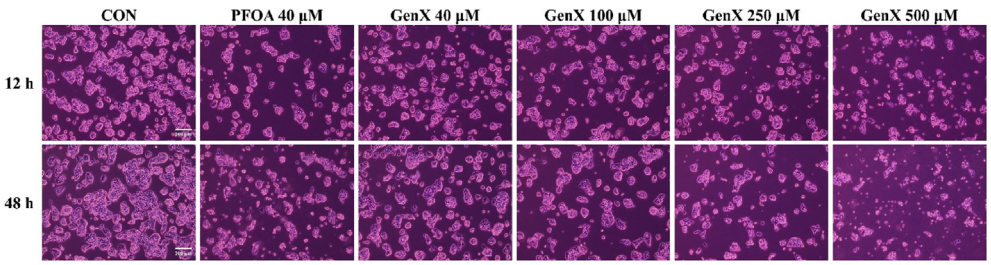

Figure 1. Morphological alterations of HepG2 cells after hexafluoropropylene oxide dimer acid (GenX) exposure. HepG2 cells were treated to the indicated concentrations of PFOA and GenX for 12 h (A) and 48 h (B). Scale bar, 400 μm. All experiments were performed in triplicate, and morphological alterations in cells were observed using an inverted microscope under the 100x magnification (IX73P2F; Olympus Optical, Tokyo, Japan).

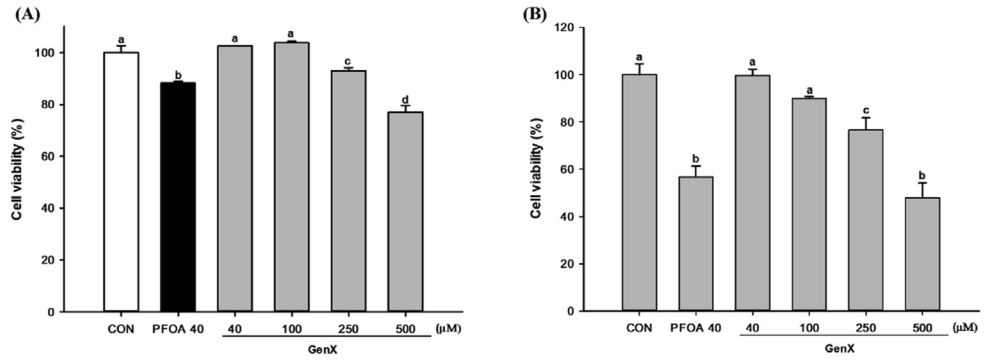

Figure 2. Cell viability of HepG2 cells after GenX exposure. HepG2 cells were treated with the indicated concentrations of PFOA and GenX for 12 h (A) and 48 h (B). Values are presented as the mean ± SD (n = 3). Significant differences ($p < 0.05$), determined using by Tukey's post hoc test, are represented by different letters (a–d) above the bars.

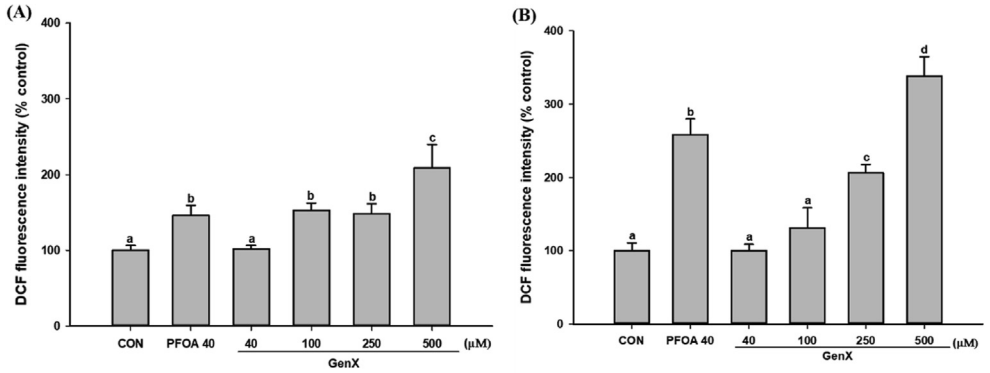

Figure 3. ROS production after GenX exposure in HepG2 cells. HepG2 cells were exposed to the indicated concentrations of PFOA and GenX for 12 h and 48 h. The DCF fluorescence intensity (% of the control) represents the relative ROS level. All DCF fluorescence values are compared to the control values (control = 100). Values are presented as mean ± SD (n = 3). Significant differences ($p < 0.05$), determined using by Tukey's post hoc test, are represented by different letters (a–d) above the bars.

that in the group treated with 40 μM PFOA (Figure 5). In the cells exposed to GenX, mRNA expression of Bcl-2 was significantly ($p < 0.05$) lower than that in cells exposed to 40 μM PFOA. Compared to untreated control cells, the mRNA levels of caspase-3 and caspase-9 were elevated in a dose-dependent manner in the cells treated with GenX, and the mRNA levels of CHOP and p53 increased significantly ($p < 0.05$) in the group exposed to GenX 250 μM and 40 μM GenX compared to the control cells.

3.6. Western blot analysis

After 12 h of treatment in HepG2 cells, we measured the protein expression of apoptosis-related factors (Figure 6), with GAPDH used as the normalization control. The Bax protein level was substantially higher in all groups exposed to GenX, whereas the protein levels of Bcl-2 decreased relative to that of the control group. The protein levels of caspase-3, caspase-9, and p53 in the groups treated with GenX were higher than those in the control group. The increase in Bax, caspase-3, caspase-9, CHOP, and p53 expression levels and the decrease in Bcl-2 protein levels after GenX treatment indicate that GenX leads to apoptosis in HepG2 cells.

4. Discussion

PFASs, such as PFOA and GenX, are compounds with carbon-fluorine bonds that are commonly used in the manufacturing industry (Prevedouros et al., 2006). However, the accumulation of PFASs in the body causes multiple toxicities, including immunotoxicity, genetic toxicity, neurotoxicity, and hepatotoxicity (DeWitt et al., 2019; Emerce and Çetin, 2018; Fletcher et al., 2019; Gaballah et al., 2020; Ojo et al., 2020; Shane et al., 2020; Sun et al., 2019; Wen et al., 2020). In this study, we used hepG2 cells to demonstrate that GenX induces apoptosis in liver cells. HepG2 cells have been extensively used to study various phenomena in the liver, including apoptosis, necrosis, and genetic toxicity (Knasmüller et al., 2004; Knowles et al., 1980; Nguyen et al., 2013). A recent study reported that GenX may induce epigenetic toxicity in HepG2 cells (Wen et al., 2020). Groups exposed to GenX showed a significant decrease in the mRNA expression levels of DNA methyltransferases. In another *in vitro* study, a comparison of toxicities between PFAS alternatives, including GenX and PFOA, on 3D spheroids was performed. The group treated with GenX displayed a significant increase in hemeoxygenase-1 (HO-1) mRNA expression, lactate dehydrogenase (LDH) leakage, and ROS content 28 d after GenX treatment (Sun et al., 2019). These results indicate that GenX could induce hepatotoxicity. In addition, the binding capacity of GenX to the human hepatic fatty acid-binding protein was higher than that of PFOA in the human liver HL-7702 cell line (Sheng et al., 2018). Interestingly, when one or more oxygen atoms are present in the backbones of hexafluoropropylene oxide homologues, such as in GenX, structural distortions are observed during their binding processes, resulting in different binding modes. Furthermore, compared to the control group, pregnant CD-1 mice exposed to GenX (2 or 10 mg/kg/d) displayed a significant increase in incidence of placental anomalies, and mice livers exposed to GenX showed abnormal ultrastructure and enlargement (Blake et al., 2020). In addition, PPARα signaling was induced in the liver during a 90-day subchronic toxicity study in which mice were treated with GenX, and the possibility of causing liver damage via the corresponding pathway was postulated (Chappell et al., 2020).

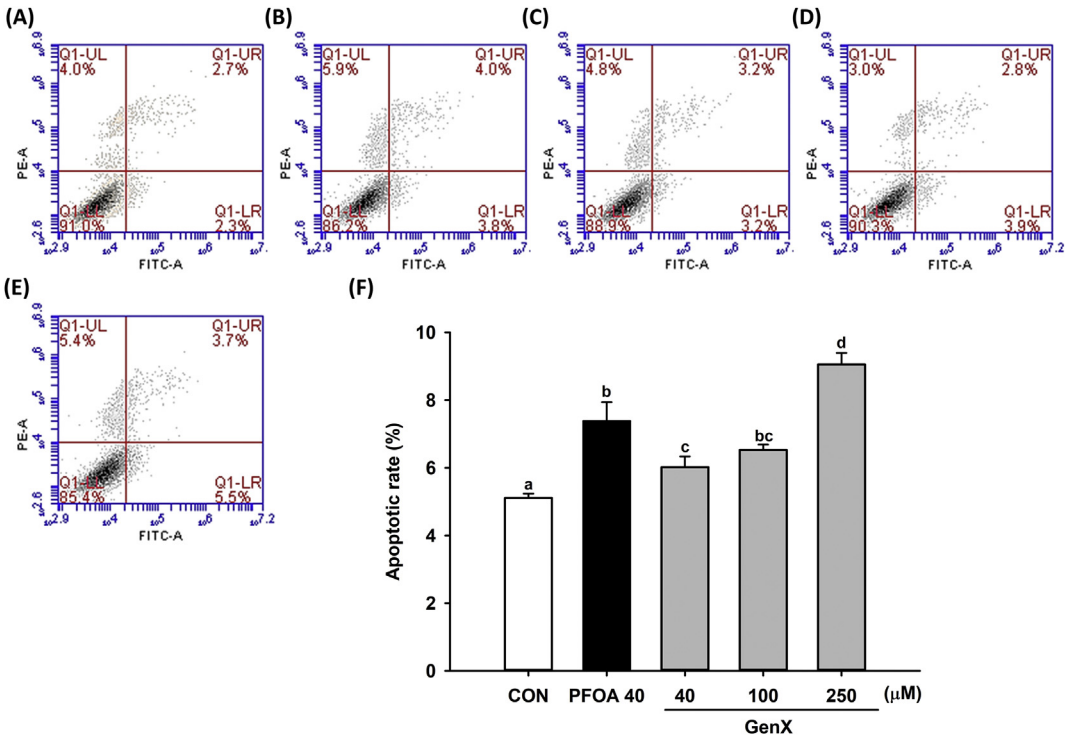

Figure 4. Apoptotic rates in HepG2 cells after GenX exposure. HepG2 cells were exposed to the indicated concentrations of PFOA and GenX for 12 h. (A): Control, (B): 40 μM PFOA, (C): 40 μM GenX, (D): 100 μM GenX, (E): 250 μM GenX, (F) Apoptotic rate (%): sum of Q1-LR and Q1-UR quadrants. Flow cytometry contour plots of HepG2 cells labelled with annexin V-fluorescein isothiocyanate (FITC) fluorescence vs propidium iodide (PI) fluorescence. Q1-UL: the upper left quadrant represents necrosis; Q1-UR: the upper right quadrant represents late apoptosis; Q1-LL: the lower left quadrant represents the distribution of living cells; Q1-LR: the lower right quadrant represents early apoptosis. Values are presented as mean ± SD (n = 3). Significant differences ($p < 0.05$), determined using by Tukey's post hoc test, are represented by different letters (a–d) above the bars.

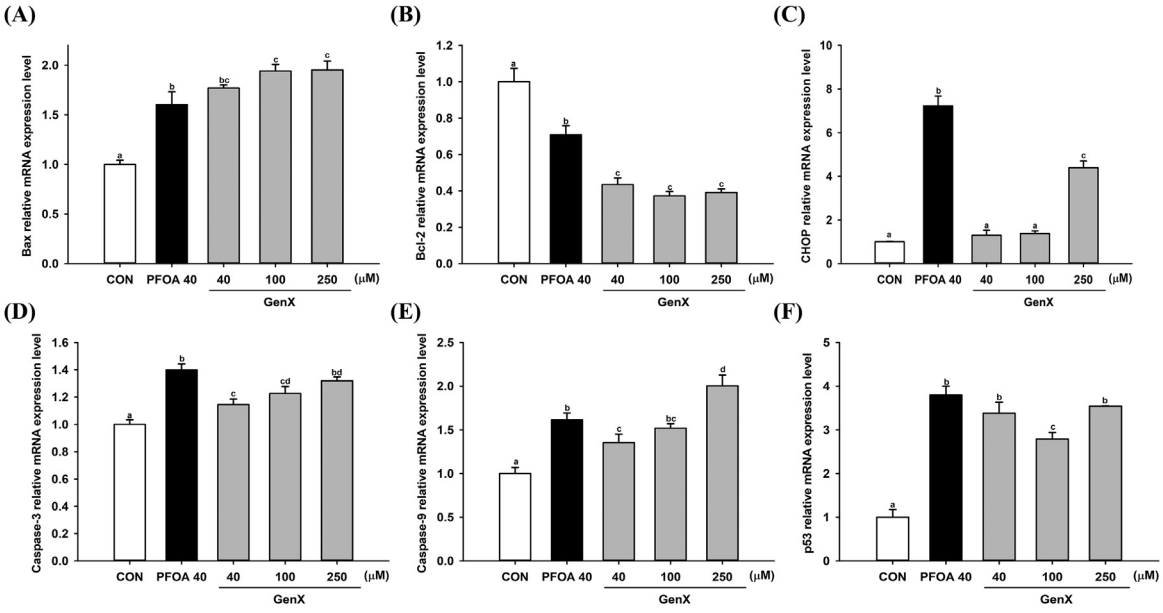

Figure 5. Levels of mRNA expression after GenX exposure in HepG2 cells. HepG2 cells were exposed to the indicated concentrations of PFOA and GenX for 12 h. All of the data are compared to the control values (control = 1). Values are presented as mean ± SD (n = 3). Significant differences ($p < 0.05$), determined using by Tukey's post hoc test, are represented by different letters (a–d) above the bars.

The possibility that GenX might cause hepatotoxicity highlights the need for further mechanistic studies.

To the best of our knowledge, it was previously unknown whether GenX could induce ROS-mediated apoptosis in liver cells. As shown in Figure 3, GenX induced an increase in ROS generation in HepG2 cells. ROS is thought to be a manifestation of the transition or an activator of the mitochondrial permeability transition (Green and Reed, 1998). If ROS-induced oxidative stress increases, changes in gene expression

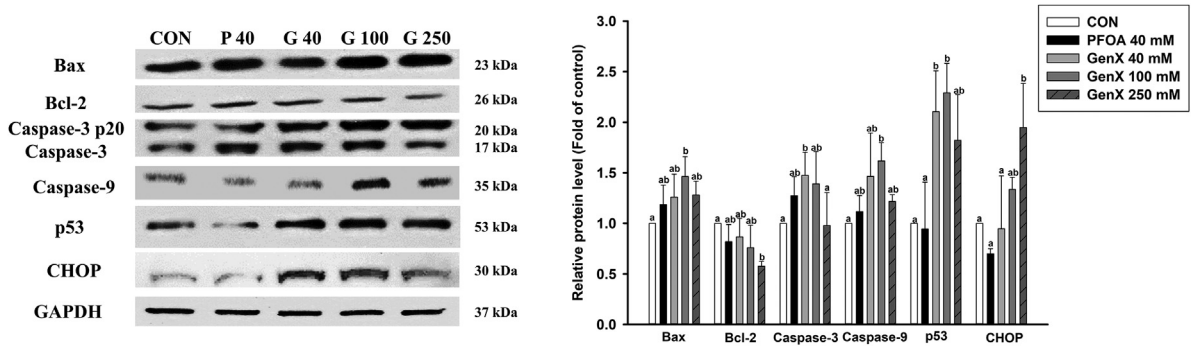

Figure 6. Effects of PFOA and GenX exposure on expression of apoptosis-related proteins in HepG2 cells. HepG2 cells were incubated with the indicated concentrations of PFOA and GenX for 12 h. CON: control; P 40: PFOA 40 μM; G 40: GenX 40 μM; G 100: GenX 250 μM; G 250: GenX 500 μM; All of the data are compared to the control values (control = 1). Values are presented as mean ± SD (n = 3). Significant differences ($p < 0.05$), determined using by Tukey's post hoc test, are represented by different letters (a–d) above the bars.

linked to signal transduction, mitogenesis, and mutagenesis may occur, resulting in apoptosis (Ermak and Davies, 2002). Additionally, to confirm that GenX induces apoptosis through the mediation of ROS in HepG2 cells, we treated HepG2 cells with GenX for 12 h and further treated them with NAC, a well-known ROS inhibitor (Supplementary Fig. 1). The mRNA expression of apoptosis-related factors, Bax, CHOP, caspase-3, and caspase-9, was substantially suppressed after NAC treatment at a level comparable to that in the untreated control group. This indicates that GenX induces apoptosis through the mediation of ROS.

Apoptosis is regulated by the expression of anti- and pro-apoptotic genes (Kiraz et al., 2016). Apoptotic cell death generally occurs when the Bax/Bcl-2 gene regulation ratio increases (Du et al., 2013). This mechanism is essential for the mitochondrial apoptotic pathway, and is known as the intrinsic pathway (Kiraz et al., 2016). Caspases play a key role in the initiation and implementation of apoptosis, and caspase-3 is known to be essential for apoptosis and DNA damage (Ahmad et al., 2012; Jänicke et al., 1998). Further, it is known that p53 increases the transcription of various genes, and this transcriptional regulation plays a leading role in the cellular response to DNA damage (Miyashita et al., 1994). Apoptotic stimuli, such as DNA damage, trigger the release of pro-apoptotic proteins into the cytoplasm (Li et al., 1998). This leads to the activation of caspase proteases, which adjusts the effective disassembly of dying cells through mitochondrial disruption (Youle and Strasser, 2008). Proteins belonging to the Bcl-2 family strongly induce this intrinsic pathway, allowing caspase-3 to be activated through caspase-9 activation (Hakem et al., 1998).

According to the findings of mRNA and protein expression, the upregulation of pro-apoptotic genes and downregulation of anti-apoptotic genes were observed in HepG2 cells after GenX exposure. The GenX-treated cells had higher levels of CHOP, caspase-3, caspase-9, and p53 mRNA and protein expression than the control cells (Figures 5 and 6). When the mRNA levels of the apoptotic marker genes were compared at the same concentration (40 μM) in GenX-and PFOA-treated HepG2 cells, GenX treatment preferentially upregulated and downregulated Bax and Bcl-2, respectively, while PFOA treatment preferentially increased the expression of other apoptotic genes, although the underlying mechanisms remain unknown.

There have been concerns that HepG2 cells may not accurately reflect hepatocyte characteristics. As a result, the mRNA expression levels of apoptosis-related genes in primary rat hepatocytes after treatment with GenX were investigated (Supplementary Fig. 2). We found that the mRNA expression of Bax, CHOP, and Caspase-9 significantly increased in a concentration-dependent manner, with the same tendency as in the previous experiments. In addition, the expression of Bcl-2 was significantly downregulated in mice treated with various concentrations of GenX relative to the control. Interestingly, the mRNA expression of rat primary hepatocytes treated with 40 μM GenX and PFOA were similar to that of HepG2 cells treated with the same concentrations of GenX and PFOA; GenX substantially down- or up-regulated the mRNA expression of Bcl-2 and Bax, respectively. The members of the Bcl-2 gene family, especially Bax and Bcl-2, play a substantial role in controlling apoptosis (Kroemer, 1997). Bax and Bcl-2 were substantially more downregulated in the GenX-treated hepatocytes than in PFOA-treated hepatocytes in the present study. This could imply that GenX is more likely to induce apoptosis in the liver than PFOA. Mechanistic research should be accompanied by a study on *in vivo* GenX exposure.

In summary, GenX induced apoptosis in HepG2 cells via mediation of ROS. The present study found that caspase-3, caspase-9, CHOP, and p53 are all involved in the mechanism of action of GenX, which induces apoptosis via an intrinsic pathway in liver cells.

5. Conclusions

Until recently, the biological toxic effects and mechanisms of GenX have not been studied. In this study, we demonstrated that GenX induces apoptosis in HepG2 cells and suggested a possible intracellular toxicity mechanism. The intrinsic pathway connects the GenX mode of action to apoptosis-related factors. Our research offers new insights into GenX-induced cytotoxic pathways and molecular mechanisms.

Declarations

Author contribution statement

Hee Joon Yoo: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Min Cheol Pyo: Analyzed and interpreted the data.

Yoonjin Park & Bo Yong Kim: Contributed reagents, materials, analysis tools or data.

Kwang-Won Lee: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2020.e03459.

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