Medit Vet J, 2026: 14-23, 2026
DOI: 10.24880/meditvetj.1771658

Research Article

Hepatocellular carcinoma cell death induced by cyfluthrin: the cellular decision between apoptosis and autophagy

Dilek Güvenç[1], Sinem İnal[2], Nilüfer Kuruca[2], Hasan Sercan Palancı[3], Oğuzhan Yavuz[4], Tolga Güvenç[2]

[1]Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ondokuz Mayis University, Samsun, Türkiye
[2]Department of Pathology, Faculty of Veterinary Medicine, Ondokuz Mayis University, Samsun, Türkiye
[3]Department of Virology, Faculty of Veterinary Medicine, Selcuk University, Konya, Türkiye
[4]Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Cukurova University, Adana, Türkiye

Key Words:
Bax
Bcl-2
Beclin-1
cytotoxicity
HepG2
synthetic pyrethroids
LC3

Received : 25 August 2025
Revised : 31 March 2026
Accepted : 03 April 2026
Published : 18 May 2026
Article Code : 1771658

Correspondence:
D. GÜVENÇ
(dguvenc@omu.edu.tr)

ORCID
D. GÜVENÇ : 0000-0003-0036-0914
S. İNAL : 0000-0002-2552-5159
N. KURUCA : 0000-0001-5601-4952
HS. PALANCI : 0000-0001-5408-9176
O. YAVUZ : 0000-0002-1419-1464
T. GÜVENÇ : 0000-0003-1468-3415

ABSTRACT

Synthetic pyrethroids are frequently used for pest control in many areas such as food, agriculture, and livestock. Therefore, its toxic effects on human health need to be investigated in more detail. The aim of this study was to investigated the hepatotoxic effects of cyfluthrin depending on time and dose in terms of the molecular mechanisms of apoptosis and autophagy in the HepG2 cell line. The IC50 value of cyfluthrin was calculated as 997.049 µmol/L, as a result of cell viability WST assay. Induction of apoptosis and autophagy was evaluated by Flow cytometry assay, PCR assay, Western Blot assay and immunofluorescence methods. The results showed that cyfluthrin induced autophagy by increasing LC3 and Beclin-1 levels on HepG2 cells at 24 h. Moreover, CYF induced apoptosis by decreasing Bcl-2 and increasing BAX levels at 48 h. In conclusion, it is considered that autophagy is survival mechanism in early time, but the main death mechanism of CYF on HepG2 cells was apoptosis.

INTRODUCTION

The type II synthetic pyrethroid insecticide cyfluthrin (CYF) is used in various formulations to control many pests in pet and food animals and the environment. In addition, it is applied to fight against pests fed by cotton, cereals, fruits, and vegetables in agriculture. CYF is also widely used in and around homes for vector control (Anadón et al., 2009; Zalom, 2010). Non-target organisms (e.g., humans, birds, and aquatic organisms) are exposed via the oral route by consuming food-containing residues and via the respiratory or skin route from the environment (Whyatt et al., 2002; Williams et al., 2003). Biomonitoring studies, especially using urine specimens, verify the CYF exposure in people in all age groups, such as children and adults, in several countries (Ding et al., 2015; Heudorf & Angerer, 2001; Gabriele Leng et al., 1997; G Leng et al., 1997; Quindroit et al., 2019; Rousis et al., 2017; Steckling et al., 2018).

Regulated cell death may occur throughout physiological and pathological processes in both single-celled and multicelled organisms. The purpose of this genetically controlled process is to eliminate cells that have the potential to damage the organism. Cell death is classified into three types, which are identified as apoptosis (type I), autophagy (type II) and necrosis (type III) according to the cellular morphological changes, cell death pathways and the mechanism of removal of cellular residues after death (Galluzzi et al., 2018). Necrosis is an uncontrolled and passive type of cell death. Unlike necrosis, apoptosis is an active and controlled type of cell death type that progresses through morphological changes. Apoptosis has been defined as programmed cell death and is initiated and inhibited by various physiological and pathological conditions (Kerr et al., 1972). Autophagy is a self-degradative and cytoprotective process that enables mammalian cells to survive under stressful conditions. It can occur in cellular starvation, as well as with the elimination of large, damaged organelles and pathogens within the cell (Wang, 2015).

Apoptotic cell death can be induced by two different signaling pathways: extrinsic (death receptor pathway) and intrinsic (mitochondrial pathway). These signaling pathways have many common features, such as caspase activation and oligomerisation. The extrinsic pathway is activated by the death receptor of tumour necrosis factor family on the plasma membrane. This pathway has an important role in tissue homeostasis and inflammatory responses (Curtin & Cotter, 2003; Roy & Nicholson, 2000). The intrinsic pathway develops in response to some stress factors such as DNA damage, endoplasmic reticulum stress and oxidative damage. Thus, intrinsic apoptosis can

To cite this article:
Güvenç D, İnal S, Kuruca N, Palancı HS, Yavuz O, Güvenç D. Hepatocellular carcinoma cell death induced by cyfluthrin: the cellular decision between apoptosis and autophagy. Medit Vet J. 2026; 2026: 14-23.

be induced *in vitro* by toxins such as tetrachlorodibenzo-β-dioxin, various heavy metals, dithiocarbamates and bacterial toxins, without activation of death receptors (Andersen & Rathmell, 2015).

In contrast, autophagic cell death is a catabolic and cytoprotective process that has cross-talk with apoptosis and necrosis. However, autophagy can mediate cell survival or death depending on different conditions, such as the type and duration of the stimulus. Autophagy is a multi-stage process beginning with the formation of autophagosomes in which cytoplasmic macromolecules are encapsulated by two layers of membrane. The autophagosomes are then transported to the lysosome, disintegrated and used to provide cellular energy and biosynthesis. In all these autophagic stages, different autophagic proteins are involved. One of these proteins is microtubule-associated protein light chain 3-II (LC3-II). It is the autophagosomal membrane-related form of LC3 and is an important autophagy marker in autophagosome formation. Another key protein is Beclin-1, which increases autophagic activity with positive and negative cofactors (Fu et al., 2013; Gozuacik & Kimchi, 2007).

The human hepatocarcinoma cell line (HepG2) is liver-derived with strong reproducibility, high availability, high production capacity and well-defined metabolic adequacy and frequently used in *in vitro* toxicity studies (O'Brien & Edvardsson, 2017; Richter et al., 2019; Shi et al., 2018). Although there are some studies showing the hepatotoxicity of CYF, hepatotoxic cellular mechanisms have not yet been clearly elucidated. The aim of this study was to investigate the hepatotoxic effects of cyfluthrin depending on time and dose in terms of the molecular mechanisms of apoptosis and autophagy in the HepG2 cell line.

MATERIALS and METHODS

Chemical and reagents

The test substance, CYF (cyano [4-fluoro-3-phenoxyphenyl] methyl-3-[2,2-dichloroethenyl]-2,2- dimethylcyclopropanecarboxylate) was provided by Sigma-Aldrich (St. Louis, MO, USA). Eagle's Minimum Essential Medium (EMEM, M0275), L-glutamine (G7513), dimethyl sulfoxide (DMSO, D2650), non-essential amino acids (M7145) and trypsin (59418c) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Penicillin/streptomycin (15140122) and fetal bovine serum (FBS) (1050064) were obtained from Gibco (Waltham, MA, USA).

Cell culture and treatments

HepG2 cells were purchased from the American Type Culture Collection (ATCC, HB-8065, Manassas, VA, USA) and seeded into 25 cm^2 flasks supplemented with EMEM containing 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin and 1% non-essential amino acids and then incubated at 37 °C with 5% CO_2. They were separately passaged with trypsinization (trypsin in phosphate-buffered saline [PBS]) every 3–4 days. For assays, cells were seeded in 96-well plates and 6-well plates at a density of 3×10^4 or 3×10^5 cells/well, respectively. Additionally, each plate contained blanks, control cells (no treatment) and vehicle (DMSO, 0.1%)-treated cells. All assays were performed in triplicate using HepG2 cells with low passage numbers (< 8) were used. The 0.1 M stock solution of CYF was dissolved in DMSO. The stock solution was diluted to various concentrations (10^{-6} to 10^{-2} M) with EMEM and the final DMSO concentration did not exceed 0.1% (v/v), so as not to affect cell viability. Cells were treated with three different doses of CYF (10, 50 and 100 μmol/L,) corresponding to 1/100, 1/20 and 1/10 of the determined inhibitory concentration IC_{50} and at two different time intervals (24 and 48 h).

Cytotoxicity (WST-1 assay)

The IC_{50} of CYF for HepG2 cells was determined by using a colorimetric assay in 96-well plates with 2–(4-iodophenyl)-3–(4-nitrophenyl)-5–(2,4-disulfophenyl)-2H-tetrazolium monosodium salt reagent (WST-1/Roche Diagnostics GmbH, Mannheim, Germany). After 24 h of culture, different concentrations (between 1 μM and 10 mM) of CYF were added under the same experimental conditions and the cells were cultured for 24 h. Ten microliters of WST-1 reactant were then added to each well and incubated for 30 min. Cell viability was measured at 450 nm in a microplate reader spectrophotometer (Tecan software, Magellan, Switzerland). The treatments were performed in triplicate and all experiments were repeated three times. Probit analysis was performed with the SPSS v21 program.

Analysis of apoptosis and autophagy using flow cytometry

The numbers of apoptotic cells were determined according to the manufacturer's instructions for the Muse Annexin V & Dead Cell Kit (Millipore, Billerica, MA, USA) Briefly, after treatment with CYF, HepG2 cells were trypsinised and the Muse Cell Dispersal Reagent was added to separate the cells. One hundred microliters of Muse Annexin V & Dead Cell Reagent were added to each tube. The samples were then placed in darkness at room temperature for 20 min for staining and then analysed with the Muse cell analyser.

Muse Cell Analyzer and Muse Autophagy LC3-antibody-based Kit (Millipore, Billerica, MA, USA) were used to determine LC3 accumulation levels. After treatment with CYF on HepG2 cells, the cells were harvested and washed twice with cold PBS (pH 7.2) and were treated with the Muse Autophagy LC3-antibody-based Kit according to the manufacturer's instructions. Autophagic cells were determined by using the Muse Cell Analyzer. All experiments were repeated three times in triplicate.

Gene expression associated with apoptosis and autophagy by real-time PCR (RT-PCR)

Briefly, HepG2 cells were cultured in 6-well plates for 24 h before treatment. After attachment, cells were treated with CYF concentrations of 10, 50 and 100 μmol/L, for 24 and 48 h. After being treated with CYF for 24 h and 48 h, the total RNA was extracted using the Triazole method according to the manufacturer's instructions. All RNA samples were reverse transcribed individually using a reverse transcription kit according to the manufacturer's instructions (iScript cDNA Synthesis kit, Bio-Rad, USA). Quantitative RT-PCR

was performed with the LightCycler 1.5 instrument (Roche Diagnostics, Mannheim, Germany) using LightCyclerTagMan Master Mix and Real Time Ready single assays primer-probe sets (04735536001, Roche Diagnostics, Mannheim, Germany). The thermal cycler protocol was as follows: 95 °C for 10 min and 40 cycles of 95 °C for 10 min, 72 °C 1 s and 40 °C for 30 s. The study was performed in three replicates. The β-actin gene was used as the control gene. Delta Ct (ΔCt) and $2^{-\Delta\Delta Ct}$ values were calculated and used for the statistical analysis.

Western blot analysis

Total protein was extracted with radioimmunoprecipitation buffer containing protease inhibitors at 4 °C for 30 min. Protein quantity was assayed with Bradford method protein assay kit (Amresco, LLC, Solon, OH, USA). Equal amounts of extracted proteins (30 µg) were loaded in 8% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, separated using electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Massachusetts; USA). This was followed with blocking 1 h at 4 °C in Tris-buffered saline-Tween 20 (TBST) containing 5 % non-fat powdered milk. After a brief rinse, the membranes were incubated with caspase 3 (bs-0081R, Bioss, Woburn, MA, USA 1:1,000), Bax (bs-0127R, Bioss, Woburn, MA, USA 1:1,000), Bcl-2 (bs-4563R, Bioss, Woburn, MA, USA 1:1,000) and β-actin (MAB1501, Millipore, 1:1,000) primary antibodies in TBST at 4 °C overnight. Horseradish peroxidase-conjugated goat anti-rabbit IgG (A9169, Sigma-Aldrich St. Louis, MO, USA, 1:5,000) and rabbit anti-mouse IgG (A9044, Sigma-Aldrich St. Louis, MO, USA, 1:5,000) were used as the secondary antibody. After incubation with seconder antibody, the protein bands were visualised using ECL western blotting detection reagent (Amersham, Buckinghamshire, U.K.) and gel documentation system (Gel Doc XR. Bio-Rad). The band intensity was measured with Image J software, and the expression of proteins normalised to the loading control (β-actin) was calculated.

Immunofluorescence staining

After the CYF treatment period, the medium was removed, and the cells were fixed with absolute ethanol and were blocked in protein blocking solution to avoid non-specific antigen-antibody reactions. The cells were then incubated with primary antibodies [Bcl-2 (bs-4563R Bioss, Woburn, MA, USA,1:100), Bax (bs-0127R, Bioss, Woburn, MA, USA 1:100), active caspase 3 (bs-0081R, Bioss, Woburn, MA, USA 1:100), Beclin-1 (bs-1353R, Bioss, Woburn, MA, USA 1:100) and LC3 (bs-8878R, Bioss, Woburn, MA, USA 1:100)] at the indicated dilution rates and periods according to their primary antibodies data pages. Secondary antibodies labelled with fluorescein isothiocyanate (FITC, F-7512, Sigma-Aldrich, St. Louis, MO, USA) or tetramethylrhodamine (TRITC, T-6778, Sigma-Aldrich, St. Louis, MO, USA) was applied to the cells. After incubating for 30 min, nuclear staining was performed with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, D9542, Sigma-Aldrich, St. Louis, MO, USA). The cells were covered with anti-fade adhesive and then they were examined with a fluorescent microscope and micro photos were taken.

Statistical analysis

Statistical analysis was performed using SPSS v21.0 Programme. The statistical data were expressed as mean ± standard error (mean ± SEM). In each time trial, the one-way analysis of variance (ANOVA) test was used for parametric data, and the Kruskal–Wallis test was used for non-parametric data, to determine differences between groups. The time-dependent changes of each parameter and group were determined using the paired samples t-test for parametric data and the Mann–Whitney U-Test for non-parametric data. The degree of the cross-group difference was considered significant when p was <0.05. IC_{50} value was calculated using probit analysis.

RESULTS

Cell viability/cytotoxicity test

The WST-1 test showed that CYF reduced the viability of HepG2 cells in a dose-dependent manner. The IC_{50} of CYF determined by probit analysis was 997.049 µmol/L (Fig. 1).

Analysis of apoptosis and autophagy using flow cytometry

A dose-dependent induction of apoptosis was observed at 24 and 48 h (Fig. 2. and Fig. 3). All treatment groups showed statistically significant changes compared to the control in

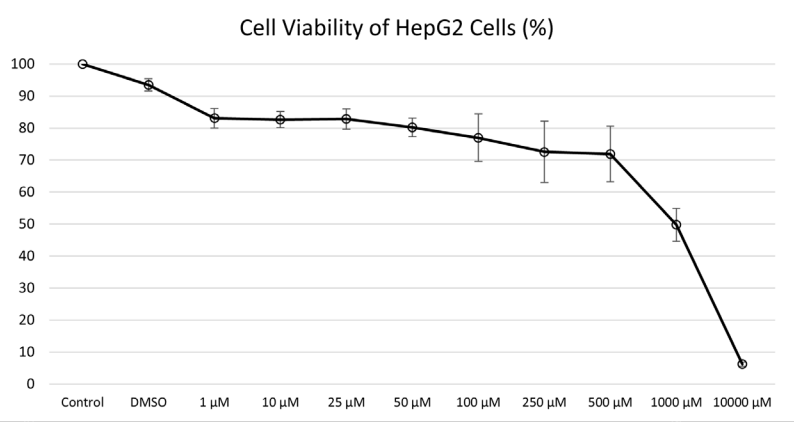

Figure 1. Cell viability of HepG2 cells exposed to Cyfluthrin concentrations ranging from 1 µM to 10000 µM for 24 hours. The data are presented as the means and standard errors.

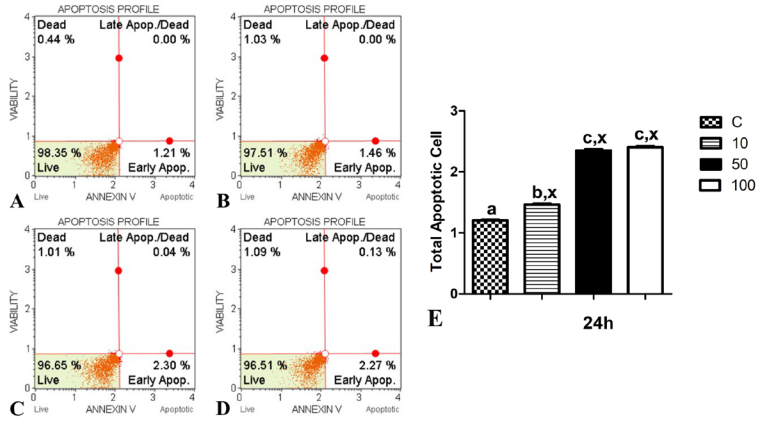

Figure 2. Cell viability of HepG2 cells exposed to Cyfluthrin concentrations ranging from 1 µM to 10000 µM for 24 hours. The data are presented as the means and standard errors.

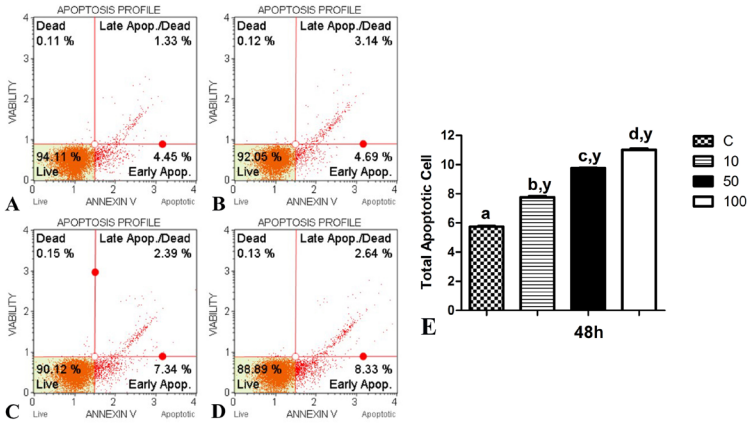

Figure 3. Total apoptotic cell percentages determined by flow cytometry in a dose-dependent manner in CYF-administered HepG2 cells for 48 h. A) Control B) 10 µmol/L CYF-administered HepG2 cells C) 50 µmol/L CYF-administered HepG2 cells D) 100 µmol/L CYF-administered HepG2 cells. E) Data of total apoptotic cell count determined by flow cytometry in a dose-dependent manner in CYF-administered HepG2 cells at 48th.

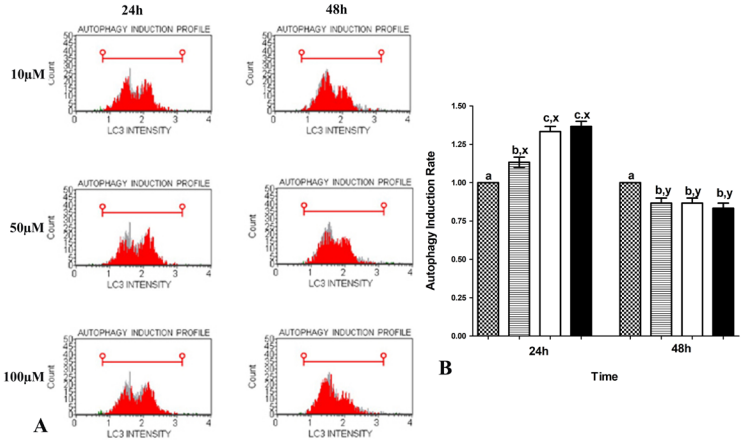

Figure 4. A) Autophagy induction determined by flow cytometry in a time and dose-dependent manner in CYF-induced HepG2 cells B) Autophagy induction rate determined by flow cytometry in a time and dose-dependent manner in CYF-induced HepG2 cells.

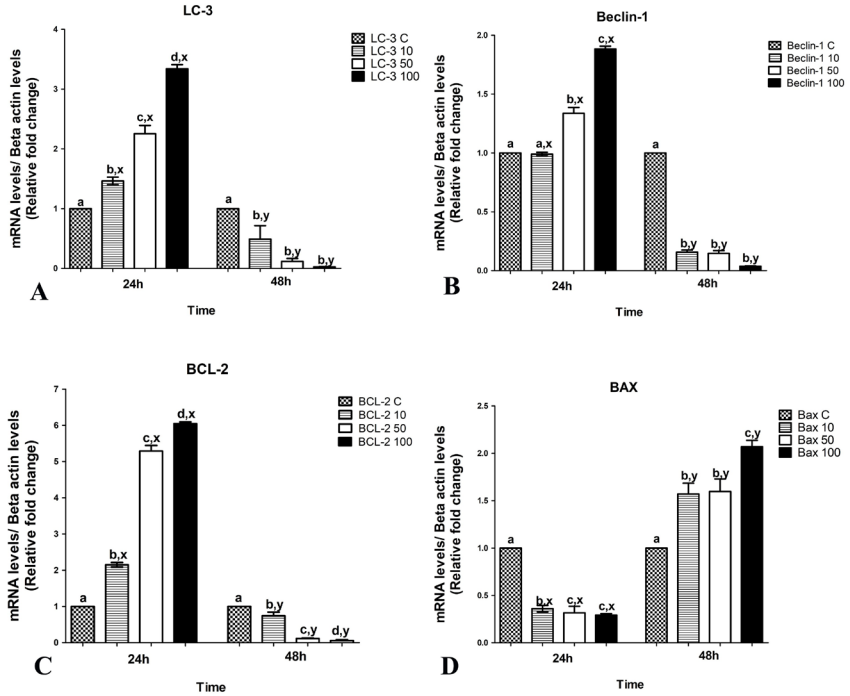

Figure 5. A) LC3 B) Beclin-1 C) BCL-2 D) Bax expression levels determined by PCR of CYF-administered HepG2 cells dose and time-dependent.
a,b,c The difference between the groups in the same time frame is statistically significant ($p<0.05$). x,y Time-dependent change is statistically significant ($p<0.05$).

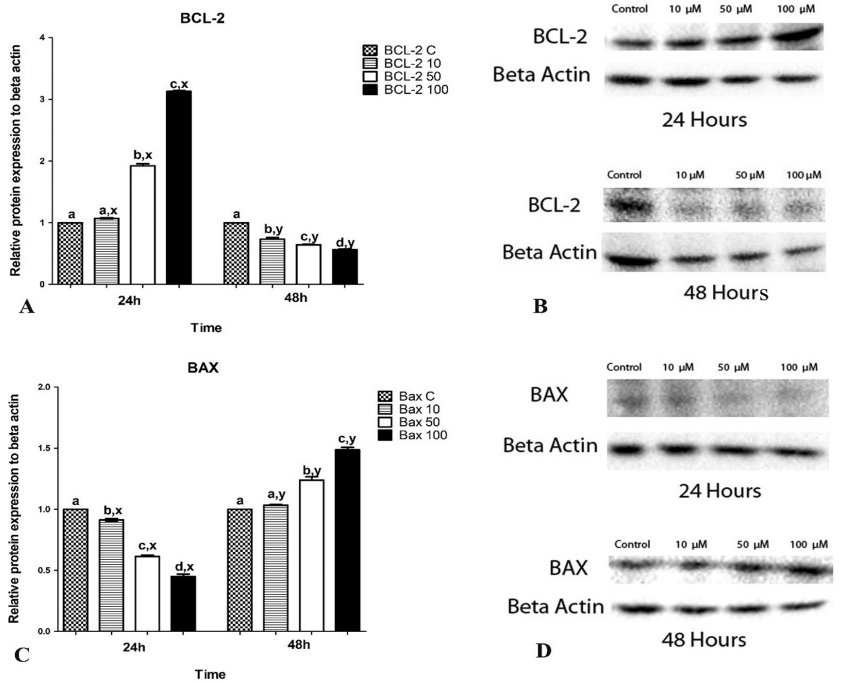

Figure 6. A) Bcl-2 expression levels determined by Western Blot in a time- and dose-dependent manner in CYF-administered HepG2 cells B) Bands of Bcl-2 protein determined by Western Blot in a time and dose-dependent manner in CYF-administered HepG2 cells C) Bax expression levels determined by Western Blot in a time- and dose-dependent manner in CYF-administered HepG2 cells D) Bands of Bax protein determined by Western Blot in a time and dose-dependent manner in Cyfluthrin-administered HepG2 cells.
a,b,c The difference between the groups in the same time frame is statistically significant ($p<0.05$). x,y Time-dependent change is statistically significant ($p<0.05$).

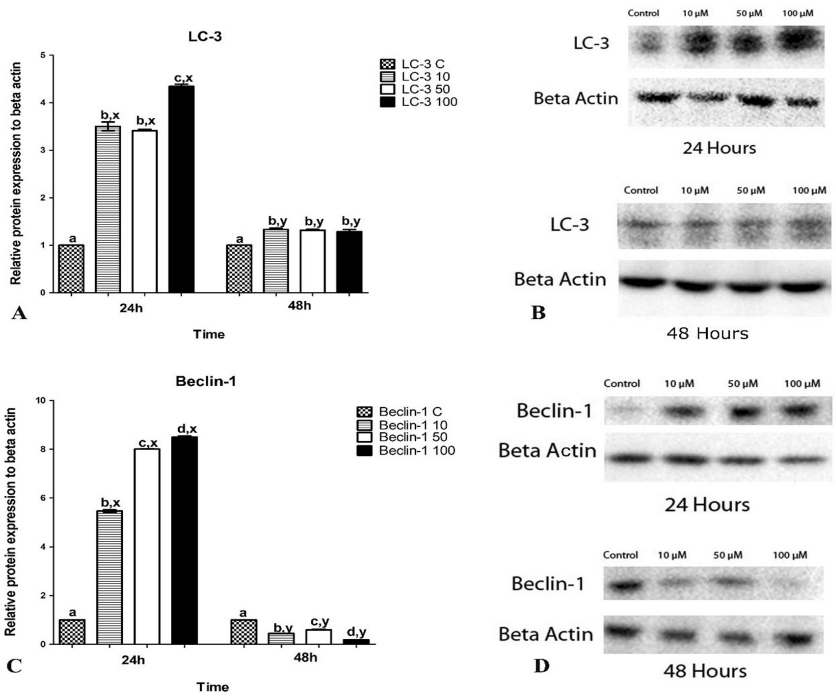

Figure 7. A) LC-3 expression levels determined by Western Blot in a timeand dose-dependent manner in CYF-administered HepG2 cells B) Bands of LC-3 protein determined by Western Blot in a time and dose-dependent manner in CYF-administered HepG2 cells C) Beclin-1 expression levels determined by Western Blot in a time- and dose-dependent manner in CYF-administered HepG2 cells D) Bands of Beclin-1 protein determined by Western Blot in a time and dose-dependent manner in CYF-administered HepG2 cells.
a,b,c The difference between the groups in the same time frame is statistically significant ($p<0.05$). x,y Time-dependent change is statistically significant ($p<0.05$).

both treatment time periods ($p<0.05$), except between the treatment groups of 50 and 100 µmol/L, at 24 h ($p>0.05$). Although autophagy was dose-dependently induced at 24 h in the treatment groups, its rate was below the basal level at 48 h. There was a statistically significant difference between the control group and all treatment groups in both time periods ($p<0.05$) (Fig. 4).

Gene expression associated with apoptosis and autophagy by real-time PCR (RT-PCR)

Although a statistically significant dose-dependent increase was detected in Bcl-2 and LC3 mRNA levels after 24 h treatment with CYF compared to the control, a decrease in the Bax mRNa level was observed ($p<0.05$). There was no change in the expression of Beclin-1 at the concentration of 10 µM ($p>0.05$), but a statistically significant increase was noted in the other concentrations ($p<0.05$). Whereas an increase in Bax expression was detected at all doses compared to the control ($p<0.05$), Bcl-2, LC3 and Beclin-1 expressions were decreased in 48 h ($p<0.05$). The fold change data of the genes is summarized in Fig. 5.

Western blot analysis

In CYF-treated (10, 50 and 100 µmol/L,) HepG2 cells, BCL-2, Bax, LC3 and Beclin-1 protein levels were determined with western blotting after 24 h and 48 h (Fig. 6 and Fig. 7). Bcl-2 protein levels were increased at 24 h compared to that in the control group. A statistically significant difference was determined by the for 50 µmol/L, and 100 µmol/L concentrations ($p<0.05$). However, differences were not detected with 10 µmol/L concentration ($p>0.05$). Compared with that in the control group, LC3 and Bcl-2 expression levels were determined to be significantly increased at 24 h in the treatment groups in a dose-dependent manner ($p<0.05$). In all treatment groups, a statistically significant decrease was detected in Bax expression at 24 h compared to the control group ($p<0.05$). At 48 h, statistically significant decreases were observed all treatment groups compare to control group for Bcl-2 and Beclin-1 protein levels. Although LC3 expression decreased in all treatment groups at 48 h when compared to 24 h, it was still higher than the control group. A significant increase in Bax expression was determined at 50 and 100 µmol/L groups in 48 h compared to the control ($p<0.05$).

Immunofluorescence staining

The effects of CYF on cell death were evaluated with an immunofluorescence assay using Bcl-2, Bax, LC3 and Beclin-1 antibodies. As shown with fluorescence staining, an increase in Bcl-2 expression occurred after 24 h of exposure, whereas a decrease in Bax expression occurred In opposition to these findings, at 48 h Bcl-2 expression decreased, whereas Bax expression increased. Despite the increase in LC3 expression at 24 h, Bax expression decreased. Although the LC3 expression decreased at 48 h, it is noteworthy that there was an increase

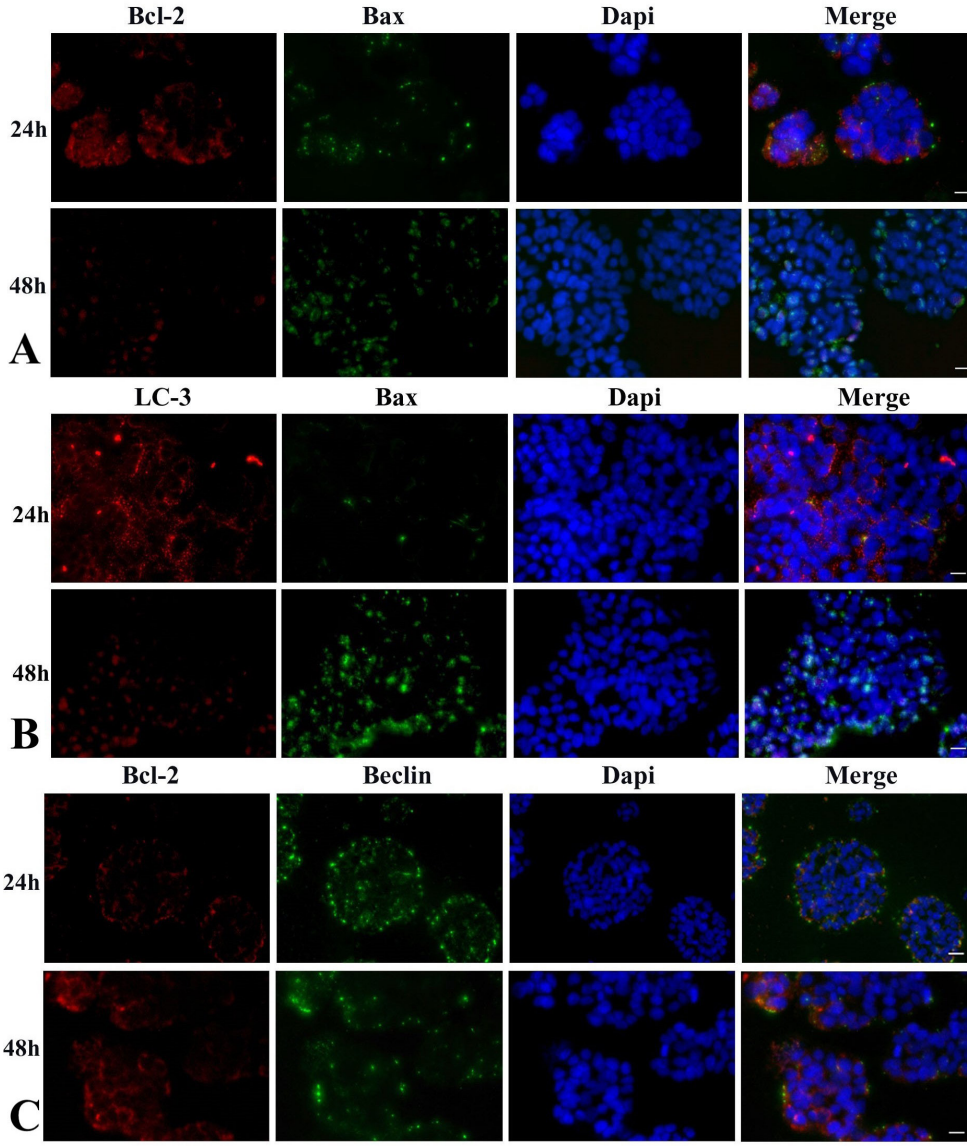

Figure 8. A) Images of Bcl-2 and Bax protein expressions in HepG2 cells at 24 h and 48 h with fluorescence microscopy. B) Images of LC-3 and Bax protein expressions in HepG2 cells at 24 h and 48 h with fluorescence microscopy C) Images of Bcl-2 and Beclin-1 protein expressions in HepG2 cells at 24 h and 48 h with fluorescence microscopy. Bar: 30µm.

in Bax expression. While an increase was observed in both LC3 and Beclin-1 expression at 24 h, very weak staining was detected at 48 h. Similarly, an increase in Bcl-2 and Beclin-1 immunofluorescence reaction was observed at 24 h. (Fig. 8).

DISCUSSION

The liver, which is involved in the detoxification of xenobiotics and related metabolites, has a high level of oxidative metabolism. This feature makes the organ vulnerable to damage from toxic substances, such as pesticides (Manfo et al., 2020). The widespread use of pyrethroids has led to the investigation of their toxic effects on human health. Accordingly, the present study investigated apoptosis and autophagy- mediated cytotoxic mechanisms in HepG2 liver cells following 24 h and 48 h of CYF exposure.

Pyrethrins and synthetic pyrethroids have a toxic effect on the nervous system of insects (Casida & Quistad, 1992). Natural pyrethrins have been reported to inhibit the viability of HepG2 cells in both a dose- and time-dependent manner (Yang et al., 2017). In one study, natural pyrethrins caused more cytotoxic and potentially genotoxic effects than beta-cypermethrin in human hepatocyte QSG7701 cells (Yang, Liu, et al., 2018). Our results demonstrate that CYF significantly reduces HepG2 cell viability in a dose-dependent manner, with an IC_{50} value of 997.049 µmol/L. Our previous study determined that the LC value of permethrin, another pyrethroid insecticide, is 1.11 mM, similar to the results of the current study (Guvenc et al., 2020). Furthermore, the cytotoxic effect of CYF in different cell lines has also been investigated. In a study evaluating the induction of oxidative stress in human neuroblastoma SH-SY5Y cells in response to CYF exposure, the IC_{50} value was calculated as 19.39 ± 3.44 µM (Martínez et al., 2019). Collectively, these data indicate that CYF exerts greater cytotoxicity in SH-SY5Y cells than in HepG2 cells.

Necrosis, apoptosis and autophagy are essential mechanisms of cell death (Edinger & Thompson, 2004). Apoptosis takes an active role in the regulation of biological processes and occurs both under physiological conditions and in pathological process such as toxicities and diseases (Corcoran et al., 1994). Previous studies suggest that synthetic pyrethroids induce apoptosis in different organs, such as liver, kidney and brain (Allam et al., 2022; Guvenc et al., 2014). In another study performed using flow cytometry, natural pyrethrins were shown to decrease HepG2 cell viability by inducing apoptosis and significantly increasing the rate of apoptotic in a dose-dependent manner for 24 h (Yang et al., 2017). In our flow cytometry study, the results showed increased induction of apoptosis in all treatment groups at 24 h, but this induction was more marked at 48 h.

In the pathway of mitochondrial-mediated apoptosis, cytochrome c is released from the intermembrane space into the cytosol, forming a cytosolic apoptosome complex with apoptosis activating factor-1 (Apaf-1) and procaspase-9. This results in activation of procaspase-9, which triggers the caspase cascade with activation of procaspase-3 (Orrenius et al., 2011). Bax and Bak proteins are proapoptotic proteins in the Bcl-2 family. They act by forming pores in the mitochondrial outer membrane and causing the release of cytochrome c into the cytoplasm. Bcl-2, an intracellular protein, exerts an anti-apoptotic effect by inhibiting cytochrome c release (Jiang & Wang, 2004). In a previous study, the level of Bax/Bcl-2 was upregulated at 6 h after natural pyrethrin treatment of HepG2 cells in a dose-dependent manner (Yang et al., 2017). Bcl-2 mRNA and protein levels increased at different concentrations and Bax expression dose-dependently decreased at 24 h in our study. Furthermore, Bcl-2 expression decreased, whereas Bax expression increased at 48 h. Moreover, according to the immunofluorescence staining results, the increase in Bax and the decrease in Bcl-2 levels indicate that apoptosis was induced in the HepG2 cell line within 48 h. This finding reveals that unlike natural pyrethrins, CYF induces apoptosis at 48 h.

Autophagy is a control mechanism that ensures the breakdown and recycling of many damaged organelles, lipids and proteins. The LC3 marker is often used to determine autophagosome formation (Kabeya et al., 2000). Also, Beclin-1 is a protein involved in autophagic activity (Fu et al., 2013). Studies on liver toxicity have reported either apoptosis or autophagy in the liver after exposure to toxic substances. When pigeons were treated with the antiparasitic drug ivermectin, expression of the autophagy markers LC3, Beclin-1 and autophagy related 5 (ATG5) increased in the liver (Wang et al., 2017). In addition, it has been determined that lindane, an organochloride pesticide, negatively affected the autophagic process in rat hepatocytes, inhibited apoptosis and caused necrotic cell death (Zucchini-Pascal et al., 2009). However, paclobutrazol is a fungicide induces apoptosis and impairs autophagy through reducing protein and mRNA levels of ATG5 and BECN1 and the conversion of LC3-I to LC3-II in HepaRG cells (Luo et al., 2021). In our study, it was observed that CYF exposure promoted autophagy in HepG2 cells at 24 h. However, it was also observed that autophagic activity decreased to basal levels at 48 h. LC3 mRNA and protein expression increased at 24 h. However, CYF induced the expression of Beclin-1 at 24 h (except the 10 μmol/L concentration) and LC3 and Beclin-1 expressions decreased at 48 h. As a result of a 6-h treatment of HepG2 cells with natural pyrethrins, autophagosome structures were observed using a confocal microscope. It was determined that the levels of the autophagy-associated proteins LC3 and Beclin-1 increased in a dose-dependent manner (Yang, Gao, et al., 2018). In contrast, the induction of autophagy was observed at 24 h in our study. This pattern suggests that autophagy may initially function as a cellular survival mechanism in the early time periods of CYF toxicity, rather than a cell death mechanism.

Pesticide studies on different cell lines have shown that apoptosis may occur with autophagy. The toxicity caused by chlorpyrifos and fipronil in SH-SY5Y cells was demonstrated by the increase in caspase 3, LC3-II and p62, in which apoptosis and autophagy were observed together (Park, Lee, Lee, et al., 2013; Park, Lee, Shin, et al., 2013). In PC12 cells exposed to deltamethrin, LC3 activation was observed at 12 h, whereas caspase-3 activation was observed at 24 h (Park et al., 2017). Consistent with these observations, our results indicate that autophagy is activated prior to apoptotic cell death in CYF-treated HepG2 cells.

CONCLUSION

Our study revealed that CYF causes autophagy and apoptosis, which are the main cell death mechanisms, resulting in dose and time-dependent hepatotoxicity in HepG2 cells. The effect of CYF on hepatocytes, inducing autophagy at 24 h and apoptotic effects at 48 h, suggests that autophagy is a stress response that ensures the survival of the cell before apoptosis. Further studies should focus on revealing more details concerning the mechanisms of autophagy and apoptosis in CYF toxicity pathways in the liver.

DECLARATIONS

Ethics Approval

This study did not require ethical approval since established commercially available cell lines were used in all experiments.

Conflict of Interest

The authors declare no conflict of interest.

Consent for Publication

Not applicable.

Author Contribution

Idea, concept and design: DG, OY, TG

Data collection and analysis: DG, Sİ, NK, HSP, OY, TG

Drafting of the manuscript: DG, Sİ, NK, TG

Critical review: DG, TG

Data Availability

The datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.

Acknowledgements

This study was supported by Ondokuz Mayis University Research Foundation (Project No PYO.VET.1901.16.013).

REFERENCES

Allam, A., Abdeen, A., Devkota, H. P., Ibrahim, S. S., Youssef, G., Soliman, A., Abdel-Daim, M. M., Alzahrani, K. J., Shoghy, K., & Ibrahim, S. F. (2022). N-Acetylcysteine Alleviated the Deltamethrin-Induced Oxidative Cascade and Apoptosis in Liver and Kidney Tissues. *International Journal of Environmental Research and Public Health*, *19*(2), 638–650. https://doi.org/https://doi.org/10.3390/ijerph19020638

Anadón, A., Martínez-Larrañaga, M., & Martínez, M. (2009). Use and abuse of pyrethrins and synthetic pyrethroids in veterinary medicine. *The Veterinary Journal*, *182*(1), 7–20. https://doi.org/https://doi.org/10.1016/j.tvjl.2008.04.008

Andersen, J. L., & Rathmell, J. C. (2015). Cell Death Pathways in Toxicological Response. In M. B. Abou-Donia (Ed.), *Mammalian Toxicology* (1st ed., pp. 75–83). Jhon Wiley & Sons. https://doi.org/https://doi.org/10.1002/9781118683484.ch4

Casida, J. E., & Quistad, G. B. (1992, August). Pyrethrum flowers: production, chemistry, toxicology, and uses. International Symposium on Pyrethrum Flowers: Production, Chemistry, Toxicology and Uses, Honolulu, Hawaii (USA).

Corcoran, G. B., Fix, L., Jones, D. P., Moslen, M. T., Nicotera, P., Oberhammer, F. A., & Buttyan, R. (1994). Apoptosis: molecular control point in toxicity. *Toxicology and applied pharmacology*, *128*(2), 169–181. https://doi.org/https://doi.org/10.1006/taap.1994.1195

Curtin, J. F., & Cotter, T. G. (2003). Live and let die: regulatory mechanisms in Fas-mediated apoptosis. *Cellular Signalling*, *15*(11), 983–992. https://doi.org/https://doi.org/10.1016/S0898-6568(03)00093-7

Ding, G., Cui, C., Chen, L., Gao, Y., Zhou, Y., Shi, R., & Tian, Y. (2015). Prenatal exposure to pyrethroid insecticides and birth outcomes in Rural Northern China. *Journal of Exposure Science and Environmental Epidemiology*, *25*(3), 264. https://doi.org/https://doi.org/10.1038/jes.2014.86

Edinger, A. L., & Thompson, C. B. (2004). Death by design: apoptosis, necrosis and autophagy. *Current Opinion in Cell Biology*, *16*(6), 663–669. https://doi.org/https://doi.org/10.1016/j.ceb.2004.09.011

Fu, L.-l., Cheng, Y., & Liu, B. (2013). Beclin-1: autophagic regulator and therapeutic target in cancer. *International Journal of Biochemistry & Cell Biology*, *45*(5), 921–924. https://doi.org/https://doi.org/10.1016/j.biocel.2013.02.007

Galluzzi, L., Vitale, I., Aaronson, S. A., Abrams, J. M., Adam, D., Agostinis, P., Alnemri, E. S., Altucci, L., Amelio, I., & Andrews, D. W. (2018). Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death & Differentiation*, 1. https://doi.org/https://doi.org/10.1038/s41418-017-0012-4

Gozuacik, D., & Kimchi, A. (2007). Autophagy and cell death. *Current Topics in Developmental Biology*, *78*, 217–245. https://doi.org/https://doi.org/10.1016/S0070-2153(06)78006-1

Guvenc, D., Aksoy, A., Gacar, A., Atmaca, E., Das, K. Y., & Guvenc, T. (2014). Evaluation of changes in monoamine levels and apoptosis induced by cyfluthrin in rats. *Toxicology Research*, *3*(5), 331–340. https://doi.org/https://doi.org/10.1039/c4tx00041b

Guvenc, D., Gulbahar, M. Y., Yavuz, O., & Guvenc, T. (2020). Dose-and Time-dependent Effects of Permethrin on HepG2 Cells: Cell Survival, Lipid Peroxidation and Antioxidant Defence System. *Kafkas Universitesi Veteriner Fakultesi Dergisi*, *26*(2), 263–268. https://doi.org/https://doi.org/10.9775/kvfd.2019.22925

Heudorf, U., & Angerer, J. (2001). Metabolites of pyrethroid insecticides in urine specimens: current exposure in an urban population in Germany. *Environmental Health Perspectives*, *109*(3), 213–217. https://doi.org/https://doi.org/10.1289/ehp.01109213

Jiang, X., & Wang, X. (2004). Cytochrome C-mediated apoptosis. *Annual Review of Biochemistry*, *73*, 87–106. https://doi.org/https://doi.org/10.1146/annurev.biochem.73.011303.073706

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., & Yoshimori, T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *The EMBO journal*, *19*(21), 5720–5728.

Kerr, J. F., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wideranging implications in tissue kinetics. *British Journal of Cancer*, *26*(4), 239–257. https://doi.org/https://doi.org/10.1038/bjc.1972.33.

Leng, G., Kühn, K.-H., & Idel, H. (1997). Biological monitoring of pyrethroids in blood and pyrethroid metabolites in urine: applications and limitations. *Science of the Total Environment*, *199*(1-2), 173–181. https://doi.org/https://doi.org/10.1016/S0048-9697(97)05493-4

Leng, G., Leng, A., Kuhn, K.-H., & Lewalter, J. (1997). Human dose-excretion studies with the pyrethroid insecticide cyfluthrin: urinary metabolite profile following inhalation. *Xenobiotica*, *27*(12), 1273–1283. https://doi.org/https://doi.org/10.1080/004982597239859

Luo, Y., Lu, S., Sun, X., Gao, Y., Sun, G., Yang, M., & Sun, X. (2021). Paclobutrazol exposure induces apoptosis and impairs autophagy in hepatocytes via the AMPK/mTOR signaling pathway. *Journal of biochemical and molecular toxicology*, *35*(10), 1–9. https://doi.org/https://doi.org/10.1002/jbt.22874

Manfo, F. P. T., Mboe, S. A., Nantia, E. A., Ngoula, F., Telefo, P. B., Moundipa, P. F., & Cho-Ngwa, F. (2020). Evaluation of the effects of agro pesticides use on liver and kidney function in farmers from Buea, Cameroon. *Journal of toxicology*, *2020*, 1–10. https://doi.org/https://doi.org/10.1155/2020/2305764

Martínez, M.-A., Rodríguez, J.-L., Lopez-Torres, B., Martí-

nez, M., Martínez-Larrañaga, M.-R., Anadón, A., & Ares, I. (2019). Oxidative stress and related gene expression effects of cyfluthrin in human neuroblastoma SH-SY5Y cells: Protective effect of melatonin. *Environmental Research*, *177*, 1–14. https://doi.org/https://doi.org/10.1016/j.envres.2019.108579

O'Brien, P. J., & Edvardsson, A. (2017). Validation of a multiparametric, high-content-screening assay for predictive/investigative cytotoxicity: evidence from technology transfer studies and literature review. *Chemical Research in Toxicology*, *30*(3), 804–829. https://doi.org/ttps://doi.org/10.1021/acs.chemrestox.6b00403

Orrenius, S., Nicotera, P., & Zhivotovsky, B. (2011). Cell death mechanisms and their implications in toxicology. *Toxicological Sciences*, *119*(1), 3–19. https://doi.org/https://doi.org/10.1093/toxsci/kfq268

Park, J. H., Lee, J. E., Lee, S.-J., Park, S. J., Park, K. H., Jeong, M., & Koh, H. C. (2013). Potential autophagy enhancers protect against fipronil-induced apoptosis in SH-SY5Y cells. *Toxicology letters*, *223*(1), 25–34. https://doi.org/https://doi.org/10.1016/j.toxlet.2013.08.015

Park, J. H., Lee, J. E., Shin, I. C., & Koh, H. C. (2013). Autophagy regulates chlorpyrifos-induced apoptosis in SH-SY5Y cells. *Toxicology and applied pharmacology*, *268*(1), 55–67. https://doi.org/https://doi.org/10.1016/j.taap.2013.01.013

Park, Y. S., Park, J. H., Ko, J., Shin, I. C., & Koh, H. C. (2017). m TOR inhibition by rapamycin protects against deltamethrin-induced apoptosis in PC 12 Cells. *Environmental Toxicology*, *32*(1), 109–121. https://doi.org/https://doi.org/10.1002/tox.22216

Quindroit, P., Beaudouin, R., & Brochot, C. (2019). Estimating the cumulative human exposures to pyrethroids by combined multi-route PBPK models: Application to the French population. *Toxicology letters*, *312*, 125–138. https://doi.org/https://doi.org/10.1016/j.toxlet.2019.05.007

Richter, L. H., Beck, A., Flockerzi, V., Maurer, H. H., & Meyer, M. R. (2019). Cytotoxicity of new psychoactive substances and other drugs of abuse studied in human HepG2 cells using an adopted high content screening assay. *Toxicology letters*, *301*, 79–89. https://doi.org/https://doi.org/10.1016/j.toxlet.2018.11.007

Rousis, N. I., Zuccato, E., & Castiglioni, S. (2017). Wastewater-based epidemiology to assess human exposure to pyrethroid pesticides. *Environment International*, *99*, 213–220. https://doi.org/https://doi.org/10.1016/j.envint.2016.11.020

Roy, S., & Nicholson, D. W. (2000). Cross-talk in cell death signaling. *Journal of Experimental Medicine*, *192*(8), F21–F26. https://doi.org/https://doi.org/10.1084/jem.192.8.F21

Shi, J., Wang, X., Lyu, L., Jiang, H., & Zhu, H.-J. (2018). Comparison of protein expression between human livers and the hepatic cell lines HepG2, Hep3B, and Huh7 using SWATH and MRM-HR proteomics: Focusing on drug-metabolizing enzymes. *Drug Metabolism and Pharmacokinetics*, *33*(2), 133–140. https://doi.org/https://doi.org/10.1016/j.dmpk.2018.03.003

Steckling, N., Gotti, A., Bose-O'Reilly, S., Chapizanis, D., Costopoulou, D., De Vocht, F., Garí, M., Grimalt, J. O., Heath, E., & Hiscock, R. (2018). Biomarkers of exposure in environment-wide association studies–Opportunities to decode the exposome using human biomonitoring data. *Environmental Research*, *164*, 597–624. https://doi.org/https://doi.org/10.1016/j.envres.2018.02.041

Wang, K. (2015). Autophagy and apoptosis in liver injury. *Cell cycle*, *14*(11), 1631–1642. https://doi.org/https://doi.org/10.1080/15384101.2015.1038685

Wang, X.-S., Liu, C., Khoso, P., Zheng, W., Li, M., & Li, S. (2017). Autophagy response in the liver of pigeon exposed to avermectin. *Environmental Science and Pollution Research*, *24*(14), 12767–12777. https://doi.org/10.1007/s11356-016-6209-0

Whyatt, R. M., Camann, D. E., Kinney, P. L., Reyes, A., Ramirez, J., Dietrich, J., Diaz, D., Holmes, D., & Perera, F. P. (2002). Residential pesticide use during pregnancy among a cohort of urban minority women. *Environmental Health Perspectives*, *110*(5), 507–514. https://doi.org/https://doi.org/10.1289/ehp.02110507

Williams, R. L., Bernard, C. E., & Krieger, R. I. (2003). Human exposure to indoor residential cyfluthrin residues during a structured activity program. *Journal of Exposure Science and Environmental Epidemiology*, *13*(2), 112. https://doi.org/10.1038/sj.jea.7500257

Yang, Y., Gao, J., Zhang, Y., Xu, W., Hao, Y., Xu, Z., & Tao, L. (2018). Natural pyrethrins induce autophagy of HepG2 cells through the activation of AMPK/mTOR pathway. *Environmental Pollution*, *241*, 1091–1097. https://doi.org/https://doi.org/10.1016/j.envpol.2018.06.049

Yang, Y., Liu, W., Wang, J., Zhang, Y., Xu, W., & Tao, L. (2018). The different effects of natural pyrethrins and beta-cypermethrin on human hepatocyte QSG7701 cells by ROS-mediated oxidative damage. *Environmental Science and Pollution Research*, *25*(24), 24230–24240. https://doi.org/https://doi.org/10.1007/s11356-018-2503-3

Yang, Y., Zong, M., Xu, W., Zhang, Y., Wang, B., Yang, M., & Tao, L. (2017). Natural pyrethrins induces apoptosis in human hepatocyte cells via Bax-and Bcl-2-mediated mitochondrial pathway. *Chemico-Biological Interactions*, *262*, 38–45. https://doi.org/10.1016/j.cbi.2016.12.006.

Zalom, F. G. (2010). Pesticide use practices in integrated pest management. In R. Krieger (Ed.), *Hayes' Handbook of Pesticide Toxicology* (3rd ed., pp. 303–313). Elsevier. https://doi.org/https://doi.org/10.1016/B978-0-12-374367-1.00008-2

Zucchini-Pascal, N., de Sousa, G., & Rahmani, R. (2009). Lindane and cell death: at the crossroads between apoptosis, necrosis and autophagy. *Toxicology*, *256*(1-2), 32–41. https://doi.org/https://doi.org/10.1016/j.tox.2008.11.004