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Necrosis factor-alpha (TNF-α) response in human hepatoma HepG2 cells treated with hepatotoxic agents

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The liver plays an essential role in xenobiotic metabolism including alcohol and drugs. Oxidative stress that usually occurs during the hepatic metabolism participates in the pathogenesis of liver disease. Inflammatory cytokines that exist in liver in both physiological and pathophysiological conditions may change the hepatic toxic response to hepatotoxicants. The human hepatoma cell line HepG2 is frequently used as *in vitro* model for biomedical studies. In this work, HepG2 cells were pre-incubated with or without TNF-α, and then treated with ethanol, acetaldehyde, acetaminophen and *tert*-butyl hydroperoxide, respectively. Cell viability was measured by MTT assay. The data showed that HepG2 cells were generally resistant to xenobiotic compounds, especially to alcohol and acetaldehyde, which may be partially caused by the absence of specific cytochrome P450 systems in these cells. TNF-α could sensitize the toxic response of HepG2 cells to those exogenous compounds, indicating the important role of TNF-α in the pathogenesis of alcohol, drugs and oxidant related liver diseases.

1. Introduction

Alcohol is a major cause of liver disease that represents a spectrum of clinical diseases and morphological changes that range from fatty liver to alcoholic hepatitis to alcoholic cirrhosis (Walsh and Alexander 2000). Drug-related hepatotoxicity is another main cause of liver failure, acetaminophen (APAP) induced hepatic injury occurs most commonly (Thiel et al. 2011). Oxidative stress that occurs during the hepatic metabolism of these exogenous compounds is a critical factor involved in the pathogenesis of related liver injury, since metabolism of chemicals in the liver can produce free radicals or electrophilic chemicals that undergo or promote a variety of chemical reactions, such as the depletion of reduced glutathione; covalently binding to proteins, lipids, or nucleic acids; or inducing lipid peroxidation. All of these have consequent effects on hepatocyte death that is the event that leads to the clinical manifestation of liver diseases (Kaplowitz 2002). Short-term *in vitro* tests for the primary identification of the hepatotoxicity caused by these xenobiotics and their corresponding metabolites are needed.
The pro-inflammatory cytokine TNF-α exerts a variety of effects on different cell types and stimulates a number of cellular responses including cell proliferation, production of other inflammatory mediators and programmed cell death. In the liver, oxidative stress that generates during xenobiotics metabolism and as endotoxin which derives from the cell wall material of Gram-negative bacteria in the gut have been implicated in the process of TNF-α production under both physiological and pathophysiological conditions (Zhou et al. 2003; Ghose et al. 2004). Inflammatory cytokines have been shown to augment the response to hepatotoxins (Pastorino and Hoek 2000; Shaw et al. 2007), whereas protective or neutral roles of cytokines in hepatotoxicity have also been reported (Boess et al. 1998; Liu et al. 2000; Simpson et al. 2000). These studies imply a controversial role of inflammation in hepatic injury, with both increased and decreased toxicity in the presence of inflammatory mediators. Therefore, the role of pre-existing inflammation in modulation of the toxic effects of exogenous compounds needs to be further investigated.
Established cell lines can be used to determine cytotoxicity, the human hepatoma cell line HepG2 is the frequently used cell system in xenobiotic and metabolism study (Knasmüller et al. 2004). In this study we intended to evaluate the hepatotoxicity of alcohol, acetaldehyde, APAP and *tert*-butyl hydroperoxide (t-BHP) in HepG2 cells, as well as to determine the role of inflammatory mediators in these agents induced hepatotoxicity *in vitro*.

2. Investigations and results

2.1. Cytotoxicity of ethanol or/and acetaldehyde in HepG2 cells

Both ethanol and acetaldehyde showed time and dose dependent toxic effects in HepG2 cells. Incubation of 100 mM of ethanol slightly inhibited cell growth while significant reduced cell viability was observed as the concentration increased to 200 mM (Fig. 1). Acetaldehyde significant inhibited cell growth only at higher concentrations of 10 mM and 20 mM after 24 h or 48 h treatment (Fig. 2). After simultaneous treatment, ethanol induced cell death was augmented by 8–32% while the effect of acetaldehyde on cell proliferation was decreased by 2–10%.

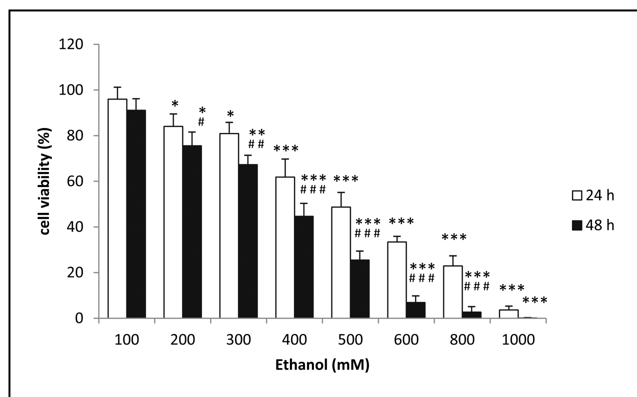

Fig. 1: Effect of ethanol on cell viability in HepG2 cells. Each value represents the mean ± SD of three independent experiments carried out in four replicates. Differences were considered significant at $^{*}P < 0.05$ *vs.* untreated HepG2 cells, $^{**}P < 0.01$ *vs.* untreated HepG2 cells, $^{***}P < 0.001$ *vs.* untreated HepG2 cells; and $^{\#}P < 0.05$ *vs.* 24 h ethanol treated group, $^{\#\#}P < 0.01$ *vs.* 24 h ethanol treated group, $^{\#\#\#}P < 0.001$ *vs.* 24 h ethanol treated group.

2.2. *Effect of TNF-α on ethanol and acetaldehyde induced cytotoxicity in HepG2 cells*

The 24 h incubation of HepG2 cells with TNF-α maintained > 90% cell viability (Table). However, TNF-α sensitized HepG2 response to ethanol and acetaldehyde, TNF-α (30 ng/mL) treatment resulted in a 6% loss of viability in ethanol exposed cells while led to 7–18% reduction of cell proliferation in acetaldehyde treated as well as acetaldehyde plus ethanol treated cells. There was also a tendency for enhanced cytotoxicity by increasing concentrations of TNF-α, with maximum effect at 30 ng/mL.

2.3. *Effect of TNF-α on APAP induced cytotoxicity in HepG2 cells*

A 24 h treatment of APAP induced a concentration dependent cytotoxic effect in HepG2 cells which was significantly increased as the concentration increased to 5 mM (Fig. 3). Pretreatment with TNF-α resulted in a moderate, though significant effect on decreased cell proliferation (10–15% decrease), whereas no significant differences were observed among cells pretreated with different doses of TNF-α.

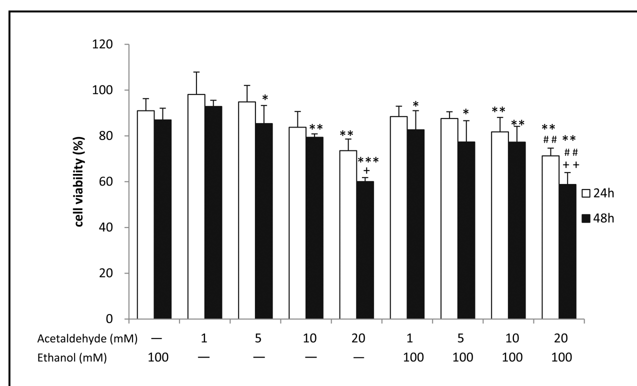

Fig. 2: Effect of combined ethanol and acetaldehyde on cell viability in HepG2 cells. Each value represents the mean ± SD of two independent experiments carried out in four replicates. Differences were considered significant at $^{*}P < 0.05$ *vs.* untreated HepG2 cells, $^{**}P < 0.01$ *vs.* untreated HepG2 cells, $^{***}P < 0.001$ *vs.* untreated HepG2 cells; and $^{\#}P < 0.05$ *vs.* corresponding ethanol treated group, $^{\#\#}P < 0.01$ *vs.* corresponding ethanol treated group; and $^{+}P < 0.05$ *vs.* corresponding 24 h treated group, $^{++}P < 0.01$ *vs.* corresponding 24 h treated group.

Table: Effect of TNF-α on ethanol and/or acetaldehyde induced cytotoxicity in HepG2 cells

EtOH (mM)		—	100	—	—	—	100	100	100
Ac (mM)		—	—	1	5	10	1	5	10
TNF-α	—	100 ± 6.2	92.8 ± 10.6	97.1 ± 11.7	94.7 ± 11.1	83.7 ± 12.7**	88.7 ± 9.1**	84.8 ± 14.1**	80.8 ± 7.7***
	10ng/mL	96.6 ± 7.2	93.3 ± 6.1	86.8 ± 4.3**,#	80.4 ± 5.5***,##	75.0 ± 8.0***	87.9 ± 12.4*	81.3 ± 21.3**	77.7 ± 19.2**
	20ng/mL	95.6 ± 4.1	87.3 ± 6.9**	87.7 ± 6.1**,#	80.8 ± 5.3**,##	72.7 ± 6.4***,#	84.7 ± 10.4**	76.7 ± 7.3**	72.4 ± 5.5***,#
	30ng/mL	95.5 ± 5.0	86.8 ± 7.0**	84.3 ± 5.7**,#	76.2 ± 5.5***,###	65.8 ± 6.8***,##	77.9 ± 3.4***,##	72.9 ± 5.3***,#	69.0 ± 3.6***,##

"EtOH": ethanol; "Ac": acetaldehyde. Each value represents the mean ± SD of two independent experiments carried out in four replicates. Differences were considered significant at $^{*}P < 0.05$ *vs.* untreated HepG2 cells, $^{**}P < 0.01$ *vs.* untreated HepG2 cells, $^{***}P < 0.001$ *vs.* untreated HepG2 cells; and $^{\#}P < 0.05$ *vs.* corresponding ethanol or/and acetaldehyde treated group, $^{\#\#}P < 0.01$ *vs.* corresponding ethanol or /and acetaldehyde group, $^{\#\#\#}P < 0.01$ vs. corresponding ethanol or/and acetaldehyde group.

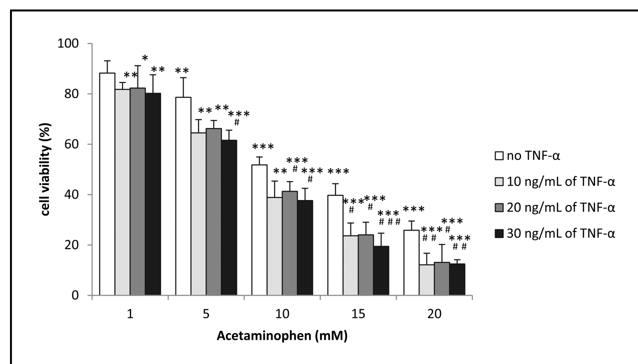

Fig. 3: Effect of TNF-α on acetaminophen induced cytotoxicity in HepG2 cells. Each value represents the mean ± SD of two independent experiments carried out in four replicates. Differences were considered significant at $^{*}P<0.05$ *vs.* untreated HepG2 cells, $^{**}P<0.01$ *vs.* untreated HepG2 cells, $^{***}P<0.001$ *vs.* untreated HepG2 cells; and $^{\#}P<0.05$ *vs.* corresponding acetaminophen treated alone group, $^{\#\#}P<0.01$ *vs.* corresponding acetaminophen treated alone group, $^{\#\#}P<0.001$ *vs.* corresponding acetaminophen treated alone group.

2.4. *Effect of TNF-α on t-BHP induced cytotoxicity in HepG2 cells*

t-BHP led to a time and dose dependent loss of cell viability, and the significant cytotoxicity was observed as the concentration of ethanol increased to 40 μM (Fig. 4). Interestingly, TNF-α pretreatment showed no effect on 3 h t-BHP exposed cells (Fig. 5A). By contrast, a significant potentiated toxic effect (10–20% potentiation) was caused by TNF-α on 24 h t-BHP exposed cells (Fig. 5B).

3. Discussion

We evaluated the cytotoxicity of ethanol in HepG2 cells, showing that cultivation of HepG2 cells with high concentration (200 mM) of ethanol produced significant loss in cell viability after 24 h treatment, suggesting that HepG2 cells are strongly resistant against the toxic effect of ethanol. This result is similar to that of a previous study showing that both acute (24 h) and chronic (7 days) ethanol (100 mM) treatments did not cause cytotoxicity in HepG2 cells (Jiménez-López 2002). In contrast, Neuman et al. (1993) reported that 60 mM of ethanol for 24 h treatment exerted a significant toxic effect on HepG2 cells, Pastorino and Hoek (2000) showed that 50 mM of ethanol for 48 h treatment caused around 20% HepG2 cell death, and Castaneda and Kinne (2000) reported that exposure to 1 mM of ethanol

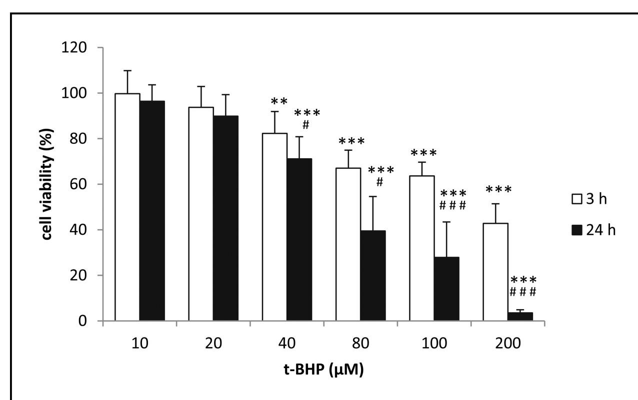

Fig. 4: Effect of t-BHP on cell viability. Each value represents the mean ± SD of three independent experiments carried out in four replicates. Differences were considered significant at $^{*}P<0.05$ *vs.* untreated HepG2 cells, $^{**}P<0.01$ *vs.* untreated HepG2 cells, $^{***}P<0.001$ *vs.* untreated HepG2 cells; and $^{\#}P<0.05$ *vs.* corresponding 3 h t-BHP treated cells, $^{\#\#}P<0.01$ *vs.* corresponding 3 h t-BHP treated cells, $^{\#\#}P<0.001$ *vs.* corresponding 3 h t-BHP treated cells.

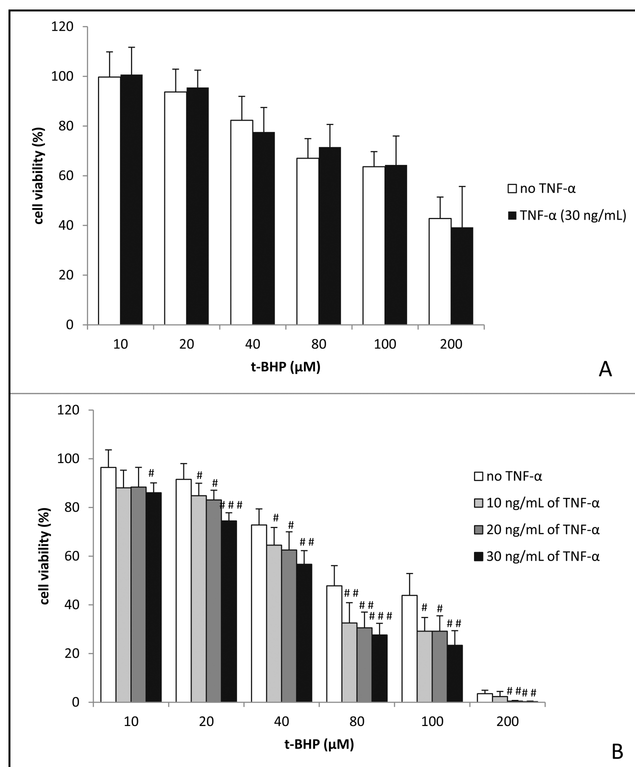

Fig. 5: Effect of TNF-α on t-BHP induced cytotoxicity in HepG2 cells. HepG2 cells were pretreated with TNF-α for 24 h and then exposed to different concentrations of t-BHP for 3 h (A) and 24 h (B), respectively. Each value represents the mean ± SD of two independent experiments carried out in four replicates. Differences were considered significant at $^{\#}P<0.05$ vs. corresponding t-BHP treated alone group, $^{\#\#}P<0.05$ vs. corresponding t-BHP treated alone group, $^{\#\#\#}P<0.05$ vs. corresponding t-BHP treated alone group.

inhibited cell proliferation in HepG2 cells by 75% after 24 h incubation and by 95% after 72 h treatment. Further investigation indicated that Fas apoptotic pathway in HepG2 cells was triggered by a low level of ethanol (Castaneda et al. 2006). Recently, long-term (9 days) ethanol (75 mM) exposure has been shown to alter the expression of around one thousand genes in HepG2 cells (Pochareddy and Edenberg 2012). Other studies demonstrated that ethanol is toxic to recombinant HepG2 cells that express cytochrome P450 2E1 (Wu and Cederbaum 1996; 1999; Wu et al. 2010) or/and alcohol dehydrogenase (Clemens et al. 2002; Donohue et al. 2006) while had no obvious effect in wild type HepG2 cells, implying the toxicity of ethanol on liver are linked to these enzyme systems involved in the oxidative metabolism of ethanol.

Acetaldehyde is a major metabolite of ethanol and is considered as a critical mediator of ethanol induced deleterious effects. A significant anti-proliferation effect of acetaldehyde was found a high concentration (10 mM) which is extremely out of the physiologically relevant range of 25–50 μM found in cirrhotic patients (Román et al. 1999) and much higher than a standard concentration of 200 μM commonly used in *in vitro* studies (Anania et al. 1999; Novitskiy et al. 2005), implying that the finding obtained with this concentration may not be of physiological significance. Although a low dose of acetaldehyde was unable to induce oxidative stress and cell death in HepG2 cells, the activation of transcription factors including activating protein 1 and nuclear factor κB has been observed (Román et al. 1999). In contrast, a lower concentration of 180 μM of acetaldehyde caused 10% HepG2 cell death after 24 h treatment (Ni et al. 2001), and 300 μM of acetaldehyde reduced 60% cell proliferation after 24 h treatment (Szuster-Ciesielska et al. 2007). This

might be due to the use of different cell status and experimental conditions.
The cytotoxicity of ethanol plus acetaldehyde was further evaluated in HepG2 cells, showing that exposure to ethanol at 100 mM did not significantly affect the cell viability, but in combination with 1 to 20 mM of acetaldehyde synergistically increased cell death. In addition, the synergistically increased effect of ethanol and acetaldehyde has been found on the paracelluar permeability in human intestinal Caco-2 cells (Geetha and Rao 2009; Elamin et al. 2012). Based on these observations, combined ethanol and acetaldehyde administration *in vitro* can result in more deleterious effects on cellular functions.
TNF-α production is a critical factor in the pathogenesis of alcoholic liver injury (McClain et al. 1998). Previous studies suggested that ethanol/TNF-α treatment greatly sensitized HepG2 cells and hepatocytes to TNF-α induced apoptosis through the depletion of mGSH and induction of mitochondrial permeability transition that ultimately lead to cell death (Colell et al. 1998; Pastorino and Hoek 2000). In a gently manner, our results showed that TNF-α slightly enhanced the toxic responses to ethanol in HepG2 cells while moderately increased the toxic responses to acetaldehyde and the combination of these two toxic agents.
A 24 h exposure of millimolar levels of APAP significantly reduced cell proliferation. Similar findings were also demonstrated in hepatoma cells (Fabre et al. 2003; Amaral et al. 2013; Zhao et al. 2013). However, compared to HepG2 cells, CYP2E1 infected HepG2 cells, rat hepatocytes, HepaRG cells and HBG BC cells expressing P450 enzymes were found more sensitive to APAP induced necrosis and apoptosis (Wang et al. 2002; Fabre et al. 2003; Bai and Cederbaum 2004; McGill et al. 2011). The APAP hepatotoxicity has been partially attributed to a cytochrome P450 generated reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI), which depletes GSH and binds to target proteins and subsequent leads to oxidative stress, mitochondrial dysfunction, and resultant necrotic cell death (Jaeschke and Bajt 2006). However, HepG2 cells, as many other hepatoma cells, have very low levels of P450 enzymes, explaining the more APAP toxic resistance of HepG2 cells than metabolizing cells.
The pre-existing inflammatory cytokine on APAP induced cytotoxicity was assessed. TNF-α can lead to moderate augment cell death in APAP exposed HepG2 cells at a relatively low dose (10 ng/mL). Previous studies have shown that co-administration of APAP and TNF-α results in sensitization to TNF-α induced apoptosis in hepatocytes which is partially mediated by activation of c-Jun N-terminal kinase (JNK) (Gadhi et al. 2010). Furthermore, Nagai et al. (2002) has shown that APAP/TNF-α treatment sensitizes primary mouse hepatocytes to TNF-α induced apoptosis due to glutathione depletion. These findings indicated that elevation of pro-inflammatory cytokines contributes to the development of pathologies of APAP induced liver injury.
t-BHP is a reactive short chain analog of lipid hydroperoxides produced from lipid peroxidation and employed as a model to study the effects of oxidative stress on cellular function and cell death pathways (Piret et al. 2004). Once inside the cell, t-BHP generates *tert*-butoxy radicals that induce several physiological alterations with consequent loss of cell viability (Panasenko et al. 2005). As confirmed in previous studies (Kim 1998; Piret et al. 2004), HepG2 cells were sensitive to t-BHP, evidenced by significantly enhanced cell death induction after short time and low dose of t-BHP exposure. Recently, NAD(P)H oxidation and ROS formation caused by t-BHP exposure, which leads to following cytochrome C release, mitochondrial permeabilization, activation of endothelial NF-kB, IKK, MAP kinase and caspases has been evidenced to contribute to t-BHP induced cell death (Piret et al. 2004; Lee et al. 2005; Rincheval et al. 2012). A prolonged toxic enhancement by TNF-α was found in 24 h t-BHP treated cells instead of 3 h t-BHP treated cells, indicating that TNF-α sensitized HepG2 cells respond to oxidant stress but after some period of time. This result suggested that inflammatory cytokines can enhance hepatic injury caused by free radicals.
It is necessary to point out that the physiological status of the used cells influence the tests and potentially lead to inaccurate or erroneous results. Generally, healthy cells are more resistant to toxic agents than poorly maintained cells or contaminated cells (Ryan 1994). Many cells undergo expression profile changes when they are stressed by culture conditions such as overcrowding and less nutritious medium. Culture contaminants, especially the unseen or undetected chemicals or biological contaminants such as mycoplasma or viral contamination, can achieve high densities altering the growth and characteristics of the cultures. Obtaining cell lines from reputable cell banks, periodically checking the characteristics of the cell lines, and practicing good aseptic technique are practices that will avoid contamination. One commonly happened but neglected issue is that reference laboratories that supply other laboratories with cell cultures without contamination testing, which may spread cell culture pollutants. Another recommendation for managing contamination is to discard and replace cell cultures after every 20 passages. The key concepts and practical strategies for keeping cell healthy conditions and managing contamination should be strictly implemented.
In summary, HepG2 cells showed relatively high resistance to xenobiotic compounds including alcohol and APAP. Besides the lack of cytochrome P450 in HepG2 cells, the healthy cells used in this study also contributed to the tolerated toxicity. Meanwhile, these findings suggested that, to some extent, human hepatocytes are resistant to toxicants especially to ethanol. Moreover, TNF-α moderately enhances the cellular injury caused by these exogenous compounds, suggesting an important role of inflammation in the pathogenesis of alcohol, drugs and oxidant related liver diseases.

4. Experimental

4.1. Chemicals

Fetal bovine serum (FBS), RPMI1640 medium, trypsin-EDTA for cell culture were supplied from Biochrom AG, Berlin, Germany. Recombinant human TNF-α was purchased from eBioscience, San Diego, CA. Ethanol and acetaldehyde were from Merck, Hohenbrunn, Germany. t-BHP and other reagents were obtained from Sigma-Aldrich, Steinheim, Germany.

4.2. Cell culture and treatment

HepG2 cells were obtained from DSMZ, Braunschweig, Germany. The cells were maintained in 25 cm^2 flasks with 5 mL of RPMI1640 medium containing 10% FBS, and incubated under an atmosphere of 95% air and 5% CO_2 at 37 °C. Cells were used for experiments between passages 4 and 20.
HepG2 cells were trypsinized, counted in a hemocytometer, seeded at 1×10^4 cells/well into 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany), and allowed to attach and spread for 24 h. The cells were then treated or untreated with TNF-α for 24 h. Followed by exposure to different concentrations of ethanol, acetaldehyde, APAP and t-BHP, respectively. The medium was refreshed every 24 h. To prevent evaporation of ethanol and acetaldehyde, the plates were placed in a plastic bag and sealed with a tape.

4.3. Measurements of cell viability

MTT assay was used to detect cell viability. Briefly, MTT was added to each well at the final concentration of 0.5 mg/mL, incubated at 37 °C for 90 min. The medium was subsequently replaced with 100 μL of DMSO. Optical density was read on an universal microplate reader (Tecan SPECTRA Fluor, Tecan Group Ltd, Mainz, Germany) at 560 nm. Cell viability was expressed as a percentage of the control value.

4.4. *Statistical analysis*

The data are presented as mean and standard deviation. Statistical differences were analyzed according to Student's t-test. Each experiment was repeated at least twice.

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