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Trinitrotoluene Induces Endoplasmic Reticulum Stress and Apoptosis in HePG2 Cells

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Background: This study aims to describe trinitrotoluene (TNT)-induced endoplasmic reticulum stress (ERS) and apoptosis in HePG2 cells.





Material/Methods: HePG2 cells were cultured *in vitro* with 0, 6, 12, or 24 µg/ml TNT solution for 12, 24, and 48 h. Western blotting was performed to detect intracellular ERS-related proteins, including glucose-regulated protein (GRP) 78, GRP94, Caspase 4, p-Jun N-terminal kinase (JNK), and C/EBP homologous protein (CHOP). Real-time PCR was used to measure mRNA expression from the respective genes.

Results: The expressions of ERS-related proteins GRP78 and GRP94 as well as mRNA and protein expression of ERS signaling apoptotic CHOP in the TNT treatment group were significantly increased. In addition, the mRNA and protein expression levels of ERS-induced apoptotic protein Caspase-4 were significantly increased. Flow cytometry revealed that after TNT treatment, the apoptosis rate also significantly increased.

Conclusions: TNT could increase the expression levels of GRP78, GRP94, Caspase-4, and CHOP in HePG2 cells; this increase in protein expression might be involved in HePG2 apoptosis through the induction of the ERS pathway.

MeSH Keywords: **Apoptosis • Endoplasmic Reticulum Stress • Trinitrotoluene**

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Background

Trinitrotoluene (TNT) is a high-energy nitro compound that has long been widely used in military ammunition, printing and dyeing, chemical engineering, and other manufacturing areas because of its simple and economical production. Therefore, the risk of exposure to TNT for workers in these sectors and ingestion due to environmental contamination for the general public is a significant concern. For example, 1.2 million tons of soil in the United States has been contaminated by military usage alone [1]. In 2007, Gong et al. reported that gene TNT-contaminated soil altered gene expression in earthworms after 28 days, which was mainly reflected in muscle contraction, nerve signaling transduction, fibrinolysis and coagulation, and transportation of iron, calcium, and oxygen; in addition, the expressions of multiple bioprocess-related genes, such as those involved in immunity, were also altered. A sublethal dose of TNT can affect the nervous system as well as cause methemoglobinemia-like blood diseases, which impair the immune system [2]. People can ingest TNT from contaminated water and soil, and this compound can easily be absorbed through the gastrointestinal tract, skin, and mucous membranes. As might be expected, TNT toxicity is most commonly observed in occupationally exposed populations, such as coal miners and workers in chemical industries [3]. TNT can lead to numerous adverse effects, including upper respiratory problems, gastrointestinal complaints, anemia, liver function abnormalities, and aplastic anemia [4,5]. A retrospective study of male workers from 8 Chinese military factories who were exposed to TNT for more than a year during 1970 to 1995 confirmed the increased relative risk of cancer, especially liver cancer [6]. The scientific community has been exploring the pathogenic mechanisms induced by TNT since Gollism first reported TNT poisoning cases in 1915. Previous reports have shown that radical production by TNT reduction, lipid peroxidation, oxidative stress, and calcium homeostasis dysfunction contribute to TNT-induced liver cell damage and cataract development [7–10]. More recently, multiple studies have implicated stress-including mitochondrial stress, endoplasmic reticulum stress (ERS), and oxidative stress as mediators of liver injury. Among these stresses, ERS has generated particular interest.

ERS had been confirmed to exist in the pathological changes of CCl_4 -induced rat typical toxic liver damages; the expression of GRP78, the marker protein of ERS inside poisoned rat liver tissues, was significantly increased, accompanied with the activation and decreased expression of Caspase-12 zymogen [11]. Unfolded protein responses, ER-associated protein degradation, and protein translation regulation mediated the recovery from hepatic ERS to stable status in the cells. Excessive ERS or prolonged exposure to ERS can lead to programmed cell death or apoptosis, further causing liver damage [12,13]. TNT toxicity in the liver causes oxidative stress. However, whether TNT

toxicity also mediates ERS in the liver is unclear. It was therefore necessary to further study the protein adducts of TNT and its metabolites with the microsomes to define the basis for the liver toxicities of TNT and its metabolites, which can help to eventually reduce the incidence of TNT toxicity. Therefore, in this *in vitro* study, we induced apoptosis in hepatocytes to determine whether the ERS apoptotic pathway was involved in the hepatic toxicity and to provide a new therapeutic target for protecting liver cells from TNT toxicity.

Material and Methods

Cell lines

The human hepatoma cell line HePG2 was derived from human hepatocellular carcinoma tissues, which exhibited proliferation kinetics similar to normal liver cells and are widely used to model normal liver cells *in vitro*. The cell line was kindly provided by the Harbin Medical University.

GRP78, GRP94, cleaved Caspase 4, p-Jun JNK, and CHOP antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA).

HepG2 cells were thawed at 40°C, recovered at 37°C, and grown in culture flasks containing DMEM with 12% FBS at 37°C and 5% CO_2 . Cells were passaged when they grew to 70–80% confluence. Pure TNT (>99.9% purity) was provided by a machinery factory, and used after recrystallization and purification.

Control were cultured in 10 mL fresh DMEM with 12% FBS. TNT-treatment groups were cultured in 10 mL fresh DMEM with 12% FBS and TNT at 6, 12, and 24 $\mu\text{g}/\text{mL}$ for 12, 24, and 48 h.

Real-time PCR

The fluorophores were added to the prepared cDNA samples in the PCR reaction system by the RT-PCR technology, and the accumulation of fluorescent signal was used to monitor the whole PCR process real-time; the unknown template was finally used to perform the quantitative analysis through the standard curve. The housekeeping gene ACTB was included for normalization.

Western blot

Cells were exposed to different concentrations of TNT (0, 6, 12, and 24 $\mu\text{g}/\text{mL}$) for 12, 24, and 48 h. Total cellular protein was extracted according to the manual, run on SDS-PAGE gels, and transferred to membranes. Blots were blocked in 5% skim milk at room temperature for 30 min and incubated overnight with the following primary antibodies at 4°C: rabbit anti-human C/

Table 1. Apoptotic indexes of each group after stimulated by different concentrations of TNT (%) ($\bar{x}\pm s$, n=6).

Group	0 $\mu\text{g/ml}$	6 $\mu\text{g/ml}$	12 $\mu\text{g/ml}$	24 $\mu\text{g/ml}$
12 h	1.47 \pm 0.42	1.86 \pm 0.83 ^a	4.73 \pm 2.01 ^{a,b}	9.76 \pm 3.55 ^{a,b}
24 h	2.32 \pm 1.52	3.80 \pm 1.76 ^a	6.32 \pm 3.78 ^a	14.58 \pm 8.83 ^{a,b}
48 h	3.67 \pm 1.76	7.35 \pm 3.45 ^{a,c}	9.55 \pm 4.23 ^{a,b,c}	27.72 \pm 5.53 ^{a,b,c}

^a Compared with the control group $P<0.05$; ^b compared with the 6 $\mu\text{g/ml}$ group $P<0.05$; ^c compared with the 24 h group, $P<0.05$.

EBP homologous protein (CHOP) (1:1000), rabbit anti-human jun N-terminal kinase (JNK) (1:1000), and mouse anti-human Caspase-4 (1:1000). Following incubation with goat anti-rabbit (1:5000) or goat anti-mouse (1:3000) secondary antibodies at room temperature for 60 min, proteins were detected by chemiluminescence.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) analysis and flow cytometry

Hepatocyte apoptosis was measured by TUNEL. Positive cells exhibiting brown-stained nuclei and apoptotic phenotypes were quantified to calculate apoptosis rates.

The cells in each group were digested by trypsin and prepared into the single cell suspension, then centrifuged at 1200 rpm for 5 min, discarded the supernatant, washed with PBS 3 times; the cell precipitate was then blown into single cells and prepared into the single cell suspension; added drops of this suspension into 2 ml of 70% ethanol (pre-cooled at 4°C), fixed at -20°C overnight; centrifuged at 1200 rpm for 5min, collected the cell precipitate, washed with PBS 3 times to clean the residual ethanol; added PI dye (1 g·L⁻¹) for 30-min staining at 4°C in the dark. Flow cytometer was then used to detect the intracellular DNA contents; the ratio of diploid cells was the apoptosis ratio. WinMDI 2.9 software was used to analyze the results.

Statistical analysis

All experiments were repeated 3 times and data are expressed as $\bar{x}\pm s$. Unpaired t-tests or ANOVA was performed on group means using SPSS19.0 software.

Results

TNT induced HePG2 apoptosis in a dose- and time-dependent manner

Apoptosis cell rates were measured in HePG2 cells stimulated with 0, 6, 12, and 24 $\mu\text{g/ml}$ TNT for 12, 24, and 48 h by TUNEL. Apoptosis gradually increased at higher TNT concentrations

and with prolonged exposure. The proportions of apoptotic cells after TNT exposure were higher than those in the controls (Table 1, $P<0.05$, Figure 1). Intergroup differences were also statistically significant ($P<0.05$).

TNT upregulated the expression of ERS markers GRP78 and GRP94

Western blot and RT-PCR were performed to detect the protein and mRNA expression of GRP78 and GRP94 in HePG2 cells stimulated with 0, 6, 12, and 24 $\mu\text{g/ml}$ TNT for 24 h. Protein and mRNA expression levels of GRP78 and GRP94 were significantly increased in TNT-treated cells (Figure 2, Table 2, $P<0.05$).

Expression of p-JNK, the ERS signaling apoptosis-regulating protein

After HePG2 cells were stimulated with 0, 6, 12, and 24 $\mu\text{g/ml}$ TNT for 24 h, the expressions of p-JNK protein and mRNA were unchanged ((Figure 3, Table 3, $P>0.05$).

Expression of cleaved Caspase-4 (C CASP4), ERS-induced apoptosis protein

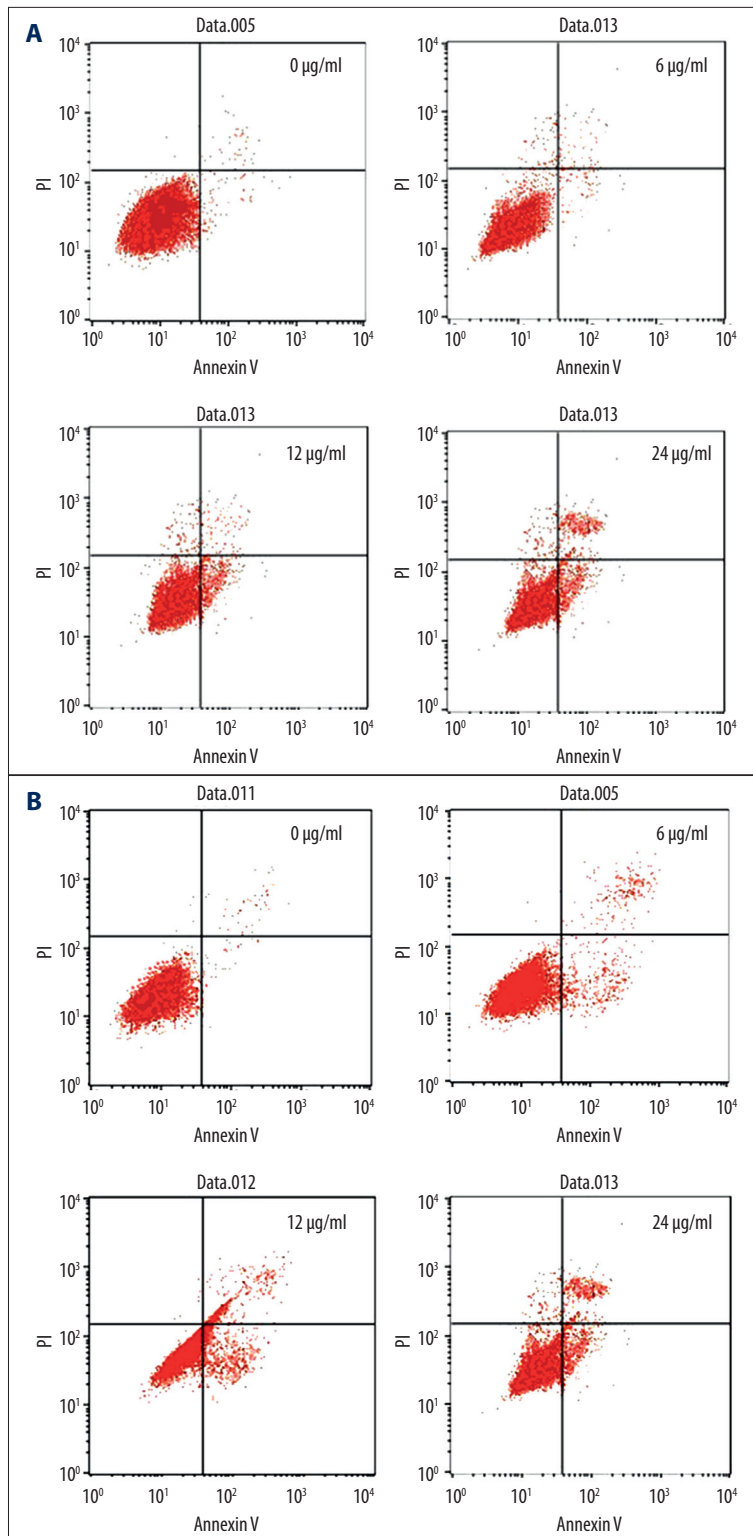
After HePG2 cells were stimulated with 0, 6, 12, and 24 $\mu\text{g/ml}$ TNT for 24 h, Western blot and RT-PCR were performed to measure C CASP4 expression. TNT exposure significantly increased C CASP4 protein and mRNA expression ($P<0.05$, Figure 4, Table 4).

Expression of CHOP, ERS- related apoptosis protein

After HePG2 cells were stimulated with 0, 6, 12, and 24 $\mu\text{g/ml}$ TNT for 24 h, Western blot and RT-PCR were performed to measure CHOP expression. TNT exposure significantly increased CHOP protein and mRNA expression (Table 4, Figure 5, $P<0.05$).

Discussion

Some research on TNT toxicity has shown that radical production by TNT reduction, lipid peroxidation, oxidative stress, and calcium homeostasis dysfunction contribute to TNT-induced liver cell damage and cataract development; they confirmed that TNT increases



peroxide and free radical production in mitochondria and microsomes that and peroxidation might be the biochemical basis for TNT toxicity. Some reports suggested that TNT causes nitro reduction and methyl oxidation in the liver and particularly affects

the smooth endoplasmic reticulum (ER). It has also been reported that TNT inhibits rat glutathione peroxidase, thus resulting in lipid peroxidation and cell membrane damage that causes toxicity.

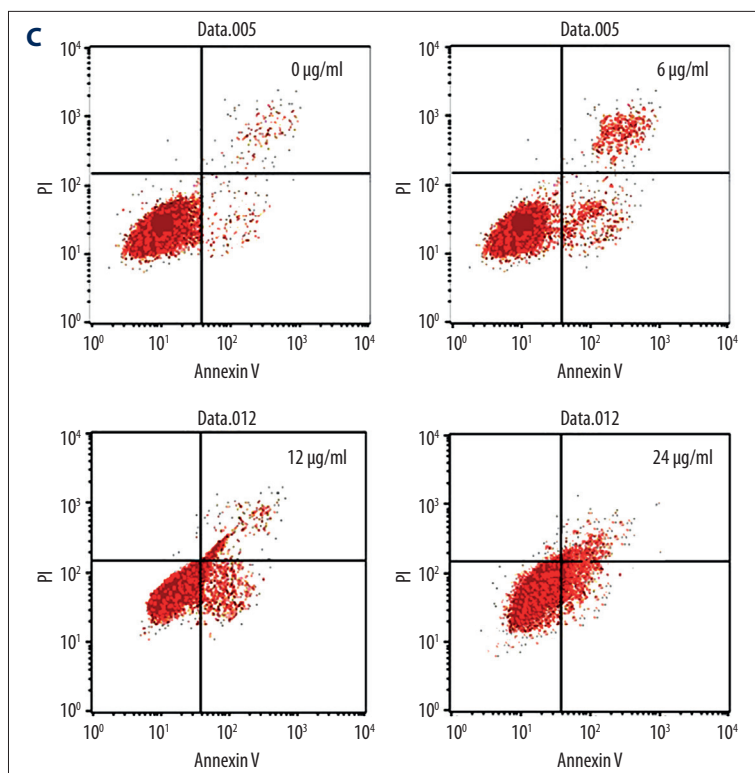


Figure 1. Apoptosis of HePG2 stimulated by different concentrations of TNT. (A) 12 h; (B) 24 h; (C) 48 h.

Table 2. mRNA and protein expression levels of GRP78 and GRP94 (pro/actin, $\bar{x} \pm s$, n=6).

Group	GRP78		GRP94	
	mRNA	Protein	mRNA	Protein
Control group	0.17±0.11	0.36±0.12	0.22±0.10	0.54±0.19
6 µg/ml	1.23±0.25 ^a	0.88±0.34 ^a	1.22±0.45 ^a	1.52±0.39 ^a
12 µg/ml	1.37±0.21 ^{a,b}	1.42±0.32 ^a	1.32±0.25 ^{a,b}	1.64±0.38 ^a
24 µg/ml	1.27±0.35 ^a	1.77±0.39 ^{a,b}	1.50±0.41 ^{a,b}	2.18±0.50 ^{a,b}

^a Compared with the control group, P<0.05; ^b compared with the 6 µg/ml group, P<0.05.

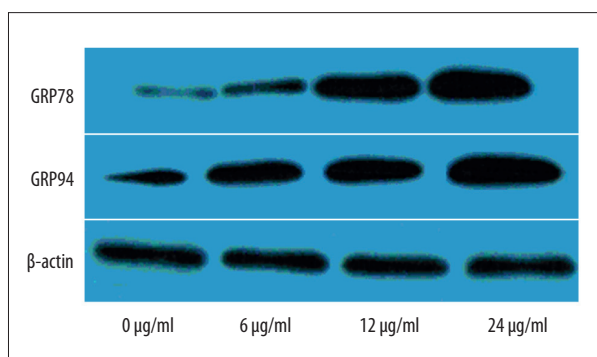


Figure 2. GRP78 and GRP94 protein levels after stimulation by different concentrations of TNT.

Research in the early 21st century found that stress might be an important aspect of liver toxicity, and it was confirmed that oxidative stress could lead to ERS [14]. Furthermore, the calcium pump in the ER was reported to be susceptible to oxidative damage, [15]. Oxidative stress and ERS can also activate one another and exacerbate apoptosis [16,17].

ERS can be caused by changes in calcium homeostasis due to oxidation, metabolic disorders, viral infections, alcoholism and drug abuse, or misfolded protein responses in the ER. However, excessive or prolonged ERS responses can cause apoptosis. ERS plays an important role in the development of many diseases. Liver cells were found to have highly developed endoplasmic reticulum and are one of the cell types most sensitive to endoplasmic reticulum stress. Various physicochemical factors, such as infections or poisoning, could cause liver

Table 3. mRNA and protein expression levels of JNK and p-JNK (pro/actin, $\bar{x} \pm s$, n=6).

Group	JNK		p-JNK	
	mRNA	Protein	mRNA	Protein
Control group	0.88±0.18	0.73±0.15	1.47±0.17	1.59±0.12
6 µg/ml	0.79±0.17	1.18±0.19	1.31±0.21	1.41±0.19
12 µg/ml	0.87±0.10	0.96±0.17	0.97±0.15	1.21±0.22
24 µg/ml	0.90±0.15	0.92±0.15	0.93±0.12	1.20±0.19

Compared with the control group, P>0.05.

Table 4. mRNA and protein expression levels of C Caspase-4 and CHOP (pro/actin, $\bar{x} \pm s$, n=6).

Group	C Caspase-4		CHOP	
	mRNA	Protein	mRNA	Protein
Control group	1.39±0.10	1.17±0.16	0.22±0.10	0.39±0.07
6 µg/ml	1.28±0.15 ^a	1.29±0.24 ^a	1.22±0.45 ^a	0.42±0.09 ^a
12 µg/ml	1.42±0.25 ^{a,b}	1.50±0.14 ^a	1.32±0.25 ^{a,b}	0.72±0.14 ^a
24 µg/ml	1.59±0.38 ^{a,b}	1.65±0.31 ^{a,b}	1.50±0.41 ^{a,b}	1.27±0.31 ^{a,b}

^a Compared with the control group, P<0.05; ^b Compared with the 6 µg/ml group, P<0.05.

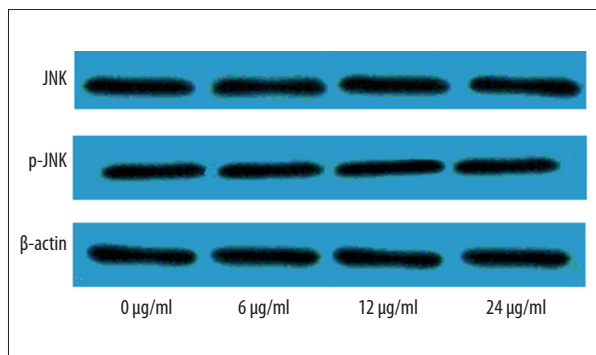


Figure 3. Expression level of JNK protein.

endoplasmic reticulum the disorders in uptake or release of Ca²⁺, processing proteins, and transportation, thus resulting in endoplasmic reticulum misfolding and aggregation of unfolded protein, as well as Ca²⁺ balance disorders; therefore, it could activate the responses of unfolded proteins, and enhance protein folding abilities through the transcriptions by certain genes, thus maintaining homeostasis and normal cell functions. When homeostasis is lost, the signaling pathway of endoplasmic reticulum stress would cause cell damage through apoptosis of liver cells [18]. In this study, we used various concentrations of TNT to stimulate HePG2 cells, and rates of apoptosis in these cells revealed that prolonged exposure increased apoptosis and that different concentrations of TNT resulted in significantly different responses, indicating that TNT could induce HepG2 apoptosis in a time- and concentration-dependent manner.

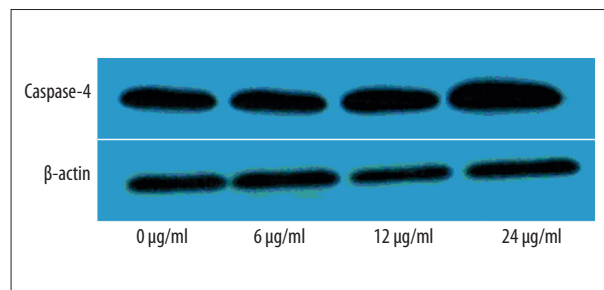


Figure 4. Expression levels of Caspase-4 protein.

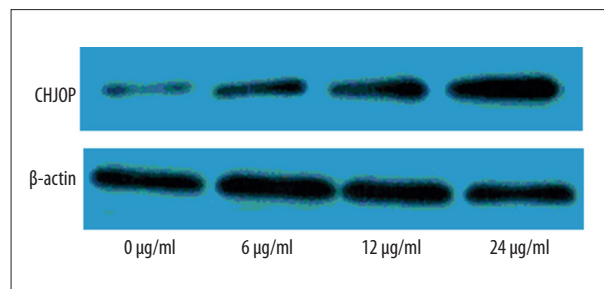


Figure 5. Expression of CHOP protein.

GRP78 and GRP94 are chaperones localized to the ER lumen. They can act to assist protein folding and transportation and are up-regulated in response to stress to maintain ER homeostasis. Therefore, their expression is widely used as a marker of ERS activation [19]. Previous studies have shown that harmful substances, including organic metabolites, drugs, poisons, and other compounds, can be processed into less toxic and easily

excreted substances in the ER. This process was also involved in the regulation of the expression of many genes. ERS contributes to the processing of the misfolded or unfolded proteins that accumulate during normal cellular functions. However, once the dysfunctions have occurred or if the stress intensity is too high or the exposure to stress is for a long duration, it might cause cell dysfunctions and death. In rat acute liver injury and alcoholic liver injury models, the expressions of GRP78 and GRP94 are increased, and the changing trends are consistent with rat hepatocyte apoptosis and pathological lesions [20,21].

In a previous study, after treatment with different concentrations of nickel (0.5, 1.25, 2.5, and 5 µg/ml) for 48 h, HepG2 cells showed a dose-dependent increase in the expressions of the promoters of ERS-marking proteins (GRP78 and HSP70) [22]. The authors observed that increase in TNT concentration or duration of treatment resulted in a significant increase in the protein and mRNA expressions of GRP78 and GRP94 in HePG2 cells, indicating that the major effects of URP appeared after TNT action began. ERS-reactive apoptosis pathways mainly consist of 4 pathways, among which 1 is involved in the activation and transcription of transcriptional factor CHOP. CHOP is the ERS-specific transcription factor; in the non-stressed condition, its expression level is low, but its expression increases drastically in ERS, eventually inducing apoptosis [23]. Another pathway has been suggested to initiate apoptosis through phosphorylating the c-Jun [24,25]. However, in this study, the protein and mRNA expressions of p-JNK showed no changes, suggesting that the TNT-induced apoptosis of HepG2 did not activate this pathway.

Caspase-12 is located on the outer membrane of the ER and is a key molecule in ERS-mediated apoptosis; Caspase-12 is

only expressed in rodents, but researchers have found that the human Caspase-4 and the rodent Caspase-12 are homologous [26]. In this study, after the HePG2 cells were treated with TNT for 24 h, Western blot and RT-PCR tests revealed that the expression of Caspase-4 was significantly increased, suggesting that it played a role in the TNT-induced apoptosis of HePG2 cells.

Conclusions

The results of this study revealed that the mechanism of TNT-originated ERS-induced programmed cell death was mediated by CHOP and Caspase-4. One limitation is that the specific roles of ERS in this study on the mechanism of apoptosis are based on *in vitro* studies; however, there have been relatively few systematic *in vivo* studies involving the use of target organs or cells of various toxic substances, thus limiting the expansion of relevant toxicity mechanism studies. Therefore, it has not been possible to identify whether the activation of ERS responses is the direct manifestation of toxicity or whether it is the protective response of the cells towards toxic substances. In order to provide a sufficient basis for the treatment of populations exposed to toxic substances, further studies are needed to elucidate this relationship.

Conflicts of interest

All of the authors declare that they have no conflicts of interest regarding this paper.

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