ORIGINAL ARTICLE

Effect Of Cadmium On Cell Viability, *GSTM1* mRNA Expression And Apoptosis Gene Expression In HepG2 Cell

Isvina Unai Zahroya[1], Naila Arum Rifkiana[1], Eko Purnomo[2], Ratih Yuniartha[3], Dwi Aris Agung Nugrahaningsih[4], Rahmi Ayu Wijayaningsih[1]

[1] Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Indonesia
[2] Department of Surgical, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia
[3] Department of Anatomy, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia
[4] Department of Pharmacology and Therapy, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia

ABSTRACT

Introduction: Cadmium (Cd) is a highly toxic heavy metal that poses severe risks to liver health. Glutathione-S-transferase (GST) enzymes is an essential enzymes working to catalyze the conjugation of glutathione (GSH) to heavy metals including Cd. While the role of detoxification enzyme mitigating these effects has been established, the detailed mechanisms of Cd-induced liver damage remain under explored. This aim of this study was to investigate the mechanistic pathways through which Cd exerts its toxic effects on liver cells, focusing on its impact on *GSTM1* mRNA expression, cell viability, and apoptosis pathways in HepG2 cells. **Methods:** HepG2 cells were incubated with Cd for 24 hours. Cell viability was measured using the MTT assay. mRNA levels of *GSTM1*, *p53*, *Bax, Caspase-3*, and *Caspase-7* were quantified via Reverse Transcription-PCR. **Results**: Cd exposure resulted in a significant reduction in HepG2 cell viability and a marked decrease in *GSTM1* mRNA expression. Additionally, Cd treatment led to an increase in p53 and Caspase-7 mRNA levels, while the mRNA levels of *Bax* and *Caspase-3* remained unchanged. **Conclusion:** This study provides new insights into the molecular mechanisms of Cd toxicity, revealing that Cd impairs HepG2 cell viability and GSTM1 expression while selectively enhancing *p53* and *Caspase-7* mRNA levels, without affecting *Bax* and *Caspase-3*. These findings contribute to a deeper understanding of how Cd-induced liver damage occurs at the cellular level.
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Keywords: Cadmium, HepG2 cell line, *GSTM1*, Apoptosis

Corresponding Author:
Dwi Aris Agung Nugrahaningsih, PhD
Email: dwi.aris.a@ugm.ac.id
Tel: +62 274 511103

INTRODUCTION

Cadmium (Cd) is a heavy metal environmental pollutant that is dangerous for human health(1,2). International Agency for Research on Cancer (IARC) classifies Cd as a first category carcinogen, meaning it is carcinogen to human (3,4). Cd contamination in humans can come from various sources such as mining activities, batteries, water and food contamination, smoking, and other industrial applications(4). In Indonesia, cases of Cd contamination were found in several places. Study Oginawati et al., (2023) found that the Cd content in drinking water sources was thought to be significantly related to the incidence of stunting in children under 5 years of age in Bandung Regency(5). Research by Yunita Dewi et al., (2022) also reported that 11 (73.3%) of 15 farmers with urine Cd > 0.8 μg/mL in Gintungan Village, Bandungan District had impaired kidney function(6).
Cd accumulation is found in the kidneys and liver(7). Research by Abu-El-Zahab et al., (2019) shows that Cd exposure increases the activity of biomarker enzymes for liver damage in mice. Cd causes liver histology damage, reduced antioxidant enzymes(1,8), and both apoptosis and necrosis(2). Oxidative stress is considered to be one of the main mechanisms of Cd-induced cell damage(9–11). However, Cd cannot produce Reactive Oxygen Species (ROS) directly but rather by inducing changes in redox homeostasis through two mechanisms, namely Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase and mitochondria (10).
Cells develop defense systems to reduce ROS-induced oxidative stress through antioxidants such as glutathione (GSH) (11). GSH is abundant in the liver and forms conjugates with xenobiotics assisted by the enzyme Glutathione-S-transferase (GSTs). Glutathione-S-transferase-Mu 1 (*GSTM1*) belongs to the cytosolic GSTs enzyme family involved in the phase II detoxification process and is highly expressed in liver tissue(9,11–13). *GSTM1* is of concern because it is highly

polymorphic(11,14). Based on research by Prayuni et al (2019) in Indonesia, the frequency of the *GSTM1* null genotype is higher in the Javanese-Sundanese ethnic population (99%) compared to the Malay ethnic population (67.2%)(15). *GSTM1* gene deletion or null mutation can inactivate the enzyme resulting in loss of function in the detoxification pathway (16). Loss of *GSTM1* can cause oxidative stress and DNA damage resulting in genomic instability (17) . Individuals with the *GSTM1* null genotype may have impaired elimination of carcinogenic compounds (18) . *GSTM1* is known to have anti-apoptotic activity in hepatocellular carcinoma (HCC) cells (13).

Apoptosis is one of the serious pathologies of liver damage caused by Cd toxicity (19). Other research shows that Cd exposure induces apoptosis through the intrinsic pathway (mitochondria) in rat liver tissue which is characterized by increased expression of pro-apoptotic proteins, namely Bcl-2-associated X Protein (*Bax*), *Caspase-3*, and decreased expression of anti-apoptotic proteins. B-cell Lymphoma 2 (Bcl-2) (19). Upregulation of *p53* mRNA expression promotes apoptosis in zebrafish (Danio rerio) liver tissue through increasing *Bax* mRNA expression and decreasing Bcl-2 mRNA expression (20). Apart from that, Cd also induces apoptosis through endoplasmic reticulum stress which is characterized by increased gene expression of *Caspase-3*, *Caspase-7*, glucose-regulated protein 78 (Grp78), C/EBP homologous protein (Chop) in chicken liver tissue (21).

HepG2 cells are a well-established human hepatoblastoma-derived cell line that retains several characteristics of normal hepatocytes and is widely used as an in vitro model for studying liver toxicity, including Cd exposure (22,23). Although many previous studies have documented the role of ROS-mediated apoptosis in Cd-induced hepatotoxicity, the specific molecular mechanisms remain incompletely understood, especially regarding the involvement of detoxification enzymes and executioner caspases. In this study, we focus on *GSTM1*, a phase II detoxification enzyme known for its antioxidant role and high polymorphism, which may contribute to individual susceptibility to Cd toxicity. Additionally, we investigate the involvement of *caspase-7*, a relatively underexplored executioner caspase in the context of Cd-induced apoptosis. Unlike the extensively studied *caspase-3*, the role of *caspase-7* in hepatocyte apoptosis remains less characterized. Therefore, this study provides novel insight into the acute effects of Cd exposure on *GSTM1* suppression and *caspase-7* activation, and how these alterations correlate with cell viability and apoptotic gene expression in HepG2 cells. These findings may enhance our understanding of molecular pathways underlying Cd hepatotoxicity and offer potential biomarkers for susceptibility and damage response.

MATERIALS AND METHODS

HepG2 cell culture
HepG2 cells were cultured in media Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Waltham, MA, USA) (10% fetal bovine serum (FBS) (Gibco, Waltham, MA, USA), 1% penicillin/streptomycin, and 0.5% fungizone (Gibco, Waltham, MA, USA)) and incubated in a 5% CO2 incubator and 37°C. After 80-85% confluence, cells were harvested using trypsinization and subcultured. Cell harvest is calculated according to needs.

MTT Assay
HepG2 cell viability was tested with 3-4(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide. Cells as many as 1x104 cells/well were planted in 96 well plates with 3 replications. Cells were incubated for 24 hours, then replaced with 100 µL/well of media containing Cd ((CdCl2.H2O) (Loba Chemie PVT.LTD, Mumbai, India.) and complete DMEM as a media control. $CdCl_2$ was diluted in distilled water to final concentrations of 19.5, 39, and 78 µM prior to treatment. Next, 100 µL/well MTT solution was added and incubated for 4 hours in a CO2 incubator. Add 100 µL of 10% SDS to each well and incubate at room temperature for 24 hours (avoid light). The results were read using an ELISA reader with an absorbance wavelength of 595 nm(24). The MTT results are used to find the Inhibition Concentration 50 (IC_{50}) value, where this value indicates the concentration of Cd that inhibits 50% of cell viability. Furthermore, the concentration of Cd used in gene expression treatment is ¼ IC_{50}, ½ IC_{50}, and IC_{50}.

Cd Treatment
$1x10^5$ HepG2 cells were planted in 24 well plates with 3 replications. Next, the cells were incubated for 24 hours and then replaced with DMEM media containing Cd concentrations of K1 (19.5 µM), K2 (39 µM), and K3 (78 µM) and 0 µM as a control (K). These three concentrations showed ¼ IC_{50}, ½ IC_{50}, and IC_{50}. Cells that had been given 500 µL of Cd were incubated for 24 hours, then harvested using the trypsinization method for RNA isolation.

Genes Detection Procedure
3x105 harvested HepG2 cell pellets were then subjected to RNA isolation according to the FavorPrep RNA isolation kit protocol (FAVORGEN® Biotech Corp, National Biotechnology Park, Taiwan). The RNA isolation results were converted into cDNA using the SMOBIO reverse transcriptase kit (SMOBIO®, Hinschu City, Taiwan). Next, PCR was carried out based on the GoTaq Green Master Mix protocol from Promega. The PCR product was electrophoresed to see DNA bands which were visualized using a B-Box blue light LED epi-illuminator. The results obtained are in the form of DNA bands that will be analyzed by densitometry

using ImageJ® software (National Institutes of Health, Bethesda, MD, USA). The sequences of the primers used are shown in the Table I. Beta-actin primers were used as housekeeping genes .

Table 1: List of the primers used in the present study.

Gene	Sequences
GSTM1	F 5′-ACTTGATTGATGGGGCTCACA-3′
	R 5′-TTGTGCTTGCGGGCAATGT-3′
Bax	F 5′-GCCGGAACTGATCAGAACCA-3′
	R 5′-GTCTTGGATCCAGCCCAACA-3′
p53	F 5′-GAGCTGAATGAGGCCTTGGA-3′
	R 5′-CTGAGTCAGGCCCTTCTGTCTT-3′
Caspase-3	F 5′ ACAGAACTGGACTGTGGCAT 3′
	R 5′ TACAAGAAGTCGGCCTCCAC 3′
Caspase-7	F 5′-GCAGCGCCGAGACTTTTAG-3′
	R 5′-GCTGCAGTTACCGTTCCCAC-3′
β-actin	F 5′-AAGAGAGGCATCCTCACCCCT-3′
	R 5′-TACATGGCTGGGGTGTTGAA-3′

Statistic analysis

Statistical analysis using SPSS software Ver.24 (IBM Corporation, Armonk, NY, USA). One-way ANOVA test to determine whether there are significant differences between groups and Tukey's test was used for further tests.

RESULTS

MTT Assay

The MTT test is used to see the viability of HepG2 cells based on a colorimetric test. The average value of % cell viability can be seen in Figure 1.

The average % cell viability in the control group was 100±1.89%, the 19.5 μM concentration was 85.96±1.62%, the 39 μM concentration was 76.66±4.75%, and the 78 μM concentration was

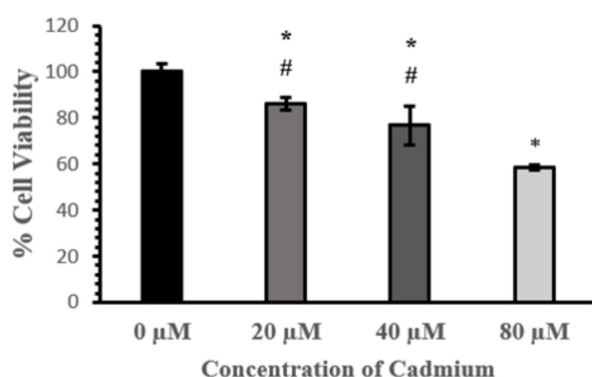

Figure 1: Percentage value of viability in HepG2 cell induced by Cd. *p<0.05 vs 0 μM, #p<0.05 vs 80 μM

58.77±0.63%. The average % cell viability in the treatment group was lower than the control group. There was a significant difference between the control group and the treatment group (p<0.05). Meanwhile, the average % cell viability in the 78 μM concentration group was lower compared to all groups. Based on data from further test results, it is known that there is a significant difference between the 78 μM concentration group compared to all groups. However, the average % cell viability in the 19.5 μM and 38 μM concentration groups did not show a significant difference.

***GSTM1* mRNA expression**

GSTM1 mRNA expression in HepG2 cells induced by Cd can be seen in (Figure 2). *GSTM1* mRNA expression in the control group was significantly higher (p<0.05) compared to the treatment groups (K1, K2, K3). There were no significant differences between treatment groups K1, K2, and K3.

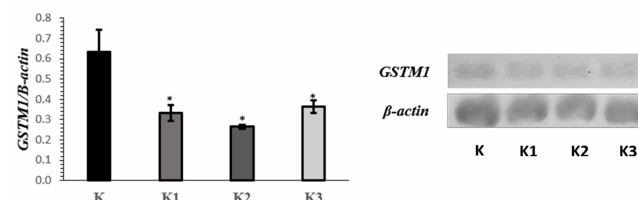

Figure 2: Expression of GSTM1 mRNA in Cd-induced HepG2 cells. (A) Relative quantification of GSTM1 mRNA. (B) Representative image of gel electrophoresis from RT-PCR product GSTM1 and β-actin (housekeeping gene). Data were shown in mean ± SD. K: control, K1:19.5 μM Cd, K2: 39 μM Cd, K3: 78 μM Cd. *p<0.05 vs K

Expression of apoptotic genes (mRNA *p53*, *Bax*, *Caspase-3*, *Caspase-7*)

p53 mRNA expression was significantly higher in the K1 group (p<0.05), K2 (p<0.05), and K3 (p<0.05) compared with the control group (Fig 3). However, *p53* mRNA expression in group K3 was significantly lower compared with group K1 (p<0.05) and group K2 (p<0.05). There were no significant differences between treatment groups K1 and K2 (Fig 3). *Bax* mRNA expression in the K3 group was higher compared to the control group although it was not statistically significant. However, *Bax* mRNA expression in group K1 and group K2 was lower compared with the control group and was not statistically significant. *Caspase-3* mRNA expression in the treatment group (K1, K2, K3) and the control group did not have a statistically significant difference (Fig 3). *Caspase-7* mRNA expression in group K1 (p<0.05), group K2 (p<0.05) and group K3 (p<0.05) was significantly higher compared to the control group. There were no significant differences between treatment groups K1, K2, K3 (Figure 3).

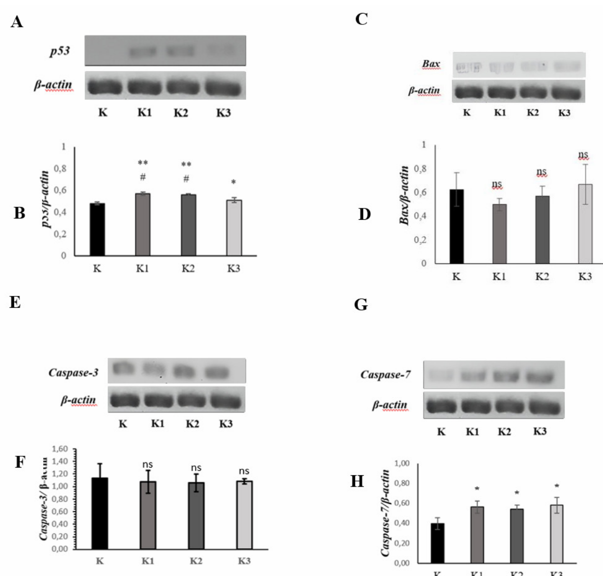

Figure 3: Expression of apoptosis genes (mRNA p53, Bax, Caspase-3, Caspase-7). (A, C, E, G) Representative image of gel electrophoresis from RT-PCR product of p53, Bax, Caspase-3, Caspase-7 and β-actin (housekeeping gene). (B, D, F, H) Relative quantification of mRNA expression of p53, Bax, Caspase-3, and Caspase-7 to the β-actin. Data were shown in mean ± SD. K: control, K1:19.5 μM Cd, K2: 39 μM Cd, K3: 78 μM Cd. *p<0.05 vs K, #p<0.05 vs K3, n.s: not significant (p>0.05)

DISCUSSION

This research was carried out in vitro using HepG2 cells to see the acute effect of Cd on cell viability, *GSTM1* mRNA expression and expression of apoptosis genes such as *p53*, *Bax*, *Caspase-3*, and *Caspase-7*. This study used three concentrations of Cd given to HepG2 cells based on previous cytotoxic test results, namely 19.5 μM (1/4 IC_{50}), 39 μM (1/2 IC_{50}), and 78 μM (IC_{50}). These three concentrations show Cd levels (1.9, 3.9, and 7.8 μg per 0.0005 L) which are still below and exceed the blood Cd levels set by the Occupational Safety and Health Administration (OSHA), namely 5 μg/L(25). The tolerable limit for human consumption of Cd contained in food is 25 μg/kg per month (26).

Based on research results, it is known that Cd can reduce cell viability. This is in line with research by Ahamed et al., (2020), exposure to Cd on HepG2 cells at a concentration of 1 μg/mL equivalent to 5 μM/μL for 24 hours significantly reduced cell viability (78.5%) compared to controls. The higher the concentration given, the more the viability decreases (27). The decrease in cell viability is likely due to increased ROS production due to Cd exposure. Induction of 2 μg/ml Cd for 24 hours increased ROS in HepG2 cells (p ≤ 0.05)(3). Cd exposure also significantly increased H2O2 intracellular and MDA enzymes which are biomarkers of oxidative stress(3). Acute Cd exposure can generate free radicals such as superoxide anion (O2•−), hydrogen peroxide (H_2O_2), hydroxy radicals (•OH) and lipid peroxides (•L) (27). Excessive ROS production causes cells to experience oxidative stress, resulting in disruption of redox control signaling and molecular damage(28). At this stage enzymes or antioxidant molecules are responsible for neutralizing ROS as radical scavengers.
In this study, *GSTM1* mRNA expression was also tested. Cd exposure can reduce *GSTM1* mRNA expression. The decrease in *GSTM1* mRNA expression in the treatment group may be related to its function as a catalyst in GSH conjugation. Cd can decrease GSH, causing increased ROS production and apoptosis(29). Increased oxidative stress can reduce antioxidant gene expression. Free radicals may cause molecular damage to these genes(30). Although *GSTM1* expression is decreased, β-actin (housekeeping gene) expression remains stable, as it is believed to have a constant expression level under various cellular, experimental and physiological conditions (31).

The decrease in *GSTM1* mRNA expression may also be due to the role of transcription factors such as Nuclear factor erythroid 2-related factor 2 (*Nrf2*). *Nrf2* functions to control the expression of various antioxidant and metabolic genes that have antioxidant response elements (ARE) sequences in their promoters. The *GSTM1* gene has a strong correlation with the transcription factor *Nrf2* (coef= 0.621, p ≤ 0.0001) (32). Choudhury Researchet al., (2021) showed that exposure to Cd in head kidney macrophage (HKM) significantly increased the expression of the *Nrf2* and Kelch-like ECH-associated protein 1 (Keap1) genes(33). Keap1 is a negative regulator of *Nrf2*. Based on research by Chanas et al., (2002), *Nrf2* knockout mice induced by butylated hydroxyanisole (BHA) showed weakened GSTA1, *GSTM1* and GSTM3 mRNA expression in the liver, thereby reducing phase II detoxification capacity(34). In HepG2 cells, induction of a combination of Cd and silver nanoparticles (AgNp + Cd) for 24 hours showed inactivation of *Nrf2* (35).

This study also looked at the expression of *p53*, *Bax*, *Caspase-3* and *Caspase-7* mRNA as markers of apoptosis. The results showed significantly higher *p53* and *Caspase-7* mRNA expression. This indicates that Cd can cause apoptosis of HepG2 cells. Based on the results of this study, Cd concentrations that are still below the levels set by Occupational Safety and Health Administration (OSHA) have shown toxic effects on liver cells. If Cd exposure continues to become chronic exposure, it can cause the development of liver disease. The tumor suppressor *p53* acts as a transcription factor that regulates several pathways such as cell cycle arrest, DNA repair, apoptosis, autophagy, and metabolism(36). The high expression of *p53* mRNA is caused by ROS and DNA damage due to Cd exposure thereby activating the *p53* signaling pathway which plays an important role in apoptosis(37). This is in accordance with the theory that during stressful conditions due to oxidative stress, DNA damage, and oncogenes, *p53* will be activated and stabilized after going through post-translational modification processes such as phosphorylation so that

it can regulate cell cycle gene transcription, DNA repair, aging, and apoptosis(36). However, in this study there was a decrease in *p53* mRNA expression in the K3 group (78 μM), this was probably due to a decrease in HepG2 cell viability caused by necrosis. This is in accordance with other research that HepG2 cells given the highest concentration of Cd experienced a significant reduction in cell viability followed by necrosis type cell death(38). Cd concentrations (10 μM) can cause apoptosis whereas high concentrations (50 μM) lead to cell necrosis(39,40). Intrinsic pathway apoptosis is mediated by the Bcl-2 family of proteins including pro-apoptotic proteins (*Bax*) and anti-apoptotic proteins (Bcl-2)(41). Based on the research results, *Bax* mRNA expression did not show a statistically significant difference. These results are not in accordance with the research of Lawal et al., (2015) Cd induction in HepG2 cells at concentrations of 5 and 10 μM for 24 hours, showing a significant increase in *Bax* protein expression compared to the control group(38). This indicates that Cd induces intrinsic pathway apoptosis not mediated by *Bax* but possibly mediated by Bak or via the extrinsic pathway (36,42). Therefore, this study also examined *Caspase-3* mRNA expression. The results showed that there was no significant difference in Cd exposure between treatment groups.

These results are in contrast to research by Ahamed et al., (2020) where Cd induction in HepG2 cells significantly increased *Caspase-3* mRNA expression compared to the control group (3). This is possibly because Cd can cause cell apoptosis through a mechanism that does not involve caspases (caspase-independent). In this pathway, apoptosis is induced by mitochondrial pro-apoptotic protein molecules (AIF) and endonuclease G (43). Based on research by Lemarie et al., (2004), Cd was proven to induce apoptosis in Hep3B cells in a caspase independent apoptotic manner (44). The mechanism is through a complex involving changes in mitochondrial homeostasis related to calcium and ROS and the release of endonuclease G and AIF. Mitochondria are the main regulators of caspase independent apoptotic. Increased mitochondrial membrane potential causes the release of AIF and Smac/DIABLO to be translocated to the cell nucleus together with endonuclease G, thereby causing DNA fragmentation and chromatin condensation in the cell. Meanwhile, Smac/DIABLO neutralizes apoptosis inhibitory proteins (45).

This research also looked at the expression of the *Caspase-7* gene as an executor caspase. Based on the research results, administration of Cd also significantly increased *Caspase-7* mRNA expression compared to the control group. Based on other studies, *Caspase-7*, Grp78, and Chop mRNA expression increased in chicken liver tissue after administration of Cd, this indicates that Cd induces apoptosis through endoplasmic reticulum stress. *Caspase-7* has an important role in the apoptotic pathway caused by endoplasmic reticulum stress (20). *Caspase-7* plays a role in apoptosis through intrinsic and extrinsic pathways in broiler chicken kidney tissue after administration of nickel chloride (46). *Caspase-7* does not have a significant role in cell apoptosis via the intrinsic pathway, but is responsible for ROS production and cell release from the extracellular matrix (47). *p53* can regulate the transcription of *Caspase-6* and *Caspase-7* genes in mouse kidney proximal tubule epithelial cells which is induced by cisplatin which results in cell apoptosis (48).

To better illustrate the proposed molecular events, a schematic diagram has been included (Figure 4). This model summarizes the effects of Cd exposure on apoptosis pathways in HepG2 cells based on our findings and relevant literature. Cd-induced oxidative stress appears to be mediated through the suppression of *GSTM1* via inhibition of the *Nrf2* pathway, which limits cellular antioxidant defense. *Nrf2* (nuclear factor erythroid 2–related factor 2) is a key transcription factor that regulates the expression of antioxidant response elements (AREs), including genes involved in detoxification such as *GSTM1*. Under normal conditions, *Nrf2* translocates to the nucleus and activates antioxidant gene expression. However, Cd exposure disrupts this process by preventing *Nrf2* nuclear translocation, thereby reducing the expression of detoxifying enzymes like *GSTM1* and enhancing ROS accumulation. This oxidative stress subsequently triggers both the extrinsic apoptotic pathway (FasL-FasR–caspase-8/10) and the intrinsic mitochondrial pathway (*p53*–PUMA–*Bax*–cytochrome c–caspase-9). Notably, our study highlights the involvement of *caspase-7*, an executioner caspase that is often overlooked in Cd-related apoptosis studies compared to *Caspase-3*. These pathways ultimately converge to activate executioner caspases and amplify apoptosis, leading to reduced cell viability. The schematic supports our conclusion that *Nrf2* inhibition, *GSTM1* suppression, and *caspase-7* activation are key contributors to Cd-induced hepatocyte damage and represent potential targets for future mechanistic and therapeutic research (Figure 4).

Cd exposure increases reactive oxygen species (ROS) generation in liver cells, primarily by suppressing the *Nrf2* pathway and reducing transcription of antioxidant-related genes such as *GSTM1*. Decreased *GSTM1* reduces glutathione (GSH) activity, resulting in oxidative stress and activation of both extrinsic and intrinsic apoptotic pathways. The extrinsic pathway involves FasL-FasR interaction and activation of *caspase-8* and *-10*, while the intrinsic pathway involves *p53* upregulation, *Bax* activation, mitochondrial membrane damage, and release of cytochrome c and AIF, which activate *caspase-9*. Both pathways converge on activation of executioner *caspases-3* and *-7*, leading to apoptosis and reduced cell viability.

The current study has several limitations. First, it focuses exclusively on gene expression without validating the

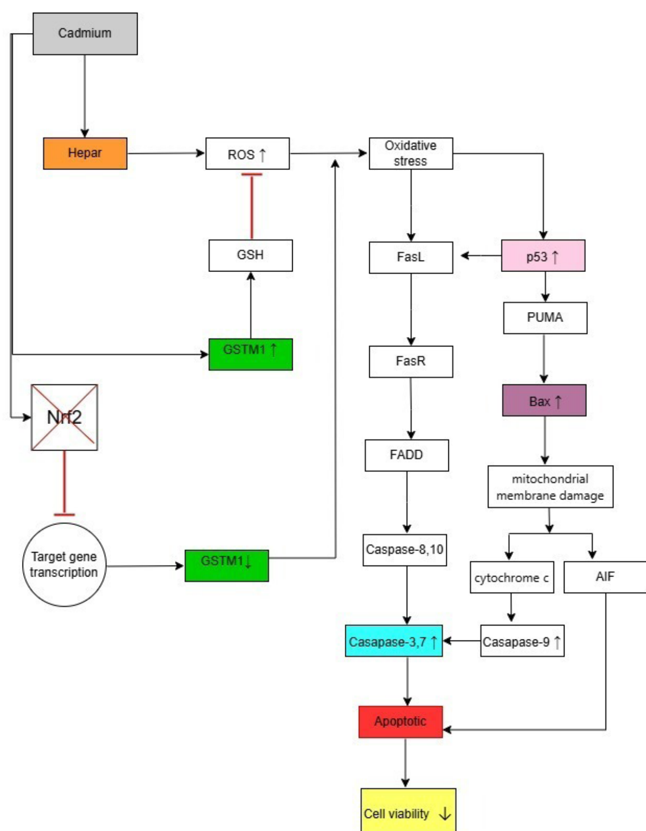

Figure 4: Proposed mechanism of Cd-induced apoptosis in HepG2 cells.

results at the protein level. Since post-translational modifications may significantly affect protein activity and function, future studies should include protein-level confirmation such as Western blot analysis. Second, the data were derived solely from acute exposure conditions. To improve translational relevance, future research should investigate chronic or repeated Cd exposure (e.g., 48–72 hours) and validate findings in in vivo models. Third, this study did not include a rescue or reversal model, which could enhance mechanistic understanding by confirming causality in the observed molecular responses. Lastly, while this study explored the roles of *caspase-3* and *caspase-7* as executioner caspases, we did not assess initiator caspases such as caspase-8, -9, or -10, which may help clarify upstream apoptotic signaling and should be investigated in future studies.

CONCLUSION

Based on the research results, it can be concluded that Cd has an effect on cell viability. The higher the Cd concentration, the lower the viability. Cd can also reduce *GSTM1* mRNA expression. Cd can also increase *p53* and *Caspase-7* mRNA expression. However, Cd had no effect on *Bax* and *Caspase-3* mRNA expression.

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