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Hydrogen Peroxide Induces Acute Injury and Up-regulates Inflammatory Gene Expression in Hepatocytes: An *in vitro* Model

(Hidrogen Peroksida Mengaruh Kecederaan Akut dan Mengatur Naik Ekspresi Gen Inflamasi dalam Hepatosit: Suatu Model *in vitro*)

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ABSTRACT

In the past, many in vitro *hepatocyte injury models developed for liver regeneration used carbon tetrachloride as irritant chemical. Recently, carbon tetrachloride usage was prohibited due to serious deleterious effects to human and environment. There is an urgent need to develop a new acute chemical-induced hepatocyte injury model using other chemical compound to replace carbon tetrachloride. In this study, we used hydrogen peroxide (H_2O_2) to induced hepatocyte injury with HepG2 as the liver cell model. HepG2 injury was established by exposing the cells to CC_{50} of H_2O_2 at the concentration of 2.4 mM, predetermined via MTT assay for 2 h exposure. Aspartate aminotransferase (AST) activity was measured to determine the extent of cellular injury and quantitative PCR was carried out to determine the expression of inflammatory genes of the cells 24 h after H_2O_2 exposure. The results showed that AST activity increased with time and peak at 24 h after H_2O_2 exposure. Quantitative PCR analysis demonstrated that expression of inflammatory genes (TGF-β1, MMP-3, NF-κβ, IL-8 and IL-6) increased significantly. In addition, the gene expression of GPX, an anti-oxidant enzyme was also increased significantly in response to oxidative stress. In summary, H_2O_2 demonstrated excellent capability in inducing oxidative injury to HepG2 and together they represent an ideal acute chemical-induced injury model that can be used for liver regeneration study. Our results also provide input for inflammatory gene expression in the hepatocyte injury model.*

Keywords: Hepatocytes; H_2O_2; inflammatory genes; in vitro; *liver injury model*

ABSTRAK

Banyak model kecederaan hepatosit in vitro *yang telah dibangunkan untuk kajian regenerasi hepar pada masa lalu adalah berdasarkan kepada iritasi karbon tetraklorida. Kebelakangan ini, karbon tetraklorida telah dilarang penggunaaannya disebabkan ia boleh membawa kemudaratan kepada manusia dan persekitaran. Maka, satu model kecederaan hepatosit akut aruhan bahan kimia yang baru untuk menggantikan karbon tetraklorida kepada bahan kimia lain perlu dibangunkan dengan kadar segera. Dalam kajian ini, kami menggunakan hidrogen peroksida (H_2O_2) untuk mengaruh kecederaan hepatosit dengan menggunakan HepG2 sebagai model sel hepar. Kecederaan HepG2 diaruh dengan mendedahkan sel kepada CC_{50} H_2O_2 pada kepekatan 2.4 mM yang telah dipratentukan melalui asai MTT selama 2 jam pendedahan. Aktiviti aspartat transaminase (AST) diukur untuk menentukan tahap kecederaan sel dan PCR kuantitatif dijalankan untuk menentukan ekspresi gen-gen inflamasi pada masa 24 jam selepas pendedahan kepada H_2O_2. Hasil kajian mendapati aktiviti AST meningkat dengan masa dan mencapai puncak kepekatan pada masa 24 jam selepas pendedahan kepada H_2O_2. PCR kuantitatif menunjukkan peningkatan ekspresi gen-gen inflammasi (TGF-β1, MMP-3, NF-κβ, IL-8 and IL-6) secara signifikan. Selain itu, ekspresi gen GPX, sejenis enzim antioksidan juga meningkat secara signifikan sebagai respons kepada tekanan oksidatif. Secara kesimpulan, H_2O_2 menunjukkan keupayaan yang baik untuk mengaruh kecederaan oksidatif pada HepG2 dan kombinasi H_2O_2 dengan HepG2 menghasilkan model kecederaan hepatosit akut aruhan bahan kimia yang ideal untuk kajian regenerasi hepar. Di samping itu, hasil kajian ini juga memberi input terhadap ekspresi gen inflamasi dalam model kecederaan hepatosit.*

Kata kunci: Gen inflamasi; hepatosit; H_2O_2; in vitro; *model kecederaan hepatosit*

INTRODUCTION

Liver is vital to maintain life as it has multiple important functions including metabolism, detoxification, protein synthesis and production of various biochemicals necessary for digestion (Mitra & Metcalf 2009). Alcohol consumption, drug intoxication, chemicals metabolism as well as autoimmune irritation can cause liver injury (Bernal et al. 2010; Galun & Axelrod 2002). Rapidly progress liver inflammation will lead to acute liver disease, whereas insidious inflammation will result in chronic liver disease (Hartley & Kelly 2010). Liver injuries cause reduction of liver functions and subsequently disrupt the normal body physiology.

Primary cultured hepatocytes isolated from whole livers or wedge biopsies have similar functionality as the *in vivo* hepatocytes. However, its usage was limited

by the short life span with limited capacity to proliferate *in vitro* and loss its liver-specific functions rapidly. Liver immortalized cell lines, such as HepG2 and HepaRG, are alternative choices of liver cells for *in vitro* study. These cell lines can multiply indefinitely. HepG2 is well differentiated transformed liver cell line that is widely used for toxicity screening before the introduction of HepaRG. HepaRG is functionally better than HepG2 and reduces the chances of underestimate toxicity due to lower expression level of phase I and phase II enzymes. However, culturing of HepaRG is much more tedious and the well differentiated hepatocytes characteristic is difficult to establish (Kanebratt & Andersson 2008; Marion et al. 2010). Thus, HepG2 as a more robust liver cellular model was selected in this study to create the acute chemical-induced hepatocyte injury model.

Liver injuries can be caused by a number of hepatotoxic agents; such as alcohol, acetaminophen, carbon tetrachloride (CCl_4), bromobenzene and dimethylnitrosanine. Most of the hepatotoxins are not directly toxic to the liver. The marked toxicity effect was usually caused by reactive intermediate metabolites and reactive oxygen species which were produced during the metabolism of hepatotoxins. These reactive intermediate metabolites and reactive oxygen species which cause oxidative damage to liver when its concentration exceeded the antioxidant capability of hepatocytes (Wu et al. 1999). Conventionally, CCl_4 was most commonly used to induce *in vivo* and *in vitro* liver/ hepatocyte injury. Metabolism of CCl_4 by CYP 450 produces reactive metabolites such as trichloromethyl (CCl_3•) and trichloromethyl peroxyl radical (CCl_3 OO•) that cause oxidative damage to hepatocytes. Concentration of ROS such as H_2O_2 also has been reported to increase with administration of CCl_4 (Sahreen et al. 2011). The usage of CCl_4 in certain countries (including Malaysia) is prohibited due to its deleterious effects on human and environment. The International Agency Research on Cancer (IARC) has graded CCl_4 as possible carcinogenic agent and it can damage the liver, kidney and central nervous system at high dose (Weber et al. 2003). Furthermore, CCl_4 is one of the causative agents for ozone layer depletion. Thus, development of a novel *in vitro* acute chemical-induced hepatocyte injury model using other types of chemical is very important to continue liver research. H_2O_2 was selected in this study as it is one of the reactive oxygen species that can cause oxidative injury to hepatocytes and less harmful to the environment.

Several reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radical (•OH) and hydroxyl ion (OH^-) were involved in liver disorders (Jeong et al. 2006; McElwee et al. 2009). Among them, H_2O_2 was thought to be the major precursor of reactive free radicals (Alía et al. 2005). Glutathione peroxidase (GPx) is an antioxidant enzyme that involves in the conversion of H_2O_2 to H_2O within the cells. ROS could cause liver disorders once its production overwhelmed the antioxidant defenses. Elevation of H_2O_2 level has been found to be the main factor that leads to liver disorders such as alcoholic liver disease, non-alcoholic steatohepatitis, viral hepatitis and hemochromatosis, (Cesaratto et al. 2004).

Oxidative stress due to imbalance between ROS and antioxidant defenses will lead to the activation of survival pathway such as NF-Kβ signaling that involves in inflammation. Activation of NF-Kβ will subsequently up-regulate IL-8 that functioned as neutrophil chemoattractant and activator (Dong et al. 1998) and IL-6 that involved in liver inflammation and regeneration (Salazar-Montes et al. 2000). TGFβ-1 is an important cytokine expressed during liver inflammation as it has profound influence on liver regeneration and fibrosis (Poli & Parola 1997). In addition, TGFβ-1 also mediated MMPs expression in acute liver injury (Knittel et al. 2000). MMP-3, a member of zinc-dependent family specifically degrades extracellular matrix, is one of the MMPs that were found to increase after liver injury.

In this study, HepG2 was treated with H_2O_2 to produce an acute chemical-induced hepatocyte injury model. Cellular damage due to H_2O_2 treatment was determined via aspartate aminotransferase activity assay and quantitative PCR was performed to evaluate the expression of inflammatory genes.

Materials and Methods

Cell Culture

HepG2 (American Type Tissue Culture Collection, ATCC), a human hepatocellular carcinoma cell line was grown in Dulbecco's Modified Eagle Medium-Ham's F12 medium (1:1) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 1% antibiotic-antimycotic (Invitrogen), 1% glutamax (Invitrogen) and 1% vitamin C (Sigma-Aldrich). Cells were incubated and maintained at 37°C with 5% carbon dioxide. Cells were passaged every 48 to 72 h.

MTT Cytotoxicity Assay

HepG2 was seeded in 96-well plate at density 7.5×10^4 cells/ well. After 4 h, the cells were treated with H_2O_2 at concentration ranging from 0-76.8 mM in serum-free medium. Serum-free medium was replaced with fresh culture medium after 2 h. After 24 h, 20 µL of 5 mg/mL MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich) was added into each well. Live cells transformed MTT into non-soluble formazan and DMSO (100 µL per well) was added to dissolve the formazan after 4 h. Afterward, the plate was read at 570 nm with ELISA microplate reader (VersaMax™, Molecular Devices, USA). Cytotoxicity was expressed as percentage of cell survival compared to the untreated control, which was calculated using the formula:

$$\text{Percentage of cell survival} = \frac{\text{OD of treated group}}{\text{OD of untreated group}} \times 100\%.$$

IN VITRO HEPG2 INJURY MODEL

HepG2 was seeded in 6-well plate at density 7.5×10^5 cells/well. The cells were left to attach for 4 h in medium added with 10% FBS. After that, the control and H_2O_2-treated group were cultured with serum-free medium and serum-free medium with 2.4 mM H_2O_2 for 2 h, respectively. This H_2O_2 concentration was chosen based on MTT assay results which showed that it caused 50% cell death (CC_{50}). After 2 h, fresh serum-free medium was added to each well.

CELL MORPHOLOGICAL

Morphology of the control and H_2O_2-treated HepG2 was observed and photos were captured at time point 0 (after 2 h exposure to H_2O_2) and 24 h. Apoptotic cells were identified based on the apoptotic features of cell shrinkage, membrane blebbing and chromatin condensation (Merrill et al. 2002).

AST ACTIVITY ASSAY

Culture medium at time point 2, 6, 12 and 24 h were collected for AST activity determination using AST cytotoxicity assay kit (BioVision Incorporated, USA). Collected culture medium was kept at -80°C prior assay.

TOTAL RNA EXTRACTION

Total RNA was extracted from the control and H_2O_2-treated HepG2 at time point 24 h using TRI reagent (Molecular Research Center, Cincinnati, OH, USA). The procedure was performed according to manufacturer's recommended protocols, which include homogenization, phase separation, RNA precipitation, RNA wash and RNA solubilization. The RNA precipitation was increased by the addition of polyacryl carrier (Molecular Research Centre).

cDNA SYNTHESIS

The extracted RNA was used for the synthesis of cDNA using TOPscript™ cDNA Synthesis Kit (Enzynomics, Korea). The reaction was carried out according to the protocol recommended by the manufacturer. The protocol conditions were 5 min at 70°C, 1 min at -20°C, 10 min at 25°C and 60 min at 50°C. The synthesized cDNA was used as template for quantitative PCR to determine the gene expression level.

QUANTITATIVE POLYMERASE CHAIN REACTION (q-PCR)

The expression of inflammatory genes including TGF-β1, MMP-3, NF-κβ, IL-6 and IL-8, and GPx, an antioxidant enzyme were analyzed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. Primers for each gene were designed using Primer 3 software based on published Gen Bank database sequences. The sequences of the primers used were listed in Table 1. PCR reaction was performed using Bio-Rad iCycler PCR machine with SYBR green as the indicator. The reaction mixture contained 2× Prime Q-Master Mix (Genet Bio, Korea), forward and reverse primers, DNase/RNase free water and cDNA. The reaction conditions were cycle 1 (1×): Step 1, 95.0°C for 30 s; cycle 2 (1×): Step 1, 94.0°C for 9 min; cycle 3 (45×): Step 1, 95.0°C for 30 s, Step 2, 53°C for 20 s and Step 3, 72°C for 20 s; cycle 4 (1×): Step 1, 95.0°C for 1 min; cycle 5 (1×): Step 1, 55.0°C for 1 min; and Cycle 6 (70×): Step 1, 60.0°C to 94.5°C for 10 s each. The specificity of the primers and PCR protocol were confirmed by melt curve analysis and 2% agarose gel electrophoresis. The expression level of each gene was then normalized to GAPDH.

TABLE 1. Primers' sequences used in Quantitative PCR

Gene	Accession No.	Primer 5'-3'	Product Size
GADPH	NM_002046	F:5'-TCC CTG AGC TGA ACG GGA AG-3' R:5'-GGA GGA GTG GGT GTC GTC GCT GT-3'	217
TGFβ-1	NM_000358	F:5'-AAC ACA TCA GAG CTC CGA GAA -3' R:5'- GAG GTA TCG CCA GGA ATT GTT -3'	141
IL-8	NM_000584	F: 5'-GTG CAG TTT TGC CAA GGA GT-3' R: 5'-CTC TGC ACC CAG TTT TCC TT-3'	196
MMP-3	NM_002422	F: 5'-TGC TTT GTC CTT TGA TGC TG-3' R: 5'-GGA AGA GAT GGC CAA AAT GA-3'	135
NF-κβ	NM_003998	F:5'-AGT GCA GAG GAA ACG TCA GAA-3' R:5'- CATTTTACCACTTGGCAGGAA-3'	163
IL-6	NM_000600	F: 5'-TAC CCC CAG GAG AAG ATT CC-3' R: 5'-TTT TCT GCC AGT GCC TCT TT-3'	175
GPX	NM_000581	F: 5'-CCA AGC TCA TCA CCT GGT CT -3' R: 5'- TCG ATG TCA ATG GTC TGG AA -3'	198

STATISTICAL ANALYSIS

All results were reported as means ± SEM. Statistical analysis was performed using Pair T-test (IBM® SPSS® Statistics), with $p<0.05$ considered significant.

RESULTS

MTT ASSAY

HepG2 were exposed to H_2O_2 at concentration ranging from 0 to 76.8 mM for 2 h. Viability of HepG2 reduced when H_2O_2 concentration increases (Figure 1). From the graph, CC_{50} was found to be 2.4 mM and this concentration was used to induce HepG2 injury in the subsequent experiments.

MORPHOLOGICAL CHANGES OF HepG2 AFTER H_2O_2 TREATMENT

Morphological changes were a simple yet sensitive method to determine the cells' health status. At time point 0 hour (2 h after treatment), control cells started to spread (Figure 2(a)), whereas the H_2O_2-treated cells remained rounded and some of the cells became apoptotic, as indicated by the observation of membrane blebbing (Figure 2(b)).

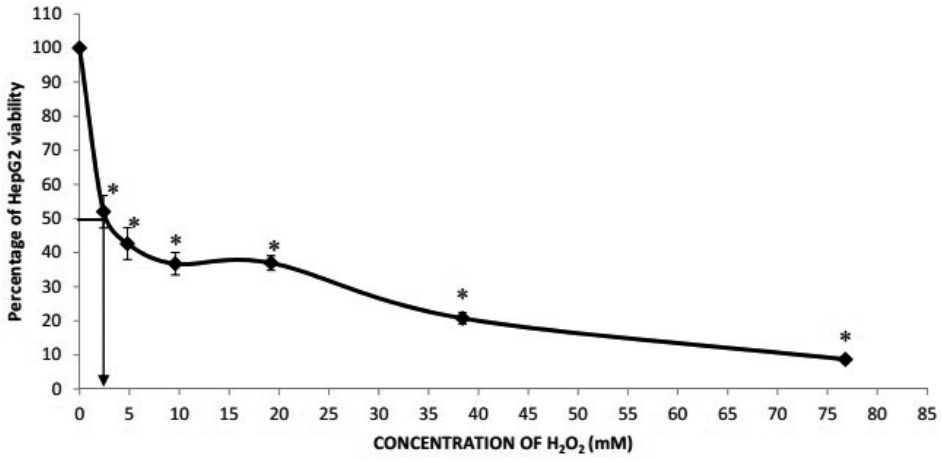


FIGURE 1. HepG2 viability at time 24 h after exposure to a series of H_2O_2 concentration. The CC_{50} was 2.4 mM

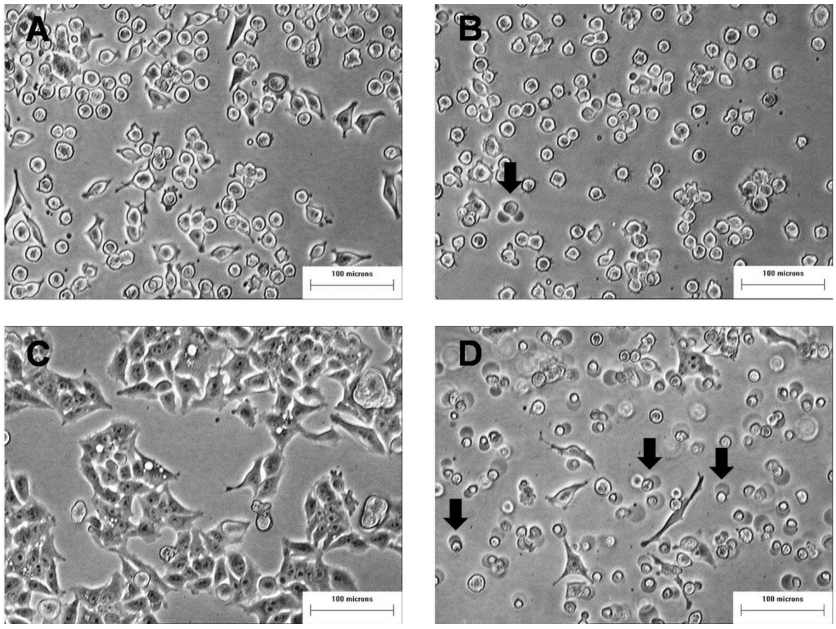


FIGURE 2. HepG2 morphology at time 0 h and 24 h after H_2O_2 induction. At time 0 h, expanded cells were seen in the control group (a) and apoptotic cells were found in the H_2O_2-treated group (b). At time 24 h, cells in the control group have a cobblestone appearance (c), while majority of cells in the H_2O_2-treated group were adherent and round with a small percentage of expanded cells and apoptotic cells (d). Arrow indicates apoptotic HepG2. (100× magnification)

At time point 24 h, the control cells proliferated, achieving 50% confluence (Figure 2(c)). Most of the H_2O_2-treated cells were round and attached, with a small proportion of floating dead cells and expanded cells (Figure 2(d)).

AST ASSAY

AST enzyme activity in culture medium was measured to determine the extent of HepG2 injury. The results showed that AST activity of the H_2O_2-treated group was low at 0, 2 and 6 h but increased significantly (p<0.05) by 4 folds at time point 12 h. Further increment of AST activity was detected at time point 24 h, which was about 50% higher compared to time point 12 h (Figure 3).

GENE EXPRESSION ANALYSIS

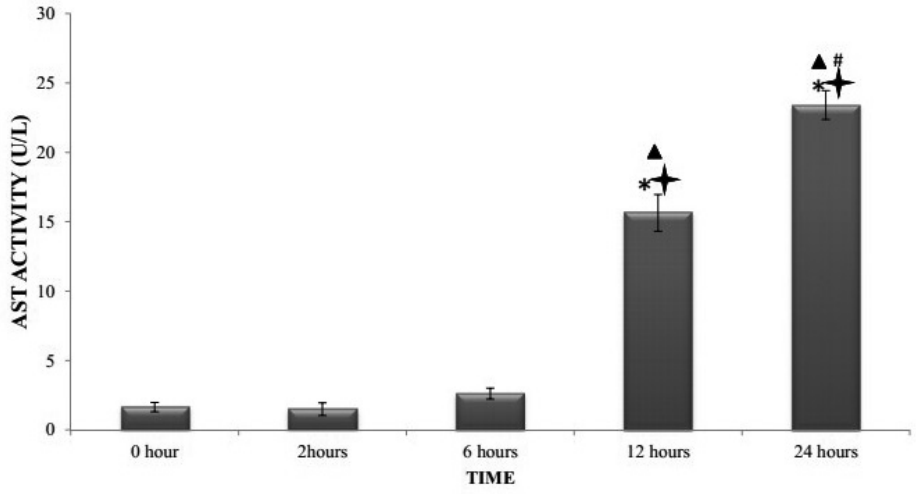
Quantitative PCR analysis of the inflammatory gene expression showed that expression of TGFβ-1 (Figure 4(a)) and IL-6 (Figure 4(d)) increased significantly (p<0.05) by 2 folds, whereas NF-κβ (Figure 4(c)) and IL-8 (Figure 4(e)) expression increased by 3 folds in the H_2O_2-treated group compared to the control. MMP-3 (Figure 4(b)) expression was very low in the control group but increased significantly by 210 folds in the H_2O_2-treated group (P<0.001). Expression of antioxidant enzyme, GPx (Figure 4(f)) was significantly higher by 5 folds in the H_2O_2-treated group compared to the control group.

DISCUSSION

Liver injury is often caused by the reactive metabolites produced during chemical detoxification. One of the reactive metabolites that cause liver injury is H_2O_2. Thus, in this study, H_2O_2 was used to induce HepG2 injury to create an acute chemical-induced hepatocyte injury model. H_2O_2 induced cell death in dose dependent manner whereby the highest H_2O_2 concentration tested (76.8 mM) killed 90% of the cells after 24 h. The CC_{50} was detected to be 2.4 mM. CC_{50} detected in this study was different with the concentration reported by Okamura et al. (Okamura et al. 2004) However, this may be attributed to the different HepG2 culture conditions, which influence the basal cellular activities (Hewitt & Hewitt 2004). We found that HepG2 exposed to H_2O_2 underwent apoptosis as supported by the presence of apoptotic features, including cell shrinkage, membrane blebbing and chromatin condensation. Furthermore, the proliferation of H_2O_2-treated HepG2 was greatly inhibited compared to the control group.

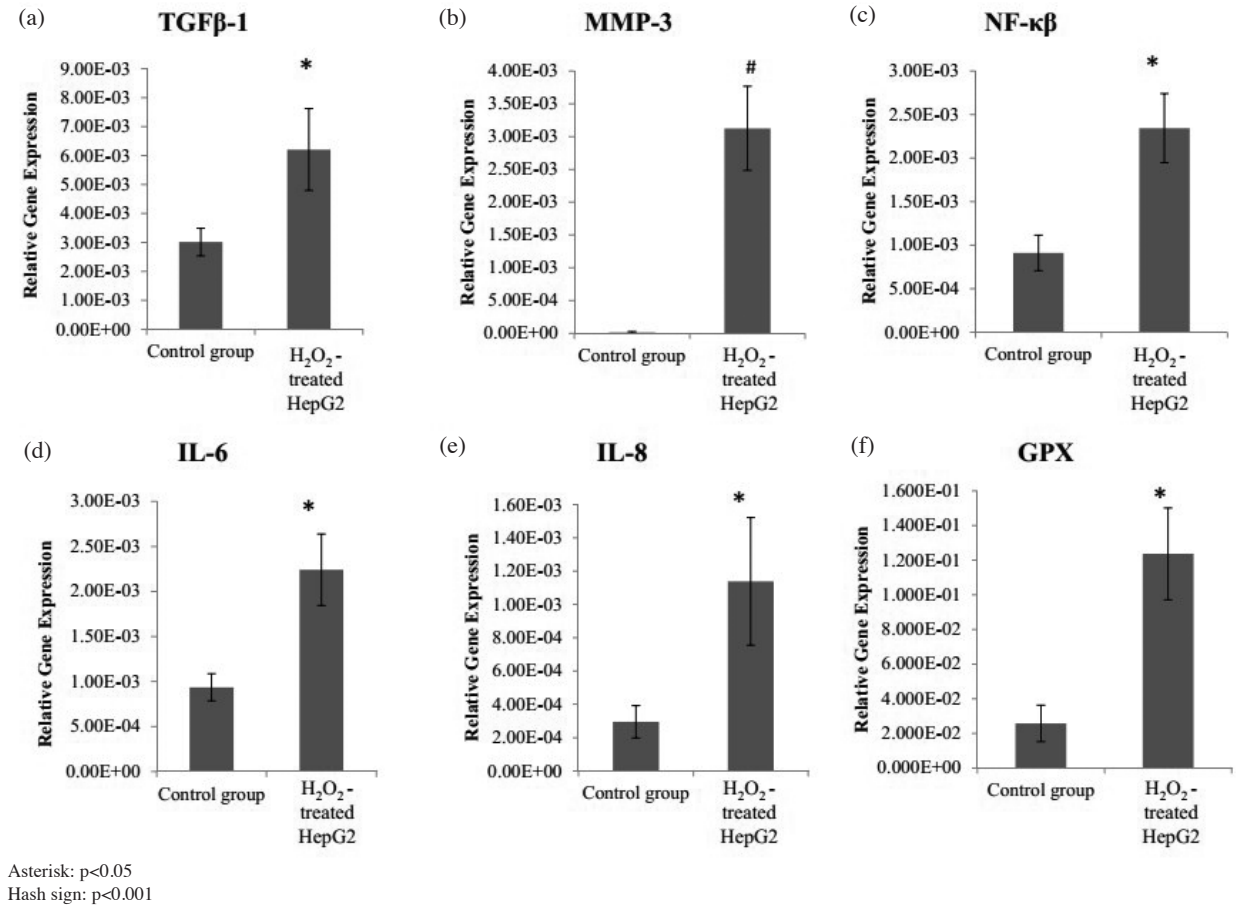
AST is an amino acid metabolic enzyme founds in cytosol and mitochondria of hepatocytes. Upon injury, AST enzyme will leak out from the hepatocytes (Herlong 1994). Thus, measurement of AST activity can give an indication on the extent of liver/hepatocyte injury. The significant increase of AST activity in the culture medium was detected 12 h after exposure. This implies that cell death did not occur immediately after 2 h exposure to 2.4 mM H_2O_2 but after a delay of 12 h. Since necrosis involved rapid loss of membrane integrity, the delay elevation of AST activities confirmed that the cell die via apoptosis.

H_2O_2 has been found to induce HepG2 apoptosis through the activation of caspases- 9 and caspases- 3 (Li et al. 2008). However, the mRNA expression of inflammation genes in injured HepG2 remained unknown. In this study, we measured the expression of inflammation genes of HepG2 at time 24 h after H_2O_2 exposure using quantitative PCR. The results showed that expression of all the measured inflammation genes were up-regulated.



Star: p<0.05 between 0, 12 and 24 h
Triangle: p<0.05 between 2, 12 and 24 h
Asterisk: p<0.05 between 12 h compared to 0, 2 and 6 h
Hash sign: p<0.05 between 12 and 24 h

FIGURE 3. AST activities in culture supernatant at time point 0, 2, 6, 12 and 24 h after treated with H_2O_2. Data denoted as mean ± S.E.M of n=6.



Asterisk: $p<0.05$
Hash sign: $p<0.001$

FIGURE 4. Relative gene expression of H_2O_2-treated and control groups. (a) TGFβ-1, (b) MMP-3, (c) NF-κβ, (d) IL6, (e) IL8 and (f) GPx. Data are denoted as mean ± S.E.M. of n=6.

H_2O_2-treated HepG2 demonstrated higher expression of TGFβ-1, which is a potent modulator of cell proliferation, differentiation and fibrogenesis in both normal and fibrotic liver (Reeves & Friedman 2002). H_2O_2-treated HepG2 also showed higher expression of MMP-3. MMP-3, also known as stromelysin-1, is an zinc-dependent enzyme that specifically degrades typr IV collagen (Jeong et al. 2006). The result of this study was consistent with the finding of Knittel et al. (2000) showed MMP-3 was produced during early hours of liver injury and inflammation.

The expression of NF-κβ, IL-6 and IL-8 was also increased in the H_2O_2-treated HepG2. NF-κβ is a family of transcription factors that form stable complexes with NF-kβ inhibitor molecules (McElwee et al. 2009). NF-κβ can be activated by stimuli such as ultraviolet, H_2O_2, heat shock and hepatotoxic agents (Poli & Parola 1997) and subsequently increased the transcription of IL-6 and IL-8 (Oliveira et al. 2012). Elevation of IL-6 has been detected in liver diseases such as hepatitis and alcoholic liver disease (Hill et al. 1992; Sun et al. 1992) and after liver dissection (Selzner et al. 2003). The presence of IL-6 is very important as it protects liver against injury and helps in liver regeneration (Bansal et al. 2005). IL-8 is a neutrophil chemo-attractant. Higher gene expression of IL-8 was consistent with the findings reported by Dong et al. (1998), which showed that primary hepatocytes and HepG2 produced IL-8 in response to oxidative damage caused by hepatotoxic agents.

Hepatocytes possess excellent antioxidant defenses as they often encountered oxidative stress induced by reactive metabolites generated during chemical detoxification. GPx, an antioxidant enzyme, was known to catalyzed the conversion of H_2O_2 into water (Sahreen et al. 2011). In this study, gene expression of GPx was analyzed to determine the capability of HepG2 to elevate its antioxidant defenses in response to H_2O_2. It was found that HepG2 can up-regulate the GPx gene expression to protect them against oxidative damage.

The results from this study clearly showed that HepG2 can response to oxidative stress in manner similar to primary hepatocytes, evidenced by the elevation of inflammatory and antioxidant gene expression. Injured HepG2 expressed higher TGFβ-1 and MMP-3 which promote liver regeneration through hepatocyte proliferation and fibrosis. At the same time, higher NF-κβ expression stimulates the increase expression of IL-6 and IL-8 which play important, if not vital role in liver protection and regeneration.

CONCLUSION

In summary, we demonstrated that H_2O_2 was capable of inducing oxidative injury in HepG2 and this represent a reliable and reproducible acute hepatocyte injury model to replace CCl_4-induced hepatocyte injury model. Furthermore, we illustrated the changes in expression of inflammatory genes in response to oxidative stress. Our H_2O_2-induced hepatocyte injury model is a promising *in vitro* model for future liver research.

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