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# DETERMINATION OF DRUG TOXICITY OF ACETAMINOPHEN USING 3D SCAFFOLD CULTURES OF IMMORTAL HUMAN HEPATOCARCINOMA CELL LINES

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**Keywords:**

APAP, Cytotoxicity, 3D PDMS scaffold culture, Hepatocarcinoma cell lines, HepG2, Huh-7

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**ABSTRACT:** Acetaminophen (APAP), well-known as paracetamol, is a safe analgesic and antipyretic agent at a therapeutic dose. However, overdoses of APAP can induce hepatotoxicity, which in turn causes severe liver injury. Various hepatic models mimicking liver architecture have been investigated to examine the potential hepatotoxic effects of chemicals and drugs, but there is always a demand for *in-vitro* high-throughput hepatic platforms for measuring the hepatotoxicity. This study aims to provide a simple, easy to fabricate and work, a micro-space three-dimensional (3D) scaffold culture system as an *in-vitro* model for APAP induced hepatotoxicity studies on hepatocarcinoma cell lines (HCC - HepG2 and Huh-7). A silicon mould based template was used to cast out polydimethylsiloxane (PDMS) hexagonal scaffolds. Cytotoxicity was performed by MTT assay. Fluorescence microscopy is used to know the differences in the morphology of the cells grown on scaffolds. mRNA levels of cytochrome P450 2E1 (CYP2E1) expressions were demonstrated through qRT-PCR technique. This study examined the characteristics and usefulness of HepG2 and Huh-7 cell lines grown on the PDMS scaffold system, a three-dimensional culture method as an *in vitro* human model for APAP-induced hepatotoxicity studies. Scaffold cultured HepG2 and Huh-7 cells showed higher expression of mRNA levels of CYP2E1, more susceptibility for APAP. We have verified that *in-house* developed PDMS scaffold culture method for APAP toxicity, by measuring cytotoxicity studies, measuring DNA proliferation, and by showing the expression of CYP2E1 in our system.

**INTRODUCTION:** Acetaminophen (APAP, paracetamol), also commonly known as *Tylenol*, is the most commonly taken analgesic worldwide and is recommended as first-line therapy in pain conditions by the World Health Organization (WHO). It is also used for its antipyretic effects, helping to reduce fever [1-2].

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However, overdoses of APAP of 10-15g can cause serious toxicity and is harmful to the liver and the kidneys [3-4]. APAP can induce different cytotoxic mechanisms on different hepatocarcinoma cell lines (HCC). It can induce caspase-dependent apoptosis on hepatoma Huh-7 and SK-Hep1 cells [5-6] and induces apoptosis and necrosis on hepatoma HepG2 cells and Hep3B cells [7-8]. Although many studies were reported, the underlying cytotoxicity mechanisms caused by APAP are still unclear.

To evaluate pathophysiological mechanisms and screen new drugs against diseases, detailed *in vivo* and *in-vitro* models of disease are essential. Most knowledge of APAP-induced liver injury has been

obtained in studies on rodents. However, differences in the pathogenesis of APAP-induced hepatotoxicity between rodents and humans appear to be considerable. To address the limitations of animal models of APAP-induced hepatotoxicity, a human *in-vitro* system may be useful. Human HCC (HepG2 and Huh-7 cells) has been proposed as an alternative to primary human hepatocytes for *in-vitro* models of normal liver cells.

The potential advantages of hepatoma cells are that as an immortalized cell line, they are readily available in large quantities, they are easy to maintain because they can be cryopreserved, and their drug-metabolizing enzyme activities do not decrease in cultivation, as happens in primary cultures of human hepatocytes. But the major disadvantage is that the mechanisms underlying drug metabolism and toxicity may be abnormal in transformed cells. Despite these issues, the hepatoma cell lines HepG2 and Huh-7 are used widely in studies of liver function, metabolism, and drug toxicity. They also possess many of the biochemical and morphological characteristics of normal hepatocytes, and hence they are widely used in studies related to toxicity by drugs [9-10].

Recent reports have demonstrated that the micro-space cell culture plate method- a three-dimensional (3D) culture system can induce hepatocyte-specific functions including CYP2E1 expression in HepG2 cells, which results in an increased cytotoxicity by acetaminophen compared with a conventional two-dimensional (2D) culture system [11]. These results suggest that 3D-cultured HCC cells may be a useful tool and serves as an *in-vitro* human model of APAP-induced hepatotoxicity. Other studies have reported on a new 3D-cultured HepG2 system using a nano culture plate (NCP) that shows higher expression of albumin and some CYP enzymes in NCP-cultured hepatoma cells compared with conventionally cultured cells [12]. However, it remains to be demonstrated whether cell injury induced by APAP in 3D-cultured HCC *in-vitro* was produced by corresponding human and rodent mechanisms of *in-vivo* APAP-induced hepatotoxicity and if antidotes such as N-acetylcysteine (NAC) or other agents also show protective potential against APAP-induced hepatotoxicity in the 3D-cultivation system [13].

This study was conducted to gain insight into 3D-cultured HCC cells as a human system and to study APAP-induced hepatotoxicity. We evaluated whether 3D-cultured HepG2 and Huh-7 cells by using PDMS scaffolds demonstrate any distinctive mechanistic characteristics such as, a) higher sensitivity against APAP-induced cell injury compared with conventionally cultured cells and b) higher expression of mRNA levels of cytochrome P450 2E1, which metabolizes APAP to a toxic metabolite.

## MATERIALS AND METHODS:

**Chemicals and Materials:** Acetaminophen (>99%), purchased from Sigma-Aldrich, PDMS (SYLGARD™ 184 Silicone Elastomer Kit).

**Scaffold Design and Fabrication:** A hexagonal template of side 20µm, with a height of 10µm, was fabricated out of silicon using Deep Reactive Ion Etching (DRIE) and the moulds were cast out of polydimethylsiloxane (PDMS) from this template. Each hexagon has two microchannels (width 3µm), one on each side of the opposite hexagon, connecting the adjacent two hexagons for enhanced perfusion of the nutrients. The hexagonal design was chosen to mimic the *in-vivo* architecture of liver cells. The detail of the scaffold fabrication process is as shown in **Fig. 1**.

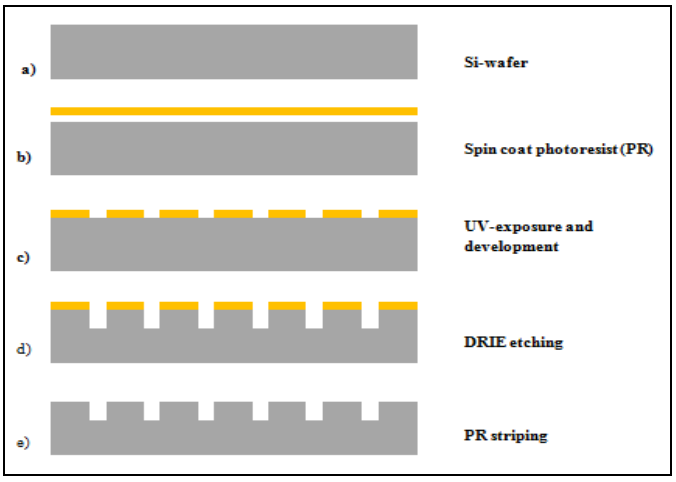


**FIG. 1: MICROFABRICATION PROCESS FLOW OF SILICON-BASED MOULD**

**Cell Culture and APAP Stock Preparation:** HepG2 and Huh-7 cells were cultured in Dulbecco's modified Eagle's medium-high glucose (DMEM, Sigma–Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo scientific, North American origin) and antibiotics

penicillin-streptomycin powder (Hi-Media) 0.4µg/ml final concentration. The cells were cultured at 37 °C, 95% relative humidity, and 5% $CO_2$. Cells from an 80% confluent dish were taken for experiments. Confluent cultures were washed with phosphate-buffered saline (PBS), pH 7.4, detached with trypsin, centrifuged, and sub-cultured. In the conventionally cultured group, the HepG2 and Huh7 cells were seeded with 1ml culture medium in 12-well plates. In contrast, 12-well plates with scaffolds incorporated were used in the scaffold-cultured group, and the cells were seeded at the same density compared with the conventionally cultured group.

The stock solutions were prepared just before use by dissolving the compound in DMSO and diluted into growth media. A controlled media was prepared, which contained the same concentration of DMSO without any compound. The maximum final DMSO concentration in experimental conditions was 0.01%.

**Processing Scaffolds for Cell Culture:** PDMS scaffolds were cut out using a circular punch of 18mm and then stuck to cleaned coverslips using a drop of PDMS. This is followed by ultra-sonicate washing in 70% alcohol and two washes with de-ionized water for 15 min each. Scaffolds are further subjected to steam sterilization by autoclaving. The scaffolds were next stuck to the bottom of the 12-well plates or 60mm culture dishes depending on the experiment and then coated with collagen by adding 700µl of 30µg/ml of Type I collagen (Rat Tail – Gibco by Life Technologies) for 4 h at 37 °C to aid cell attachment. Excess collagen is removed from the culture wells/dishes by washing with PBS prior to cell seeding.

**Staining and Fluorescence Microscopy:** Scaffolds were fixed in 4% paraformaldehyde for 10min at room temperature after 72 h of culture, followed by permeabilization with 0.5% TritonX-100 for 10 min at 37 °C. Nucleus was stained with DAPI (4,6-diamidino-2-phenylindole, dihydro-chloride) (Molecular Probes) for 10 min at 37 °C. Images were acquired with a Leica AF 6000 inverted microscope series.

**Cytotoxicity and Cell Viability Assay:** MTT [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay [14-16] was performed to determine the cytotoxicity levels of APAP. Hepatoma cells (HepG2 and Huh-7) were taken from an 80% confluent flask. Cells were trypsinized, and approximately 5000 cells in 200µl medium per well were plated in 96-well plate. After 24 h of incubation, cells were treated with different concentrations of APAP and incubated for 48h. After the incubation period, drug and medium were pipetted out from the wells. MTT reagent was added and incubated for 4h. The MTT reagent was removed, and DMSO added to dissolve the formazan formed in live cells. Absorbance at 570nm was measured using a Micro-plate reader. The results were produced from independent experiments, and each experiment was performed in triplicate for each cell line. The concentration required for a 50% inhibition of viability ($IC_{50}$) was determined graphically. The effect of the samples on the proliferation of cell lines was expressed as the % cell viability, using the following formula:

% of cell viability = Absorbance of treated cells/Absorbance of control cells x 100 …. (1)

% of cell inhibition = 100 – % of cell viability …..(2)

A similar procedure was followed for scaffolds in 12-well plates as it is very difficult to incorporate PDMS scaffolds into 96-well plates.

**DNA Quantification:** After 24 h of cell attachment in 12 well plates with and without scaffolds. The wells are treated with 15mM (HepG2) and 10mM (Huh-7) APAP for 48h except for control wells. The proliferation of the cells in control and treated conditions was evaluated by measuring the cellular DNA content using the Picogreen dsDNA quantification kit (Invitrogen), as described recently [17]. Briefly, cells were lysed using lysis buffer (0.02% SDS with Proteinase K 0.2mg/ml). The lysate was mixed with the picogreen dye to determine DNA content by measuring the fluorescence intensity in a plate reader with 485nm excitation and 528 nm emission.

**Quantitative real-time PCR Analysis:** Cells (HepG2 and Huh-7) were grown in a 60mm Petri-plates with and without scaffolds for 72 h. Quantitative reverse transcription-polymerase chain reaction technique (qRT-PCR) was performed to measure the mRNA expression levels as previously

described with minor modifications [22]. Briefly, total RNA was extracted using an RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. The qRT-PCR for human cytochrome P450, family 2, subfamily E, polypeptide 1 (CYP2E1). Total RNA was transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and qRT-PCR analysis was then performed in Bio-Rad CFX qPCR System using Power up SYBR Green master mix (Thermo Scientific) with 10ng of the cDNA as a template.

**TABLE 1: PRIMER SEQUENCES USED FOR qPCR**

| Class | Name | Primer forward | Primer reverse |
|---|---|---|---|
| CYP | CYP2E1 | CATGAGATTCAG CGGTTCATC | GGTGTCTCGGG TTGCTTCA |
| Liver marker | Albumin | TGCTTGAATGTG CTGATGACAGG | AAGGCAAGTC AGCAGGCATCT CATC |
| House-keeping | GAPDH | ATTTGGCTACAG CAACAGGG | CAACTGTGAGG AGGGGAGA |

The PCR quality and specificity were verified using melting curve analysis. Gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Fold change was calculated using $2^{-\Delta\Delta ct}$. Liver marker albumin (Alb) was used for gene expression study. The details of primer sequences used are shown in **Table 1**.

**Statistical Analysis:** The experimental data were reported as mean ± SEM of three parallel measurements and were performed using GraphPad Prism ver 5.01 (GraphPad Software, San Digo, CA, USA). One way analysis of variance (ANOVA) was analyzed using Tukey multiple range test.

## RESULTS AND DISCUSSION:

**SEM Images of PDMS Scaffolds: Fig. 2** shows the scanning electron microscopic images of the PDMS scaffolds. They were fabricated in silicon using DRIE, and moulds were cast out of PDMS from this template. Each hexagon has two micro-channels (width 3µm), one on the opposite side of the hexagon, connecting the adjacent two hexagons for enhanced perfusion of nutrients.

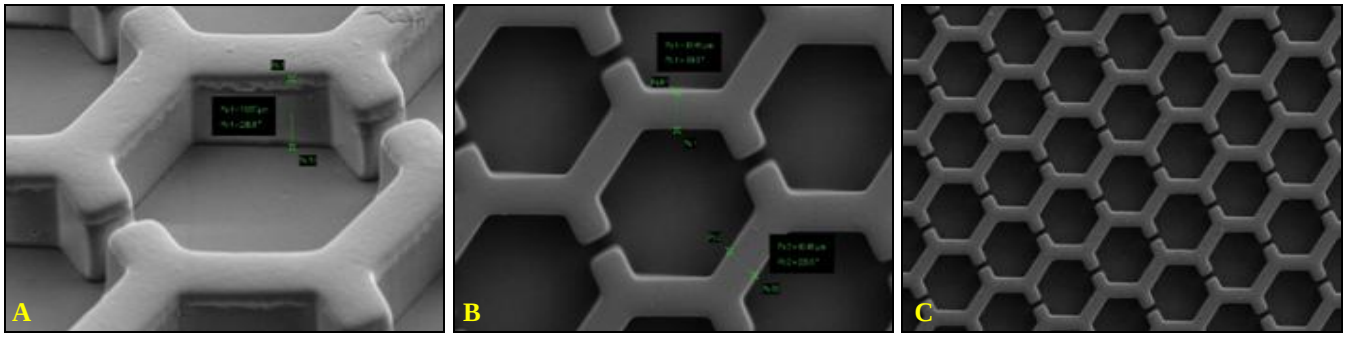


**FIG. 2: SEM IMAGES OF PDMS SCAFFOLDS (A) ENLARGED VIEW OF THE HEXAGONAL TEMPLATE (B) ARRAY OF HEXAGONAL (C) 10µm HEIGHT SCAFFOLD**

**Cell Morphology: Fig. 3a, 3b,** and **Fig. 3c, 3d** represents HepG2, and Huh-7 cells cultured on 10µm PDMS scaffolds for 72 h, respectively. In conventionally cultured cells, cells have adhered to the bottom of the plate, whereas in PDMS scaffold culture cells were aggregated and grow in clumps.

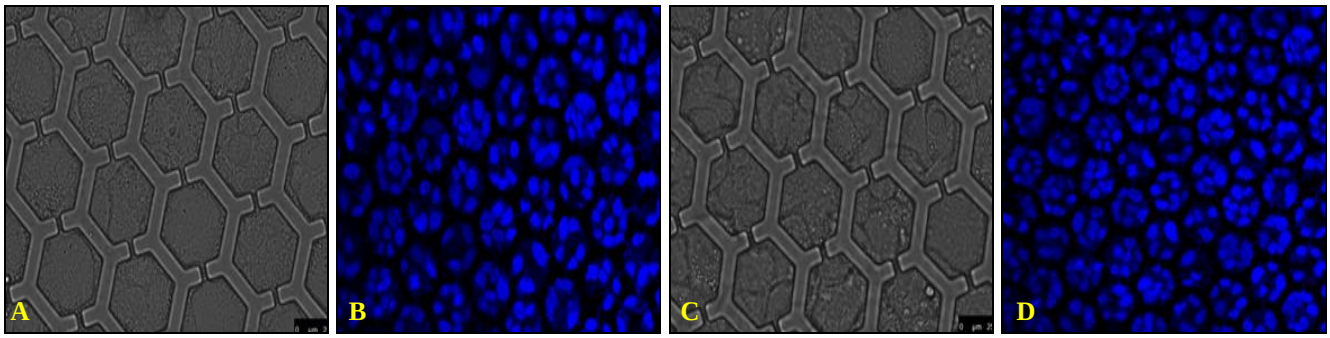


**FIG. 3: CELL MORPHOLOGY ON SCAFFOLDS A). BRIGHT-FIELD IMAGE OF HepG2 CELLS GROWN ON 10µm SCAFFOLDS B). HepG2 CELLS STAINED FOR NUCLEUS WITH DAPI ON 10µm SCAFFOLDS C). BRIGHT-FIELD IMAGE OF Huh-7 CELLS WAS GROWN ON 10µm SCAFFOLDS D). Huh-7 CELLS STAINED FOR NUCLEUS WITH DAPI ON 10µm SCAFFOLDS**

**Cytotoxicity and Cell Viability Assay:** We compared the effects of APAP on cell viability in HepG2 and Huh-7 cells 48 h after APAP exposure in conventionally cultured groups. The results revealed that a dose-dependent exposure of APAP on both cells lines **Fig. 4a** and **4b**. 50% of cells were viable at 15mM and 10mM concentration of APAP cells on HepG2 and Huh-7 cells, respectively.

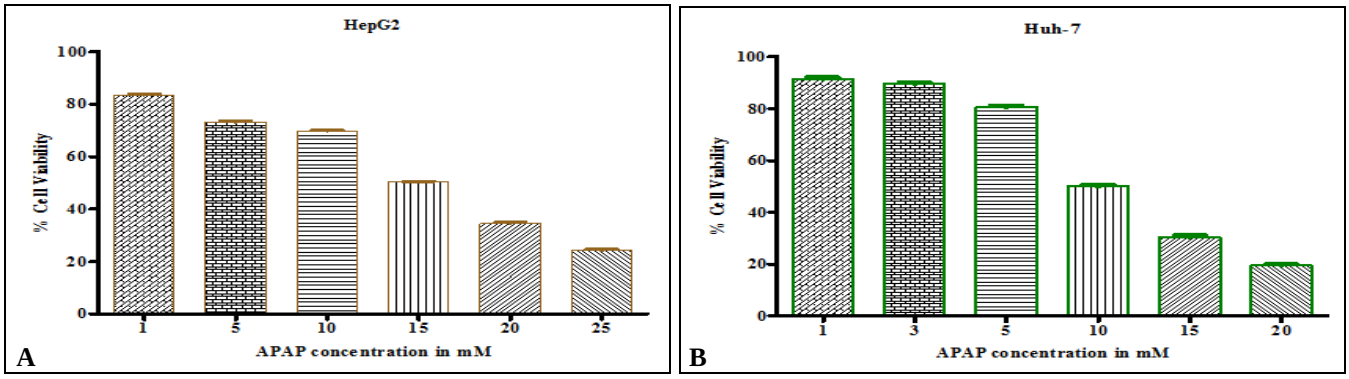


**FIG. 4: CYTOTOXICITY OF APAP: DOSE-DEPENDENT EFFECT ON A) HepG2 AND B) Huh-7 CELL LINES. MTT ASSAY WAS PERFORMED TO DETERMINE THE CYTOTOXICITY LEVELS IN HCC CELL LINES. CELLS WERE TREATED WITH DIFFERENT CONCENTRATION OF APAP AND INCUBATED FOR 48 h.** Each bar represents the Mean ± SE (n=3).

HepG2 and Huh-7 cell lines grown on PDMS scaffolds were subjected to three (10mM, 15mM, 20mM) different concentrations of APAP for 48 h and cytotoxicity (MTT) were performed on scaffold cultured cells. **Fig. 5a** (HepG2) and **5b** (Huh-7), there was a difference in the percentage of cell viability in both the cell lines between two types of cultures. Cell viability was slightly but significantly different between the two-culture system, *i.e.*, Conventional monolayer cultures and PDMS scaffold cultures. Cells grown in the presence of scaffolds showed slightly lower cell viability than with a conventionally cultured type. This shows scaffold cultures have a higher susceptibility to APAP-induced cell injury compared with conventional monolayer plate cultured cells.

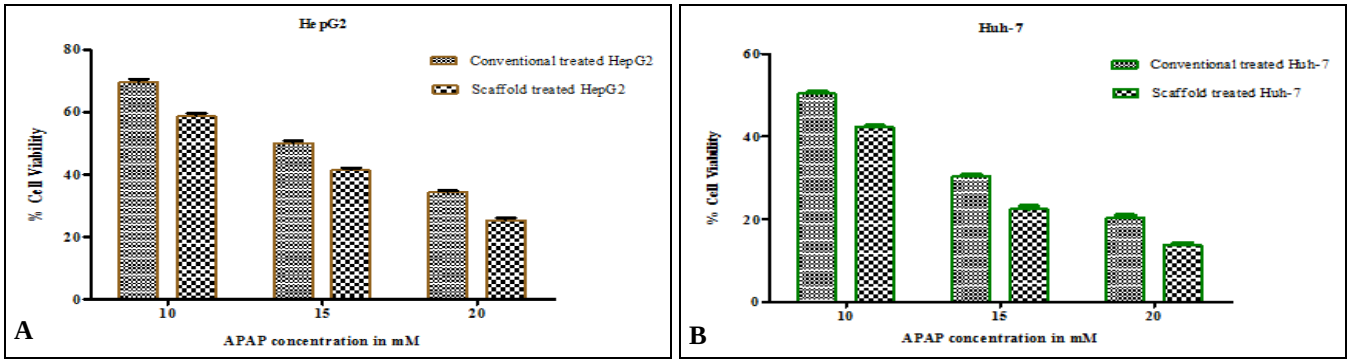


**FIG. 5: SENSITIVITY TO APAP-INDUCED CELLULAR INJURY A) HepG2 AND B) Huh-7 CELL LINES. CELL LINES GROWN ON PDMS SCAFFOLDS WERE SUBJECTED TO THREE DIFFERENT CONCENTRATION OF APAP (10, 15, 20mM) FOR 48 h AND CYTOTOXICITY (MTT) PERFORMED ON SCAFFOLD CULTURED CELLS. CELLS GROWN IN THE PRESENCE OF SCAFFOLD SHOWED SLIGHTLY LOWER CELL VIABILITY THAN WITH THE CONVENTIONAL CULTURE TYPE IN BOTH CELL LINES. THIS SHOWS THAT SCAFFOLD CULTURES HAVE HIGHER SUSCEPTIBILITY TO APAP-INDUCED CELL INJURY.** Data represent Mean ± SE (n=3).

**DNA Quantification:** Both cell lines grown on scaffolds upon APAP treatments lead to a decrease in DNA content (**Fig. 6a** for HepG2 and **6b** for Huh-7). In comparison to the conventionally cultured plates after APAP treatment for 48h results in lower DNA content suggesting enhanced cell death. HepG2 cells were treated with 15mM APAP concentrations and Huh-7 cells with 10mM concentrations for both scaffold and conventional cultures. DNA quantification, in turn, confirms the more susceptibility to APAP from scaffold culture system.

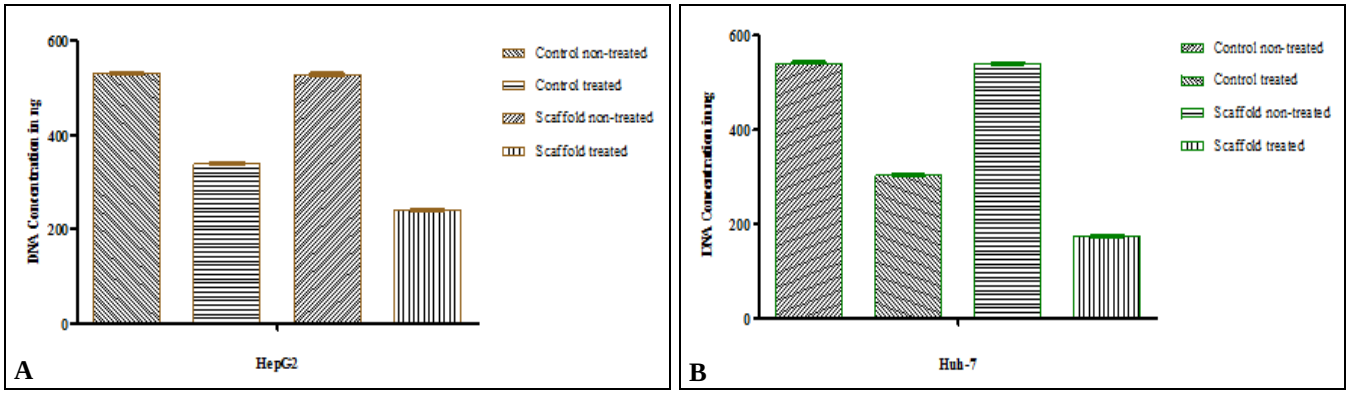


**FIG. 6: DNA QUANTIFICATION ON A) HepG2 AND B) Huh-7 CELL LINES. THE 12 WELL PLATES WITH AND WITHOUT SCAFFOLDS ARE TREATED WITH 15mM (HepG2) AND 10mM (Huh-7) APAP FOR 48 h EXCEPT FOR CONTROL WELLS. THE PROLIFERATION OF THE CELLS IN CONTROL AND TREATED CONDITIONS WAS EVALUATED BY MEASURING THE CELLULAR DNA CONTENT USING PICOGREEN dsDNA QUANTIFICATION KIT. BOTH THE CELL LINES GROWN ON SCAFFOLDS UPON APAP TREATMENTS LEAD TO A DECREASE IN DNA CONTENT WHICH IN TURN, CONFIRMS THE MORE SUSCEPTIBILITY TO APAP FROM SCAFFOLD CULTURE SYSTEM. WHEREAS DNA CONTENT IN CONTROL (2D AND 3D) WERE ALMOST THE SAME, WHICH SHOWS 3D (WITH SCAFFOLDS) SUPPORTS CELL PROLIFERATION AS 2D.** Data denotes Mean ± SE (n=3).

**Quantitative Real-Time PCR Analysis:** As shown in **Fig. 7a** in the case of HepG2 cells and **7b** in Huh-7 cells, the mRNA expression level of CYP2E1 in PDMS scaffold cultured group was 8-fold and 6-fold greater than that of the conventionally cultured group, respectively. Next, we compared the expression level of liver marker Albumin (Alb) expression levels; the results show 4-fold increased expression levels in the HepG2 cell line and 6-fold greater expression in the presence of scaffold than that of the conventional culture system.

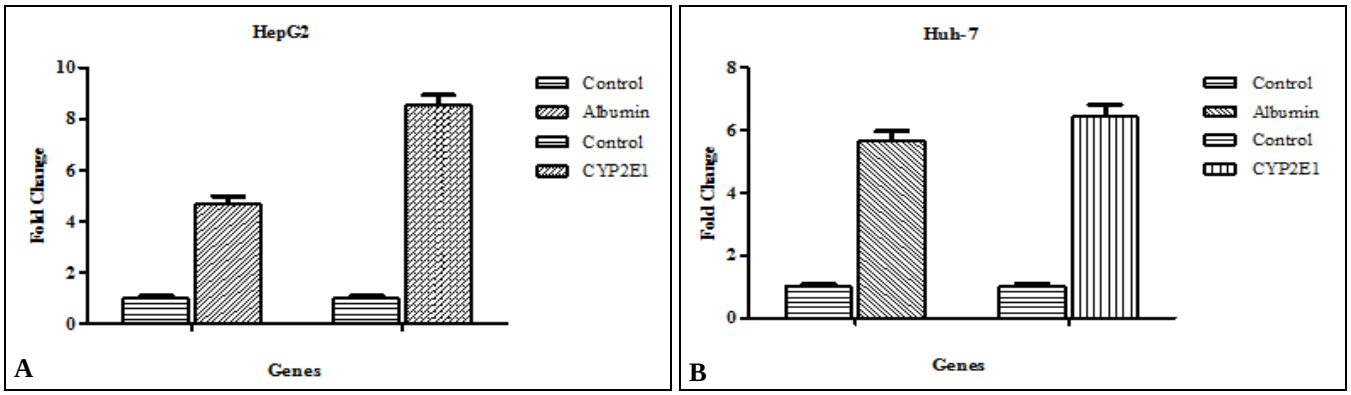


**FIG. 7: GENE EXPRESSION PROFILE GENERATED FROM CONTROL AND SCAFFOLD CULTURES A) HePG2 AND B) HUH-7. CELLS WERE GROWN IN A 60mm PETRI-PLATES WITH AND WITHOUT SCAFFOLDS FOR 72H AND qRT-PCR WAS PERFORMED TO MEASURE THE MRNA EXPRESSION LEVELS. THE mRNA EXPRESSION LEVEL OF *CYP2E1* IN PDMS SCAFFOLD CULTURED GROUP WAS 8-FOLD (HepG2) AND 6-FOLD (Huh-7) AND ALSO, ALBUMIN (Alb) EXPRESSION LEVELS SHOWED 4-FOLD (HepG2) AND 6-FOLD (Huh-7) GREATER EXPRESSION IN PRESENCE OF SCAFFOLD THAN THAT OF CONVENTIONAL CULTURE SYSTEMS.** Data represent Mean ± SE (n=3).

In general, HCC cell lines have been considered an inefficient *in-vitro* model to study the mechanisms of APAP-induced liver injury. Recently, Nakamura *et al.*, [18] demonstrated that the 3D-culture method enhances hepatocyte-specific functions of hepatocytes, including drug-metabolizing enzyme activities in HepG2 cells. These cell lines also identified higher sensitivity to acetaminophen cytotoxicity in the 3D-cultured system compared with conventional monolayer cultured cells and suggested that this 3D-cultured hepatoma cell system may be useful as a human model of APAP-induced hepatotoxicity. Molecular mechanisms of APAP-induced hepatocyte cell death have been evaluated by many researchers and seem to have become clearer [19-21]. The effects of APAP toxicity in the liver had not previously been defined. The Huh-7 model recapitulated mitochondrial dysfunction, DNA damage, and loss of viability, which characterized aspects of APAP toxicity in

people previously [22-23]. Various cell lines have been used for APAP toxicity studies, although outcomes may depend in part on gene expression range of cells, the duration, and amount of drug exposure [24].

There is an increasing amount of evidence showing that when primary cells are grown in 3D culture, they retain physiological characteristics for a longer period compared with when the same cells are grown using classical (2D) cell culture techniques. Regardless of that immortal cells are transformed and may contain numerous chromosomal aberrations, it appears that they are able to recover at least some of these physiological attributes when grown in 3D, apparently repeating their differentiation pathway. Growing cells in 3D culture systems have not yet been extensively practiced. This is probably because of the additional tasks in working with 3D cultures that are not present when using the classical 2D culture systems. It is a known fact that 3D cell culture allows the cells to develop a more intricate extracellular matrix and better intercellular communication, and this leads to recovery or maintenance of *in-vivo* function. Three-dimensional (3D) structure, therefore, will provide *in-vitro* models that appropriately mimic *in-vivo* conditions, and it serves as a better platform for drug toxicity related studies [25-26].

In the present study, we have developed a simple fabrication method to get PDMS scaffolds. PDMS is a silicone polymer that is biocompatible and has high permeability for gases. Furthermore, these PDMS structures are easy and inexpensive to manufacture and reusable since they can be autoclaved.

We demonstrated that scaffold-cultured HepG2 and Huh-7 cells showed higher susceptibility to APAP-induced cell injury compared with conventional monolayer plate cultured cells, as well as higher expression of CYP2E1 mRNA. The PDMS scaffold system, a 3D-cultured method, may explain mechanisms of cytotoxicity more accurately than conventionally cultured methods and be more useful in toxicological and pharmacological safety assessment. Based on these facts, we believe that the PDMS-scaffold system, which requires neither specialized techniques nor expensive apparatus, may be a valuable tool as a convenient human *in-vitro* model of APAP-induced hepatotoxicity.

**CONCLUSION:** In summary, we have demonstrated that PDMS-scaffold cultured hepatoma cell lines show key mechanistic features of APAP-induced hepatotoxicity, such as increased susceptibility to APAP doses and elevated levels of CYP2E1 expression compare to conventional culture method. Our future studies will be aiming to measure intracellular GSH and mitochondrial membrane potential, activation of JNK, and cellular injury, after APAP treatment. Including and demonstrating the antidotes of APAP-induced hepatotoxicities, such as NAC, SP600125, and CyA. However, the present study suggests that cellular injury induced by APAP treatment using the PDMS-scaffold is a useful human model to study toxicity mechanisms and to screen drug candidates against APAP-induced hepatotoxicity.

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**CONFLICTS OF INTEREST:** Authors declare no conflict of interest.

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