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# A GEL CASTING TOOL FOR FABRICATION OF THREE DIMENSIONAL SANDWICH IN VITRO CULTURE SYSTEM FOR CYTOCOMPATIBILITY EVALUATION OF HYDROGELS

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**Keywords:**

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**ABSTRACT:** Two-dimensional (2D) culture systems are indispensably used as *in-vitro* tools to improve our perception and understanding of cell biology, tissue engineering, tissue morphology, mechanisms of diseases, and drug action. However, the conventional 2D culture systems suffer from limitations such as alterations in cell morphology and lack of interactions between extracellular and cellular environments. As a result, three-dimensional (3D) cell cultures have received remarkable attention in studies such as drug discovery and development; the sandwich model being one of the unique cellular models widely used for analyzing tissue metabolism and functions. In this study, we devised a novel Gel Casting Tool (GCT) for *in-vitro* toxicity analysis. Liver cells need complex 3D micro-architecture to express tissue-specific functions and were suitable for this study. Human Carcinoma Cell line (HepG2 cells) with Gelatin methacrylamide (GelMA) hydrogel was used to check the efficacy of the GCT. The results showed that GCT Sandwiched HepG2 exhibited high cell viability, proliferation, and tissue-specific functions such as albumin and urea over 7 days. The major advantage of this culture model is the maximized availability of cells for observation under microscope and imaging cells in one plane. This culture system has potential application in *in-vitro* toxicity testing, early drug screening, and evaluation of scaffolds for tissue engineering.

**INTRODUCTION:** Cell-based in vitro testing is the first and mandatory analysis that is followed in drug discovery and cytotoxicity evaluation. Cell-based *in-vitro* models represent a simple but very effective test.

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System in all disciplines of *in-vitro* analysis. Conventional two-dimensional (2D) cell culture relies on the adherence of cells to a flat treated surface, which provides mechanical support to the cells. However, the flat treated surface was a stiff platform that offered unnatural growth kinetics and cell attachments and did not represent the natural microenvironments of the cells.

Hence, despite the simplicity of analysis, the bioactivity of the cells in 2D is affected. Recently, significant research efforts by many groups have resulted in improvements in the form of better *in-*

*vitro* cell culture models that resemble *in-vivo* conditions. Three-dimensional cell culture models are such products with a better capacity of mimicking tissue physiology in multi-cellular organisms [1]. It is well known that cells in the 3D culture environment differ morphologically and physiologically from cells in the 2D culture environment and are thus preferred to 2D culture as it represents a cellular microenvironment similar to the tissues [2]. Three-dimensional culture systems serve as excellent *in-vitro* models, allowing the study of cellular responses in a setting that resembles *in-vivo* environment [3].

Three-dimensional culture systems are classified into scaffold-based and scaffold-free techniques. Scaffold-based approaches include extracellular matrix-based cell sandwich culture and cell encapsulation within matrix, whereas the scaffold-free technique includes use of self-assembled cell aggregates called spheroids. Cell encapsulation system provides cells with three-dimensional structures by immobilization of the cells within a hydrogel that helps to maintain the metabolic activity and normal cellular function [4]. On the other hand, sandwich culture systems provide 3D model by tissue-like micro-environment within the dual layers of extracellular matrix [2]. *In-vitro* evaluation system requires very reliable data acquisition related to cell viability, imaging, cell - proliferation and cell-specific functions. Cell entrapped systems have variation in reliability and reproducibility because of the non-uniform distribution of cells and difficulty in imaging cells (in 3D axis) entrapped in thick constructs. On the other hand, cells entrapped in sandwich systems maintain a better cell-cell contact as well as allow easy and reliable data acquisition and feasibility.

For these reasons, sandwich models are a well-established *in-vitro* model for the study of hepatobiliary drug transport, transport drug-drug interactions and hepatotoxicity [5]. The reliability and repeatability of the acquired data in a sandwich culture model depend largely on the factors such as the thickness of the encapsulation, materials used for encapsulation, and the nature of the encapsulated cells. To address these concerns, we developed a device that could readily/consistently cast a uniform gel for encapsulation and ensure the encapsulated cells are available on a single plain. This study aims at the preparation and evaluation of a sandwich model prepared using a unique Gel Casting Device (GCT). To evaluate the device, hepatocarcinoma cells (HepG2) was encapsulated in Gelatin methacrylamide (GelMA) using GCT.

**MATERIALS AND METHODS:** Gelatin (Type A, 175 bloom from porcine skin), methacrylic anhydride (MAA), dialysis tubing (12-14 kDa), Fluorescein Diacetate (FDA), Cell Counting Kit (CCK-8) were purchased from Sigma, India. 2-Hydroxy -4′-(2–hydroxyl lethoxy) -2 methyl propiophenone) (Irgacure 2959) was purchased from Ciba Specialty Chemicals, Tarrytown, NJ. Propidium Iodide (PI) was procured from Hi-Media, India. Minimum Essential Medium (MEM), Penicillin-Streptomycin (Pen Strep - 10,000 U/ml), Trypsin-EDTA, Fetal Bovine Serum (FBS) were from purchased from Gibco, Thermo scientific, USA. Human liver carcinoma cells (HepG2) were procured from National Center for Cell Sciences, India. All cell culture grade plastic ware was procured from Eppendorf India Limited.

**Sandwich Model:** Gelatin Methacrylamide (GelMA) was synthesized by previously reported method [6]. Gelatin methacrylamide solution was prepared by dissolving 10% (Wt/V) GelMA and 1% (Wt/V) Irgacure in MEM-SFM medium at 37 °C. The GelMA-Irgacure solution was subsequently cross linked with UV light (Biogrow UV lamp, USA) of 365 nm wavelength at an intensity of 6 mW/cm$^2$ for 3 min to form hydrogels. A conventional sandwich model (Sw) was created by entrapping HepG2 in sandwich culture. Briefly, 100 µl GelMA solution was added to each of the wells of a 96 well plate and irradiated by UV lamp at 365 nm UV light for 3 min, to ensure cross-linking. HepG2 cells ($1\times10^5$) were seeded on the cross-linked GelMA. The second layer of 50 µl GelMA solution was added to the cell-layer and cross-linking process repeated to entrap cells in a sandwich fashion. A sufficient cell culture medium was added, and sandwiched cells were incubated at 37 °C, 5% $CO_2$ for 1, 3, 5, and 7 days. The cell viability, proliferation, and liver-specific function was analyzed at the end of these time periods,

**Design and Fabrication of Gel Casting Tool:** Gel Casting Tool, consisting of a piston and shell, was fabricated from Teflon **Fig. 1**. When the piston is

inserted into the shell it creates a precise "Casting cup" at one end. Hydrogel poured into it, and cross-linked will serve as the first layer of sandwich culture. The cast hydrogel construct is 5 mm in diameter with 1 mm wall thickness and 1 mm base thickness. This creates an inner base area of 19.625 mm2 for cell seeding, which can accommodate approximately $1\times10^4$ to $1\times10^5$ cells. The hydrogel added inside the casted chamber forms the second layer of hydrogel in the sandwich.

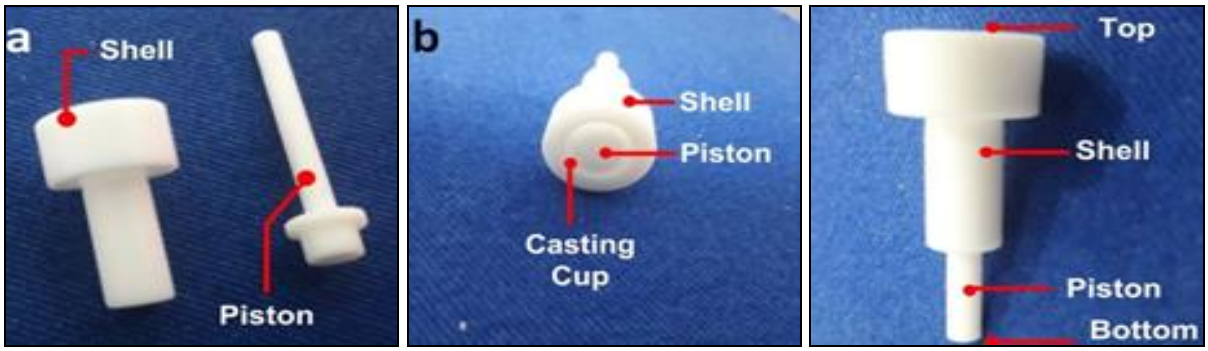


**FIG. 1: THE DESIGN OF THE GEL CASTING TOOL. (A) THE TWO COMPONENTS OF GCT, THE SHELL, AND THE PISTON. (B) ASSEMBLED GCT SHOWING THE "CASTING CUP" AT ONE END WHERE HYDROGELS WILL BE CROSSLINKED. (C) SIDE VIEW OF GCT PLACED IN UPRIGHT POSITION.**

**Fabrication of Gel Casting Tool Sandwich:** Another sandwich culture was created using a GCT, hereafter called as Gel Casting Tool Sandwich (GCTSw) model, as illustrated in **Fig. 2.** To fabricate the hydrogel using GCT, approximately 100 µl of Gel MA-Irgacure solution was added to "cast cup" formed at the end of the GCT **Fig. 2C**and cross-linked using UV. The GCT was inverted, and the piston is pushed to release the casted hydrogel to a 6 well plate **Fig. 2F.** Around $1\times10^5$ HepG2 cells were seeded inside the cup-like structure and incubated for 2 h in a $CO_2$ incubator. The cells were sandwiched by adding a second layer of 50 µl Gel MA-Irgacure solution and again cross-linked using UV. A sufficient cell culture medium was added, and sandwich systems were incubated for 7 days. The hydrogel was cast using the GCT in a sequence of steps **Fig. 2.**

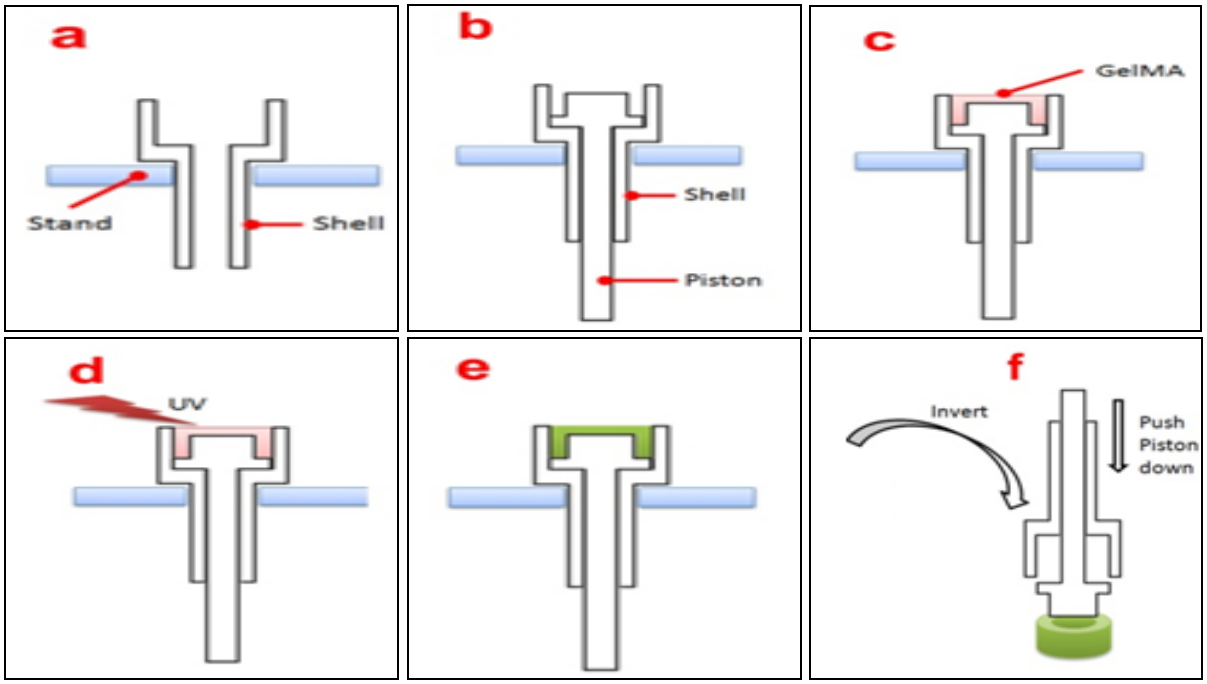


**FIG. 2: SCHEMATIC REPRESENTATION OF WORKING WITH GEL CASTING TOOL. (A) SHELL KEPT ON THE STAND. (B) PISTON INSERTED INTO THE SHELL TO COMPLETE THE ASSEMBLY. (C) APPROXIMATELY 100 µL OF GELMA-IRGACURE SOLUTION WAS TRANSFERRED INTO THE "CASTING CUP". (D) UV EXPOSURE (365 NM FOR 1MIN) FOR CROSSLINKING. (E) INVERT THE GCT ASSEMBLY. (F) THE CROSSLINKED HYDROGEL IS PUSHED DOWN USING THE PISTON**

**Efficacy Evaluation of Gctsw System:** The efficiency of GCTSw was evaluated using HepG2 cells obtained from NCCS, Pune. The cells were grown in MEM supplemented with 10% FBS, 10000 µ/ml pen/strep, 7.5 g/ml $NaHCO_3$ and 3 mM L-glutamine at 37 °C in a 5% $CO_2$ atmosphere.

**Histology:** Sandwich system and Gel Casting Tool Sandwich system was removed from the culture medium, placed into a 24-well plate (Eppendorf), and fixed with 4% paraformaldehyde for 2 h. The construct was washed with 1 × PBS for 5 min. The construct was sequentially incubated in sucrose infiltration solutions of 15% and 30% (w/v) for 2 h at room temperature and 24 h at 4 °C, respectively. Construct was then removed from the infiltration solutions, soaked in Jung Tissue Freezing Medium® (Leica microsystems) for 30 min and transferred to cryomolds. Each cryomold was then filled with Jung Tissue Freezing Medium® and frozen by floating in cold isopentane with liquid nitrogen. After solidification, cryomolds were removed from the slurry and stored at -80 °C until cryosectioning. Cryosectioning was performed on the frozen block using a cryostat (Leica CM 3050 S) at -22 °C. For each block, 20 micron sections were collected on Star Frost glass slides (Waldemar Knittel). The slides were then air-dried before staining. Cryosections were stained with toluidine blue stain (0.1%) for 2 min. The sections were washed twice with distilled water and air-dried. The sections were then overlaid with DPX mounting medium (Sigma-Aldrich) and covered with a coverslip. Images of stained sections were captured using an alight microscope (Nikon Eclipse TS2-FL/TS2, Japan).

**Cell Viability:** Sandwich system and Gel casting tool system with HepG2 cells were incubated for 24 h, and the viability of the cells was analyzed by live/dead staining. Cells were incubated with a combination of Fluorescein Diacetate (FDA) and Propidium iodide for (PI) 5 min and observed under a confocal microscope equipped with filters suitable for green and red emission. The live cells and dead cells were observed under a confocal microscope (U-RFL-T; Olympus, Tokyo, Japan).

**Cell Proliferation:** The proliferation analysis of HepG2 cells in Sw and GCT-Sw system sandwiched using GelMA was carried out using CCK-8 assay according to manufacturer's instruction. A sufficient cell culture medium was added to entrapped cells and was cultured for 7 days with proliferation analysis on every alternate day.

**Tissue Function Analysis of the Sandwich Culture:** Sandwich model and GCT-Sw system construct with HepG2 cells were maintained for 7 days with medium change on 1, 3, 5, and 7 days. The culture medium from both the GCT-Sw system and Sw Model was constructed at regular intervals and stored at 80 °C until used for estimating liver-specific functions such as albumin and urea synthesis. Albumin secreted by HepG2 cells was quantified by a commercially available enzyme-linked immunosorbent assay kit (Bethyl ELISA Kit, USA). The cell's ability to detoxify ammonia was estimated by quantifying urea synthesis using a urea assay kit (Biochain, USA) as per manufacturer's protocol. The functional ability in the GCT-Sw system was compared with that of Sw.

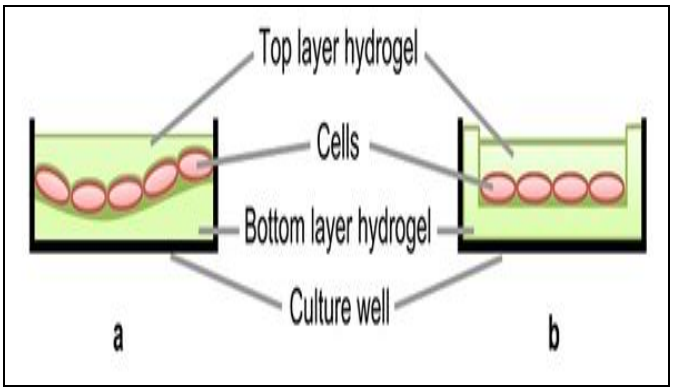


**FIG. 3: SCHEMATIC REPRESENTATION OF SANDWICH MODELS A SW AND B GCTSW MODEL**

**RESULT AND DISCUSSION:** Normal sandwich system in which manually sandwiching liver carcinoma cells (HepG2) between two layers of GelMA (Gelatin Methacrylamide) in a multi-well cell culture dishes [7]. Gel casting tool is devised with the two components of the tool, the first layer of GelMA was prepared in the form of an open trough-like structure, and to this trough, HepG2 cells were seeded and filled with a second layer of GelMA. In this study, we evaluated a novel gel casting device for fabrication of sandwich culture that provides consistent output with respect to cell localization and distribution that translates to enhanced tissue function *in-vitro*. A gel casting tool is devised to cast a cup like a construct having 5 mm inner diameter with 1 mm height using a cross-

linkable hydrogel. HepG2 cells are sandwiched within the 'cast cup' construct. In GCT, cells are provide with a 3D environment and are arranged in one plane that improves cell-cell interaction **Fig. 3.**

**Efficacy Evaluation of Gctsw System:** Sandwich culture is a widely accepted *in-vitro* model for improving cell functions and hence is useful as in vitro toxicity test system [8]. The conventional method of improving liver function *in-vitro* is to sandwich hepatocytes between collagen gels [9]. However, for cross-linkable hydrogels such as alginate, modified gelatin, fibrinogen, etc, the preparation process greatly affects the model system. Hence we propose a GCT that allows fabrication of sandwich culture and provides in vivo like three-dimensional environment and improved cell-cell interaction via localization of cells in a planar region [6]. This system also makes microscopic imaging more reliable as cell distribution is localized to one plane. The device proposed here is economically viable and very easy to fabricate **Fig. 4**.

The efficiency of the GCT-Sw system was compared with the conventional method of sandwich (Sw) culture. The GCT-Sw localized cells to a plane without variation in the distribution of cells. This is important in the case of hepatocyte culture, as the cells lose their viability, cell polarity and cease functional activity within 72 h [2].

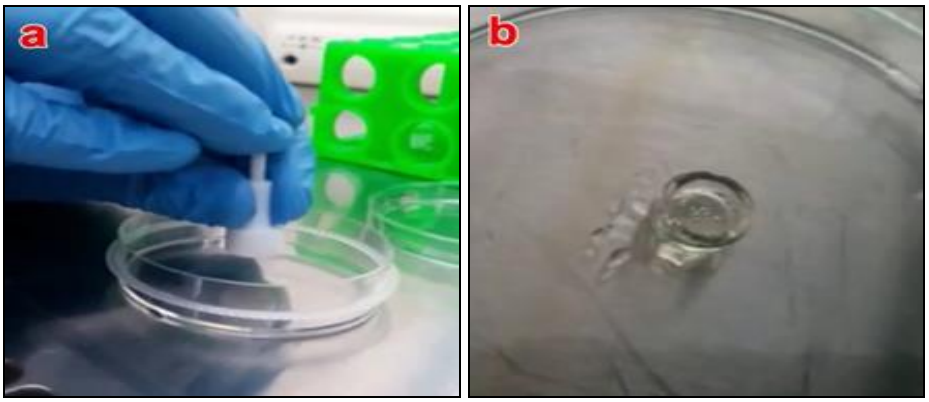


**FIG. 4: (A) PHOTOGRAPH SHOWING THE RELEASE OF CROSSLINKED HYDROGEL FROM THE GCT. (B) PHOTOGRAPH OF THE GELMA CAST USING THE GCT. THIS FORMS THE FIRST LAYER OF SANDWICH CULTURE**

**Histology:** Cell distribution and morphology inside both sandwich culture systems were analyzed by the sections stained with toluidine blue. It is an acidophilic meta-chromatic dye that binds to the DNA content of the cells [10]. It was observed that the cells were uniformly distributed over a plane in GCT Sw construct **Fig. 5** but the cells in Sw were not uniformly distributed over the plane they adhered towards the rim of the constructs revealed by toluidine staining. Further, the pore size of the bottom layer was reduced compared to the top layer, which can be attributed to dual exposure to the UV light during cross-linking.

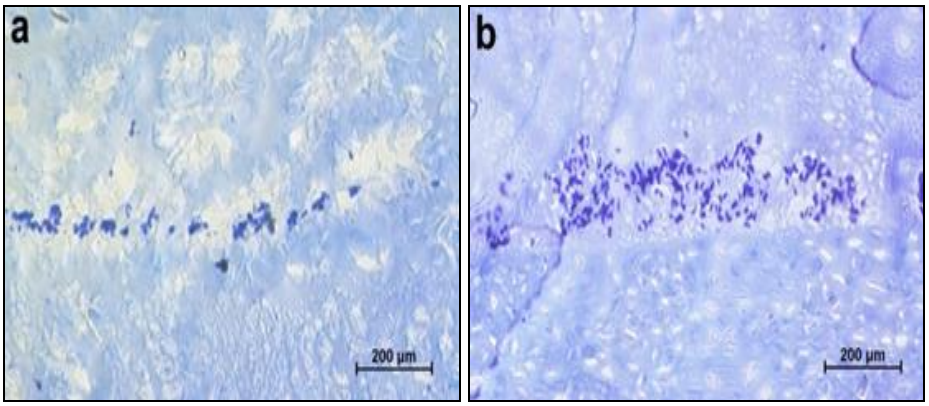


**FIG. 5: CRYO-SECTIONS OF (A) SW AND (B) GCT-SW CONSTRUCT WITH HEPG2 CELLS AFTER TOLUIDINE BLUE STAINING**

**Cell Viability:** Generally, the sandwich culture is prepared in routine multiwell plate or petridishes using the ECM relevant for the cell or tissue type. But depending upon on the type of culture ware used to cast the gel, the size and shape of the sandwich system vary. Moreover, it is difficult to distribute cells in one plane. The biological evaluation of hydrogels requires a battery of tests done on encapsulated cells. To enhance the cell functions, researchers have thought about natural as well as synthetic sandwich culture [11]. The synthetic system has two different components, such as a PET-Gal with a GRGDS-modified PET TE membrane top support. IT is difficult to image distributed cells in a hydrogel for routine analysis such as viability staining, immune fluorescence staining *etc*. The device works with the category of cross-linkable hydrogels that are non-toxic to cells before, during and after cross-linking. Viability assessment is a key parameter to describe the efficiency of sandwich culture [12]. This study analyzed cell viability by combining fluorescein diacetate (FDA) and Propidium iodide (PI) staining.

Live cells uptake FDA and convert it to the fluorescent product by the activity of non-specific esterase. Propidium iodide is only permeable to the plasma membrane of dead cells, and it gets intercalates to DNA [13]. HepG2 cells sandwiched within GelMA hydrogel were viable and localized to a single plane. Normal fluorescent microscopic analysis was possible to elucidate the cell viability as the cells can be visualized in one place. Though the same number was used in Sw and GCT-Sw, the microscopic analysis was more eminent compared to Sw.

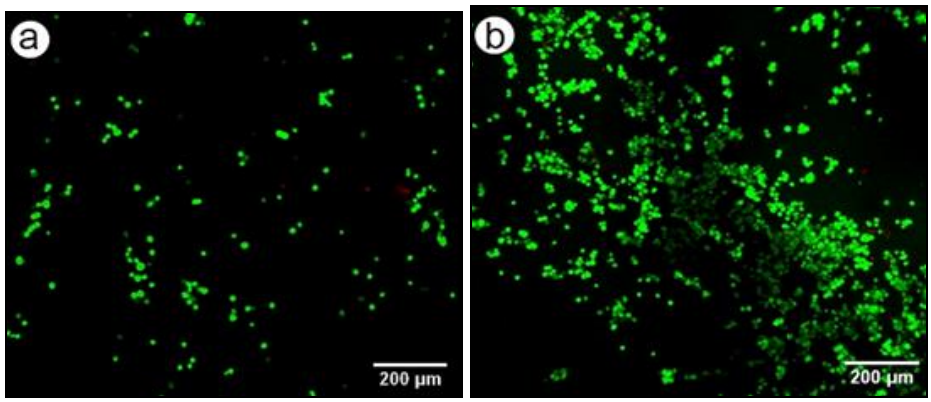


**FIG. 6: VIABILITY OF HEPG2 CELLS IN A) SW AND B) GCT -SW MODEL.CELLS ARE VIABLE (GREEN) AND DEAD CELLS(RED) IN BOTH THE SYSTEMS. CELLS LOCALIZED IN THE GCT SW AS COMPARED TO NSW**

**Cell Proliferation CCK-8 Assay in Gctsw System and (Sw) Model:** The proliferation of HepG2 cells in GCT-Sw and Sw were compared by analyzing the cell activity by CCK-8 Assay. It has been reported that HepG2 cells have significant proliferation in initial 3 days in GelMA sandwich models [14]. The GCT-Sw model expressed similar cell activity compared to that of Sw **Fig. 7.** During initial 3 days, GCT-Sw culture showed significant differences on cell activity compared to Sw**.** However on subsequent incubation till 7 days there were no significant differences of cell activity in Sw and GCT-Sw. This variation could be due to the uneven distribution of cells in Sw, whereas it is localized in a plane in the case of GCT-Sw. Hence to collect reliable data on the initial days of the experiment, GCT-Sw would be a better system.

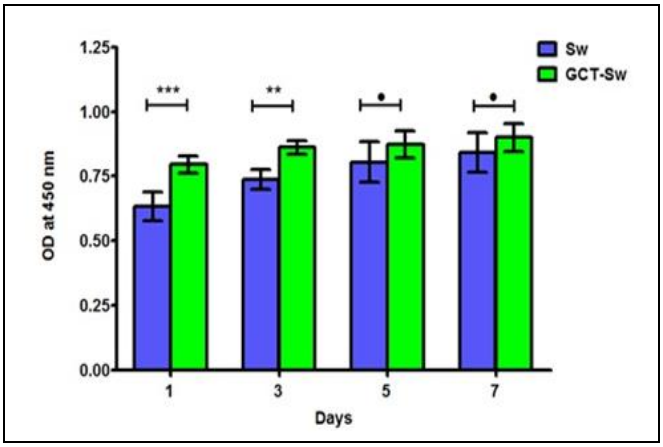


**FIG. 7: CELL PROLIFERATION ANALYZED BY CCK-8 ASSAY. THE GCT-SW SHOWED SIMILAR CELL ACTIVITY AS COMPARED TO SW. ALSO THERE IS STEADY STATE IN CELL ACTIVITY FROM DAY 1 TO DAY 7. ** = P≤0.005, **= P≤0.001, □•= NON SIGNIFICANT**

**Tissue Function Analysis of the Sandwich Culture:** Liver Function is an important *in-vitro* system of a toxicity model. The liver-specific functions such as albumin and urea synthesis have been commonly used to analyze the functions in vitro liver models. Albumin and urea synthesis by HepG2 cells in Sw and GCT-Sw was evaluated on 1, 3, 5 and 7 days. HepG2 cells derived from hepatic carcinoma are often used in the toxicity models to analyze liver-specific functions *in-vitro* as it shares similar properties of hepatocytes [15, 16]. Our results showed that GCT–Sw with HepG2 secreted significantly more albumin than Sw system throughout the experiment. The albumin secretion from the third day onwards was double the amount compared to Sw. A higher level of albumin secretion in GCT –Sw can attribute to an increase in hepatic function 16**. Fig. 8A.** Albumin secretion is often used as the marker for hepatic metabolic activity *in-vitro* and as an indicator of the functional liver [17]. The albumin estimated in the culture medium of Sw model was 16.8 ± 3 on Day 1, which increased only upto 23 ± 1 ng/ml in 7 days of culture. The albumin synthesis by GCT-Sw model was significantly higher to Sw. The GCT Sw system exhibited 23.16 ± 1 (Day 1) to 47.7 ± 11 (Day 7) ng/ml. The albumin secretion pattern observed in this study correlates with previously published studies that showed the ability of HepG2 cells to secrete albumin at different conditions [18]. Urea secretion is another important hepatic detoxification parameter of functional hepatocytes. Though urea synthesis is low in HepG2 cells due to lower expression of ornithine transcarbamylase, HepG2 cells are still considered versatile cells in routine hepatotoxicity testing [19]. The urea synthesis by Sw and GCT-Sw showed no significant differences **Fig. 8B.**

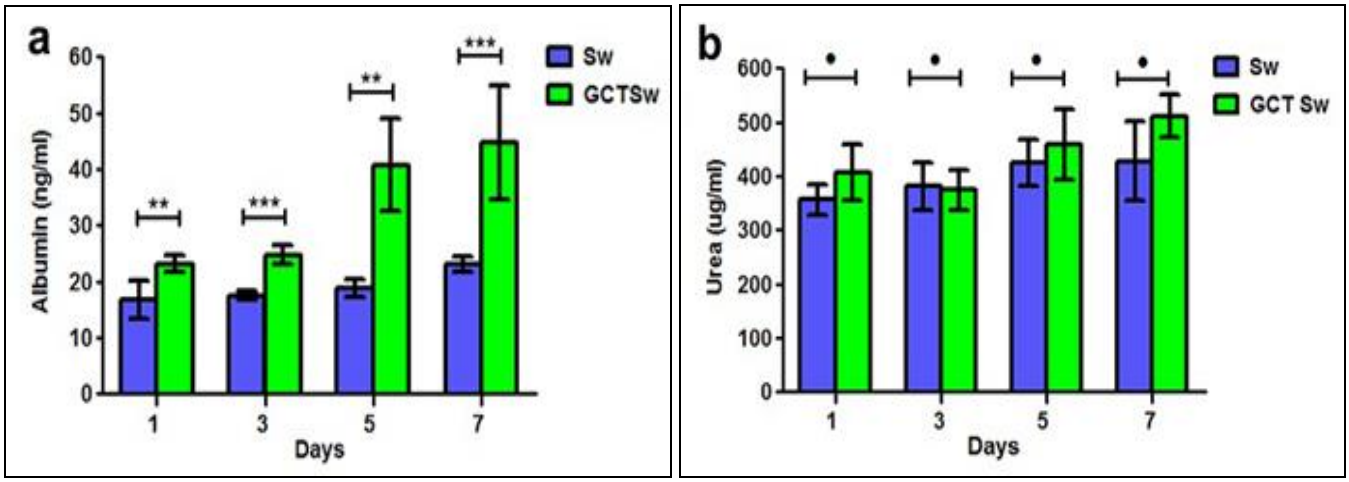


**FIG. 8: FUNCTIONALITY ASSESSMENT OF HEPG2 CELLS IN SW AND GCTSW COMPARED WITH CONTROL MONOLAYER. (A) ALBUMIN SECRETION BY CELLS IN GCT SW WAS HIGHER TO SW AND SHOWED AN INCREASING TREND OVER 7 DAYS IN CULTURE. (B) UREA SYNTHESIS INDICATING THE DETOXIFICATION FUNCTION SHOWED NO SIGNIFICANT VARIATION. ** = P≤0.005, **= P≤0.001, □•= NON SIGNIFICANT**

**CONCLUSION:** Three-dimensional cell cultures had great potential as a tool for drug discovery- from disease modeling to target identification to screening to lead identification. Gel Casting Tool device that can give better and consistent results in the *in-vitro* evaluation of The device has been proved to be useful with cross-linkable hydrogels. Functional benefits of this culture system include the localized cell distribution, which may enhance cell-cell contact and cell-extracellular contact and helps in increased functionality of cells. Ease of Microscopic images -As cells are localized in one place, it is easy to image cells in one frame and also easy to handle, and also the results are reproducible /reliable. Furthermore, the GCT- Sandwich culture is a better culture system than the normal sandwich culture and potential implications in clinical studies. GCT-sandwich culture system enhances hepatic functions and cell viability better than in the normal sandwich culture, which implies that it can lead to the study of liver cell functions and interactions.

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## CONFLICTS OF INTEREST: Nil

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