RESEARCH ARTICLE

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Design, Manufacture and Characterization of a Liver-Chip Model: A Platform for Disease Modeling and Toxicity Screening

Baddal and Mammadov. Liver-Chip Model for Disease Modeling and Toxicity Screening

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Abstract

BACKGROUND/AIMS: Drug research and development processes often fail in human clinical trials due to the inability of current *in vitro* cell culture and *in vivo* experimentation platforms to accurately predict toxicity and drug efficacy. There is an increasing need for human relevant systems which will enable studies on target tissue models prior to clinical trials. Drug-induced liver injury (DILI) is an important cause of acute liver failure. Species differences in liver toxicity and the limited predictability of traditional models represent a primary barrier to drug development for DILI. The aim of this study is to establish and characterize a dynamic microfluidic liver-chip with a physiological secretion function, continuous flow and compatibility with monitoring toxicity reactions.

MATERIALS AND METHODS: A biocompatible polymer-based prototype liver chip was designed using 3D stereolithography printing, CNC milling technology and molding. The chip with two microchannels, an upper channel for hepatocytes, and a lower microvascular channel for endothelium was produced by 3D bioprinting. The liver chips pre-coated with collagen type I were seeded with HepG2 cells and cell proliferation was monitored via microscopy. Toxicity was measured using a lactate dehydrogenase (LDH) assay. Albumin, alpha-fetoprotein (AFP), ALT and AST secretion was also investigated.

RESULTS: LDH readings demonstrated that the designed microfluidic chip was non-toxic to human hepatocytes. Albumin, AFP, ALT and AST secretions were detected in cellular secretions at physiologically-relevant levels.

CONCLUSION: Overall, this study demonstrates the design and manufacture of a physiologically-relevant microfluidic liver chip model, which can be used in drug-monitoring and toxicity studies.

Keywords: Microfluidic system, 3D bioprinting, organ chip, drug monitoring, personalized medicine

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INTRODUCTION

Drug development and disease simulation studies include both *in vitro* and *in vivo* stages. During these stages, multiple repetitions are performed to obtain the desired accurate result, leading to the consumption of large amounts of consumables and experimental animals. As a major drawback, incompatibilities between the two stages may occur. A disease model or a treatment that can be simulated at the cellular level can sometimes not be replicated in the animal model (1). Therefore, scientists have sought models that can be studied both on a cellular level basis and closely represent the model organism. In the search for this model, organ-on-a-chip (OOAC) technology has recently come in use. OOAC systems contain engineered or natural human-relevant miniature tissues grown inside microfluidic chips. To better mimic human physiology, the chips are designed to control cell microenvironments and maintain tissue-specific functions (2). It is a promising technology for effective drug discovery and drug screening for precision medicine. OOAC systems have made a significant advancement in the last 10 years. The OOAC was initiated with a lung chip, and has subsequently evolved into systems in which many different organs can be simulated (3-7). The OOAC technology combines both cell culture and microfluidic systems. The most important distinguishing feature of these systems is that the cell culture medium is not static; it is stimulated with a continuous dynamic fluid flow and mechanical stimuli can be given if required. These mechanical stimuli can be in the form of stretching, mimicking the human organs such as the simulation of the human respiratory movements via the stretching in a lung chip or peristalsis in a gut-chip. In addition, the dynamic flow of the culture medium in the OOAC eliminates the need for daily medium changes by the researchers, and the amount of consumables is considerably reduced due to the small size of the microfluidic channel (8).

Liver is the largest internal organ of the human body, with its complex microarchitecture and function that plays a critical role in drug metabolism. Hepatotoxicity and drug-induced liver injury (DILI) are the main causes of drug failures (9). Moreover, liver diseases are among the leading causes of death in the world, with new cases occurring each year. Although animal models have been traditionally used to investigate human drug metabolism and toxicity prior to

clinical trials, rat and dog models have been reported to predict only 71% of drug toxicities observed in humans (10).

The aim of the current study was to design a three-dimensional (3D) microfluidic liver chip model which would reflect the characteristics of human liver physiology and pathophysiology; recapitulate the sinusoidal structure of the liver, maintaining high cell viability and cellular phenotypes, and mimic natural liver functions. The designed liver chip would allow the simulation of different disease phenotypes and aid in the development of safer and more efficient personalized treatments.

MATERIALS AND METHODS

Chip Design, Production and Assembly

For mold design, a two-piece mold system which allows the production of six chips was designed using the Fusion 360 (Autodesk Inc, USA) software. The production of the molds was accomplished both by using biocompatible resin in Prusa SL-1 (Prusa Research, CZ) SLA 3D printer and CNC machining of aluminum. Quality control of the molds and canal diameter verification was performed with DinoLite (DinoLite Inc, USA) digital microscopy. Mold surfaces were cleaned with alcohol and distilled water. PDMS (Sylgard 184, Dowsil, USA) and curing agent were mixed at a ratio of 10:1 and placed in a vacuum desiccator device for 30 minutes to remove air bubbles. This mixture was then poured into molds and was incubated in an oven at 100 degrees for 30 minutes. Two halves of the chip were then removed from the mold after cooldown and stored in dust-proof sealed boxes. Corning Transwell polyester membrane (10-micron thickness, 0.4 micron pore width) (Sigma-Aldrich, St. Louis, MO, USA) was used as the separating membrane between the chip channels. The membrane was cut from the transwell wells with a scalpel to form strips that would coincide with the middle channel of the chip. Two pieces separated from the molds and the previously prepared membrane in the form of upper part-membrane-lower part were applied to all surfaces with Plasmatreat FG5001 (Plasmatreat GmbH) plasma activator device (IL, USA). As an alternative method, the surfaces of the upper part and the lower part were treated with uncured PDMS to adhere to each other and then cured in an oven at 60°C for 12 hours. Subsequently, each chip was connected to the Syringe Two (Elveflow, FR) microperfusion pump and checked for watertightness. Cross-sectional controls of the first chips were also performed with DinoLite (DinoLite Inc, USA) digital microscopy.

Cell Culture

For cell culture, HepG2 hepatocellular carcinoma cell line (catalog no: HB-8065, ATCC, Virginia, USA) was used. Vials containing 1 ml of frozen cell suspension at passage 10 were removed from the liquid nitrogen tank, thawed in a 37°C water bath and added to 14 ml of cell culture medium (DMEM, 10% FBS, and 1% penicillin-streptomycin) (Invitrogen, USA). The cell suspension was centrifuged at 1500 rpm for 5 minutes. After the centrifugation, the supernatant was gently decanted without disturbing the cell pellet. The cells were resuspended in 5 ml culture medium, and transferred into a 75 cm^2 flask (VWR, USA) containing 15 ml culture medium. After 24 hours of incubation at 37°C5% CO_2, the medium was refreshed and placed in the incubator. Cells were observed daily under Olympus IX53 inverted microscope and until 80% confluency was reached. Then, the culture medium was removed and cells were washed with phosphate buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA). Three ml of 0.05% 1x Trypsin-EDTA solution (Thermo Fisher Scientific, MA, USA) was added to the culture dish and was incubated at 37°C for 10 minutes. After incubation, the cells were suspended in 10 ml of DMEM with 10% FBS and centrifuged at 1500 rpm for 5 minutes. The cell pellet was resuspended in 10 ml of cell culture medium in a new cell culture dish and transferred to a 37°C

incubator with 5% CO^2. This procedure was repeated twice a week to ensure the continuity of the cell line.

Chip Loading with HEPG2

Hydrogen peroxide gas plasma sterilization was performed for all manufactured chips. Chip channels were treated with 1 mg/ml Sulfo-SANPAH (sulfosuccinimidyl 6-(4'-azido-2' nitrophenylamino) hexanoate) solution (Thermo Fisher Scientific, MA, USA) with 50 µl in the upper channel and 30 µl in the lower channel. Activation was performed using 365 nm UV light for 20 minutes. Post activation, Sulfo-SANPAH was removed and the channels were washed first with 200 µl of sterile H_2O, and later with 200 µl of 70% ethyl alcohol. The channels were washed 3 times with 200 µl of sterile PBS in order to avoid ethyl alcohol residues. Subsequently, the upper and lower channels of the chips were coated with ~30 µl of 1 mg/ml collagen type I (Sigma-Aldrich, St. Louis, MO, USA) previously prepared with 0.25% acetic acid, and kept at 4 °C overnight. At the end of the incubation period, the collagen in the channels was removed, the channels were washed once with 200 µl of HepG2 culture medium and equilibrated for cell culture.

HepG2 cells, when reached 80% confluency, were removed from the culture medium and washed with 5 ml of PBS. Then, 3 ml of 0.05% 1 x Trypsin-EDTA solution was added to the culture dish and after shaking gently, the culture dish was incubated at 37°C for 10 minutes. After incubation, the cells were suspended in 10 ml of DMEM with 10% FBS and centrifuged at 1500 rpm for 5 minutes. Then, cells resuspended in 300 µl of DMEM medium with 10% FBS were stained with 10 µl of 0.4% trypan blue (Sigma) and counted on a Neubauer chamber. Cells were seeded at a concentration of 3 x 10^6/ml cells (~30 µl) into the upper channel of a chip previously coated with type I collagen. In order for the cells to adhere to the chip, the chips were placed in an incubator at 37 °C with 5% CO_2 and incubated for 2 hours. After the incubation, the chips were examined with an inverted microscope. The chips were consequently connected to the syringe pump and the flow rate was set to 30 µl/hr. Input reservoir containing 10 ml culture medium and empty waste output reservoir, chips and perfusion pump were placed into the incubator at 37°C with 5% CO_2 for the duration of the experiment (Figure 1).

Chip Characterization

HepG2 cells were examined with an inverted microscope at different incubation times including 6h, 24h, 36h, 48h, and 72h in order to monitor cell proliferation. Lactate dehydrogenase (LDH) measurement was performed using a colorimetric assay (Sigma-Aldrich, St. Louis, MO, USA) to test for cellular cytotoxicity. The culture medium containing cell secretions collected in the output reservoir was used for the LDH assay. In addition, the albumin, alpha-fetoprotein (AFP), alanine transaminase (ALT) and aspartate aminotransferase (AST) levels in the output culture medium were measured using ARCHITECT ci4100 Analyzer (Laboratories, Abbott Park, IL, USA) and compared with DMEM culture medium without cells.

Statistical Analysis

No statistical analysis was required for the study.

RESULTS

Primarily, two mold designs were investigated in this study. It was determined that the first design caused an air bubble problem that could prevent the flow of the culture medium, and therefore the study proceeded with design number two (Figure 2). The molds produced from resin by SLA 3D printing prevented complete curing of the PDMS thus leading to the adhesion of PDMS to the mold surface after post curing. To solve the issue with resin, the molds were subjected to both heat (60°C) for 12 hours and a second UV (365 nm) treatment for 30 minutes.

After this treatment, the PDMS was easily released from the mold. However, it was noted that the PDMS partially lost its transparency feature. On the other hand, PDMS chips that were cured at 100°C for 30 minutes using an aluminum mold were easily released and showed proper transparency. Another failure was encountered during the air plasma activation of the chip halves to induce adhesion. The parts did not adhere after air plasma treatment, and therefore this method was discarded for this study. Instead, the chip halves were treated with a thin layer of uncured PDMS on the inner surfaces, the polyester membrane was placed in between, chips were assembled and cured at 60°C for 12 hours. With this method, it was noticed that the two surfaces adhered to each other in a watertight manner (Figure 3).

The results indicate that, during the liver-chip culture, HepG2 cells were able to adhere to the upper channel of the liver chip at 1-hour post seeding as shown in Figure 4. Monolayer formation was examined under the microscope on the 3rd, 4th and 6th days post seeding (Figure 5). Culture medium containing cell secretions was collected in the output reservoir for LDH cell cytotoxicity measurement. The levels of LDH in the liver chips were found to be comparable with DMEM culture medium without cells. (Table 1). The cells, which formed a monolayer in the chip were found to secrete albumin, ALT and AST. In addition, AFP level in cells was found to be high and concordant with regenerating hepatic cellular physiology.

DISCUSSION

Time and cost are two significant parameters for the R&D of new drugs. The development of a new drug takes about 12 to 15 years and costs about 800 million US dollars. In the R&D process, which consists of four phases including discovery and research, preclinical studies, clinical studies and treatment approval, 10 of approximately 10,000 compounds taken into animal testing reach clinical trials and only 1 is approved by the pharmaceutical agencies (11). In this approach, only preliminary physiological and pathological effects and toxicity results of the tested drug in tissue culture models can be observed. If the *in vitro* results are promising, the effects of the developed compound are further investigated using animal models. However, these two models have significant disadvantages. For example, *in vitro* models cannot fully represent complex cell-cell and cell-matrix interactions, and this incomplete interaction may lead to erroneous study results (12). Drugs compounds shown to be effective *in vitro* may be ineffective during *in vivo* studies. Therefore, unexpected toxicity and low efficacy not detected during *in vitro* studies represent one of the most common and important causes of drug development failures. In addition, the highest rate of failure in drug development occurs in the second and third phases of clinical trials conducted in the final stages. For this reason, critical studies performed in the earlier stages significantly reduce the cost and time spent in the process. The main reasons for the failure of new drug candidates can be stated as the ineffectiveness of the existing *in vivo* and *in vitro* models, and the inability to detect serious side effects through these models (13). Although the current market size of organ-on-a-chip technology is not as much as its potential, it was 7.5 million USD worldwide in 2016. According to the estimates, the market size will increase rapidly and may reach 163 million USD in 2023 with the completion of the existing R&D projects and the increase in the acceptance rate of organ chips in pharmacology (14). Considering the use of the animals as well as the high costs, there is an increasing need for an innovative alternative research process that can achieve success in a shorter time, and that can be investigated using target tissue models before starting clinical studies, increasing the reliability of the R&D process. Recently developed bioengineered devices that mimic human organs or tissues have emerged as OOAC technology in the literature (15). OOAC is an *in vitro* technique in which the mechanical and physiological responses of human tissues and organs are recreated

and simulated by three-dimensional (3D) microfluidic and other micro-scale supported structures (16,17). Cell and tissue cultures made with standard cell culture flasks, petri dishes and well plates have many disadvantages such as being static, lack of extracellular matrix, deficiencies in modeling human tissue physiology, pathophysiology and microstructure. Microfluidic organ-chips enable 3D cell culture in designed microchannels, and by better modeling complex tissues with multi-cell culture, enable assays that cannot normally be performed with existing experimental methods can be performed (18). When previous studies on OOAC are examined, it can be observed that the importance of OOAC which can mimic the functions of various living organs, including kidney, liver, lung, brain, skin, bone marrow and intestine, has increased in recent years (19-22). If the liver is taken as an example, the role of both new drug candidates and chemicals such as food additives in liver metabolism should be examined before human use. It is not possible to fully understand human liver tissue cells through animal models. Since *in vitro* cell lines proliferate on a single surface, they cannot be successfully simulated with liver tissue. With the organ-on-a-chip design, it is possible to place hepatocytes, endothelium, Kupfer, stellate and iPSC cells into the microchannel for the physical simulation of the liver, or it is possible to create a spheroid, which is a 3D cell culture form from hepatocytes, and model 3D liver tissue within microchannels with these spheroids (23, 24).

In the current study, the first organ-chip study was established in our region, and although it is a pilot organ-chip study, the results obtained are promising for further studies. In this study, it has been microscopically observed that HepG2 cells continue their physiological development on the OOAC platform created, and the cells have been demonstrated to physiologically secrete proteins. Although HepG2 cells are hepatic carcinoma cells and are more disadvantageous compared to normal hepatocytes due to their proximity to the fetal liver, they represent a good model for proof-of-concept studies. During the study, significant potential advantages of microfluidic systems over conventional methods were observed. The most important of these is that the cells do not need intermittent media replacements due to the dynamic flow, and the positive effects of dynamic flow on cell physiology compared to the static environment. The flow of dynamic DMEM or other culture media solutions in the chip channel has been proven to mimic the physiological blood or extracellular fluid flow of cells, and has also been shown to trigger growth. Although not included in the results, an increase in AFP secretion was observed within days in our study. It is also likely that the risk of deterioration of the sterile environment will be reduced as it requires less intervention. However, the most important advantage of microfluidic systems can be stated as the use of very limited amounts of biomaterial. This allows a higher number of experiments to be performed with the same amount of material consumption compared to the conventional methods

In the context of recent literature, a plethora of studies have been performed in order to demonstrate the superiority of microfluidic liver-on-a-chip systems over conventional cell culture methods in terms of their recapitulation of the physiology, mechanisms, and functionalities of liver cells in an *in vitro* environment. In particular, the use of primary hepatocytes obtained from liver biopsies or non-transplantable livers have been stated as the gold standard for the development of human-relevant *in vitro* liver models, yet human induced pluripotent stem cells (hiPSCs), non-parenchymal cells and hepatic cell lines, which was the choice of cell source in the current study, are also being used as alternative choices. In addition to monolayer cultures, matrix-free spheroids/organoids-on-chip, scaffold/hydrogel-based 3D liver OOACs and 3D liver OOACs using bioprinting have been considered as the emerging models of liver chips (25). Furthermore, models such as PhysioMimix liver-on-a-chip has been

recently proposed and its utility has been shown to be effective for the exploration of multiple drug metabolism applications (26). A hybrid polymer-based microfluidic platform, including cyclic olefin copolymer and PDMS, has also been developed for culturing hepatocytes and has been demonstrated to retain the functionality and metabolic activity of perfusion culture as assessed by the secretion rates of albumin, urea, and cell viability visualization. The hybrid platform has proved to produce comparable biomarker levels similar to published studies on other *in vitro* models (27). More recently, a customizable microfluidic origami liver-on-a-chip model which simplifies the laboratory-scale fabrication of organ-on-chip models and hence speeds up chip development and optimization without reducing structural and functional features has been proposed (28).

In the current study, the levels of albumin, AFP, ALT and AST values of HEPG2 cells were also measured. Butterfield et al. has previously demonstrated that HEPG2 cells cultured in 24-well plates produced AFP at 606 ng/ml/106 cells at 24 h (29). In our study, the amount of AFP secretion was found to be 978 ng/mL, which was comparably higher in the liver chip. Similarly, albumin secretion by HEPG2 cells in tissue culture has been reported to be between 1.7-2 µg/ml, which is lower than the albumin values obtained in the liver chip (15 µg/ml) (30, 31). On the other hand, ALT and AST levels secreted by HEPG2 cells in dishes or multi-well plates have been reported to be 3 IU/L and ~5 IU/L in literature, respectively (32). While these results are not superior to cells in dishes, our results still demonstrate the presence of these secretions important for physiological relevance.

Study Limitations

Under current conditions, experiments can be concurrently performed on four chips with a single channel or two chips with two channels. However, more equipment is required to perform experiments with higher numbers of chips. The liver chip secretions were only compared with cell culture medium without cells and not with secretions from cells grown without the chip. Another limitation of our study was the lack of immunostaining. This limitation did not pose a major problem, as we biochemically and microscopically demonstrated that cell viability was preserved.

CONCLUSION

The liver chip model established in this study successfully demonstrated viability of hepatocytes and physiologically-relevant secretions under constant flow. It is predicted that, in the near future, the developed OOAC models will replace experimentation on animal models and offer cheaper and faster potential new diagnosis and treatment methods. Therefore, future studies to improve and develop the technology are of paramount importance for advancing research.

MAIN POINTS

- There is an increasing demand for human-relevant microphysiological systems which can accurately model and predict human diseases and aid in drug development processes.
- A microfluidic human liver-chip for modeling drug-induced liver injury was developed using 3D stereolithography printing, CNC milling technology and molding.
- The liver-chip model was non-toxic to human hepatocytes. HepG2 cells secreted albumin, ALT and AST at physiologically-relevant levels. AFP level in cells was found to be high and concordant with regenerating hepatic cellular physiology.
- The developed model represents an advancement towards drug-monitoring and toxicity studies.

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ETHICS

Ethics Committee Approval: Ethics committee form was not required. The study followed the principles of the Declaration of Helsinki.
Informed Consent: A consent form was not required for this study as the cells were purchased commercially.
Authorship Contributions
Concept: B.B., E.M., Design: B.B., E.M., Data Collection and/or Processing: B.B., E.M., Analysis and/or Interpretation: B.B., E.M., Literature Search: B.B., E.M., Writing: B.B., E.M.

DISCLOSURES

Conflict of Interests: The authors have no conflicts of interest to declare.
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Table 1. Comparison of HepG2 liver chip and DMEM culture medium for cellular secretions

	Liver Chip	DMEM without cells
LDH	45 U/L	37 U/L
Albumin	15 mg/L	7.41 mg/ L
AFP	978 ng/mL	0
ALT	<6 U/L	ND
AST	3 U/L	ND
ND: Not detected		

Figure 1. Incubator setup (chip, chip tray, perfusion pump, connectors, and output vials)

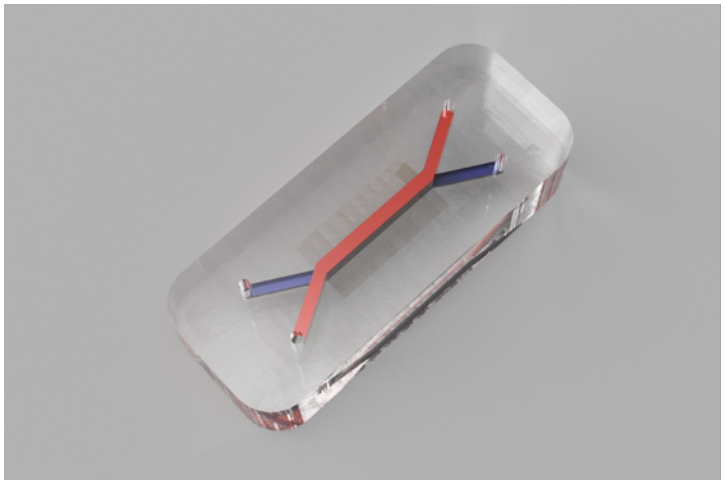

Figure 2. Chip design which was selected for the study [rendered in Autodesk Fusion360 (Autodesk Inc, USA)]

Figure 3. Assembled chip after watertightness test (different dye colors indicate separate channels)

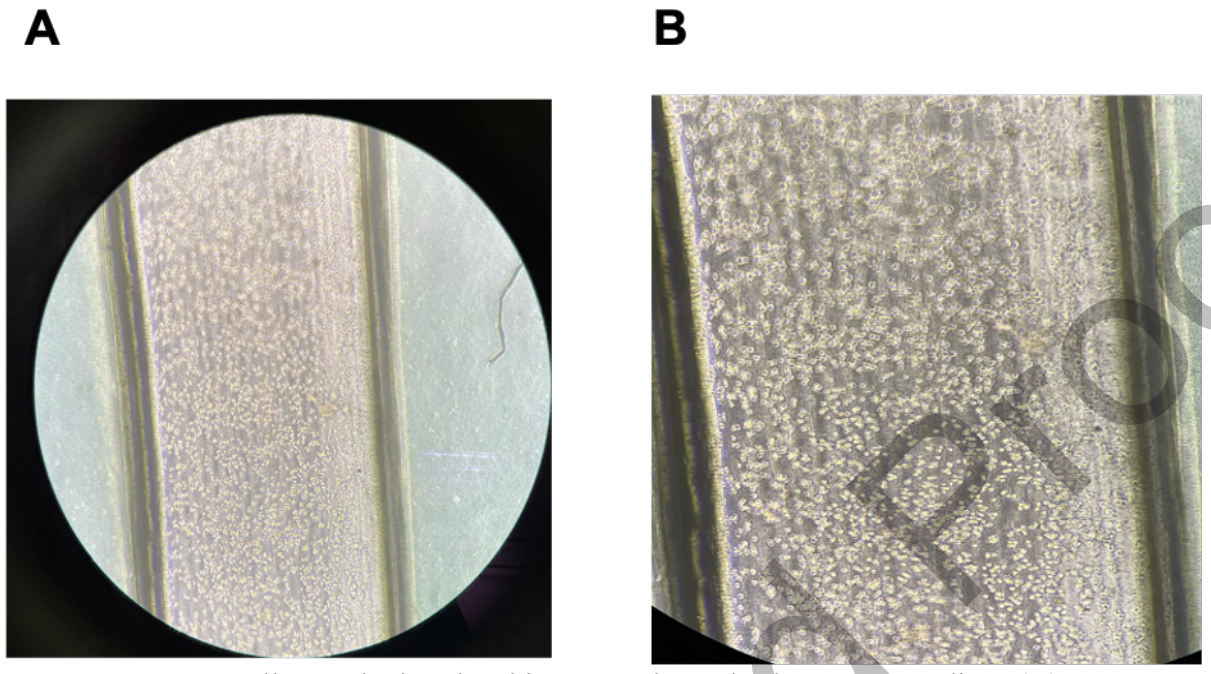

Figure 4. HEPG2 cells attached to the chip upper channel 1-hour post seeding [(A) 10x magnification, (B) 20x magnification]

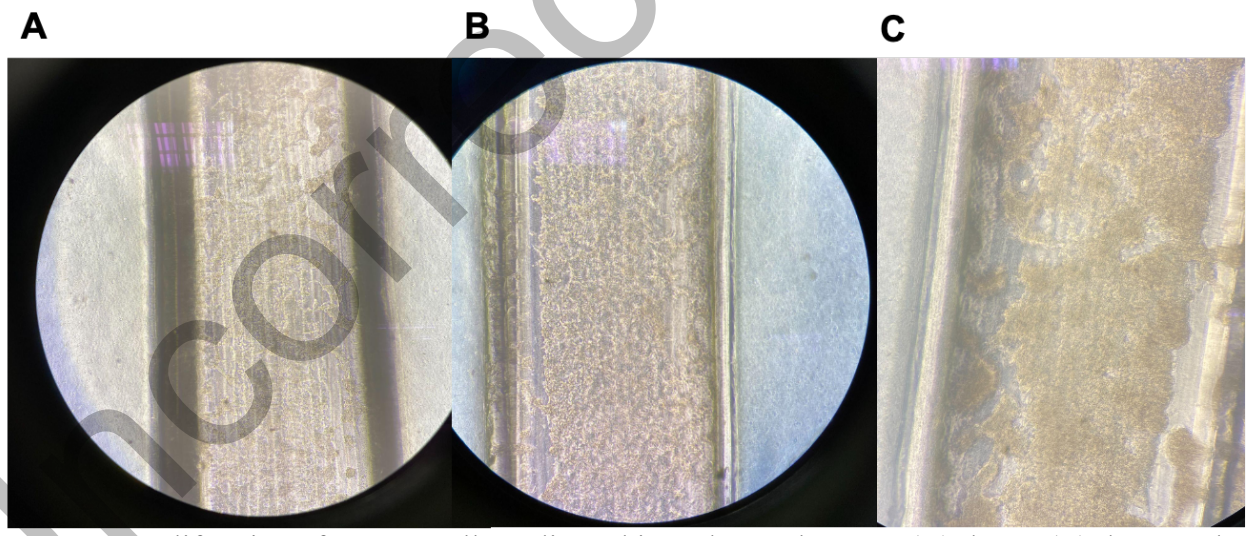

Figure 5. Proliferation of HepG2 cells on liver chip and monolayer on (A) day 3, (B) day 4 and (C) day 6 (10x magnification)