Comprehensive Physiology

COMPREHENSIVE PHYSIOLOGY
Interorgan communication in health and disease

american physiological society

RESEARCH ARTICLE

Geltrex-Enhanced Two-Dimensional Culture as a Viable Alternative to Primary Rat Hepatocyte Sandwich Models

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Received: 2 September 2025 | **Revised:** 24 November 2025 | **Accepted:** 28 November 2025

Keywords: albumin | CYP3A4 | extracellular matrix | Geltrex | hepatocyte | liver | sandwich culture

ABSTRACT

Primary hepatocytes rapidly lose viability and function in conventional two-dimensional (2D) cultures due to the absence of a physiologically relevant extracellular matrix (ECM). The collagen sandwich method improves polarization and function but creates a diffusion barrier that limits nutrient and signal exchange. This study investigates whether daily supplementation of a diluted, non-gelling Geltrex layer can sustain hepatocyte function and viability in 2D culture, offering a practical alternative to the sandwich method. Primary rat hepatocytes were cultured for 15 days under four conditions: monolayer (ML), monolayer with Geltrex (ML + GT), sandwich (SW), and sandwich with Geltrex (SW + GT). Cell morphology, confluency, viability (CCK-8, live/dead staining), and functionality (urea synthesis, albumin production, CYP3A4 activity) were assessed. The ML group showed significant declines in confluency, viability, and functional markers over time. Geltrex supplementation preserved confluency (~97% at day 15), improved viability, and maintained higher albumin production and CYP3A4 activity compared to ML. Functional outputs in ML + GT were comparable to SW and SW + GT groups, without the diffusion limitations of the sandwich top gel. Daily supplementation with low-dose Geltrex creates a biochemically enriched, diffusion-permissive microenvironment that supports long-term viability and function of primary rat hepatocytes in 2D culture. This method represents a simple and effective alternative to traditional sandwich cultures for liver cell studies and drug testing applications.

1 | Introduction

Two-dimensional (2D) cell cultures provide a simplified and controlled environment for modulating cellular conditions, enabling straightforward observation and measurement (Duval et al. 2017). They are easier both to establish and use compared to three-dimensional (3D) cell cultures and organ-on-a-chip systems. Nevertheless, 2D cultures lack the structural and biochemical cues of the native tissue microenvironment, which may compromise cell viability, differentiation, and long-term functionality (Joseph et al. 2018). Despite their simplicity and widespread use, such limitations hinder the use of 2D cell cultures toward recapitulating interorgan interactions, which is becoming increasingly important in the face of regulatory shifts that discourage the use of animal models (Zushin et al. 2023).

In vivo, organ functions are orchestrated not only by intrinsic cellular mechanisms but also by complex and dynamic interorgan interactions between multiple tissues and organs (Ni et al. 2025). These interorgan interactions are central to maintaining systemic homeostasis, coordinating metabolic pathways, and regulating drug responses and disease progression (Ingber 2022). As a result, the ability to accurately recapitulate physiologically relevant interorgan interactions in vitro has become increasingly critical for developing predictive platforms for drug discovery, toxicology, and human disease modeling (Picollet-D'hahan et al. 2021). However,

the successful establishment of such sophisticated, multilayered organ-on-a-chip systems depends on the availability of stable and functionally competent 2D cell culture models that can serve as a robust foundation for developing next-generation multi-organ platforms (Leung et al. 2022).

Cellular interactions with the extracellular matrix (ECM) and neighboring cells are essential for maintaining tissue viability and supporting differentiated morphology and polarization (Rozario and DeSimone 2010; Huang et al. 2017). For example, hepatocytes, form distinct apical and basal domains, necessary for functions such as bile canaliculi formation and ECM anchoring (Gissen and Arias 2015). Although hepatocytes perform key roles in human liver metabolism (Alamri 2018; Hou et al. 2020; Jones 2016; Adeva-Andany et al. 2016; Rui 2014; Coleman 2020), they rapidly lose their function and viability in 2D cell culture due to a lack of ECM-mediated signaling (Karsdal et al. 2015).

Various methods have been developed to sustain liver viability and functionality in cell culture to overcome this limitation (Khetani and Bhatia 2008; Andria et al. 2010; Yu et al. 2017). One widely used approach is the sandwich culture approach, in which hepatocytes are cultured on an ECM-coated surface and subsequently overlaid with an additional gelled ECM layer upon culture stabilization (Dunn et al. 1989, 1992; Berthiaume et al. 1996). This configuration facilitates and maintains apical-basal polarity and promotes 3D cellular organization (Baker and Chen 2012). Among ECM materials, Collagen I (300 kDa, 1.25 mg/mL) gels are most commonly used in sandwich cultures, typically forming a dense matrix that is approximately 0.5 mm thick. Although this matrix provides robust mechanical support, it can hinder the diffusion of nutrients and signaling molecules (Berthiaume et al. 1996). Oxygen and nutrient transport in collagen hydrogels has been shown to decrease with increasing thickness, leading to higher cell death in thicker regions (Ishida-Ishihara et al. 2022). Additionally, ECM can impede the removal of waste products, which in turn increases cellular sensitivity to both artificial and self-generated stress-inducing substances (LeCluyse et al. 2012). Furthermore, it has been reported that nanoparticle diffusion may be restricted by the ECM, depending on its composition and density (Cahn et al. 2024). Collectively, these studies support the notion that ECM thickness in sandwich cultures may impose diffusion-related functional limitations. Moreover, these gels are limited solely to purified type I collagen and lack the diverse ECM components necessary to mimic native microenvironments.

In contrast to collagen gels, Geltrex—a basement membrane extract derived from murine Engelbreth-Holm-Swarm tumors—offers a more physiologically relevant ECM composition. It contains key ECM components such as laminin, collagen IV, entactin, and heparan sulfate proteoglycans, which collectively promote cell attachment, differentiation, and maintenance of differentiated phenotypes (Acun et al. 2022; Suominen et al. 2023; Broeders et al. 2015). Geltrex, due to its biochemical diversity and complexity, has been used in co-culture systems with human induced pluripotent stem cell-derived hepatocyte-like cells (iPSC-HLCs) and human umbilical vein endothelial cells (HUVECs) where it supported hepatocyte polarization and sustained functionality while enabling HUVECs to form vascular networks (Suominen et al. 2023). Additionally, Geltrex has facilitated a stable co-culture of other cells of different tissue origins in 3D culture models (Fang et al. 2013; Grützmeier et al. 2023; Ivers et al. 2014; Jalilian and Shin 2023). These findings underscore the versatility of Geltrex in advanced culture applications for various tissue types and highlight its potential to facilitate in vitro modeling of interorgan communication.

In this study, we investigate a simpler alternative to the gold-standard collagen sandwich method for culturing primary rat hepatocytes (PRHs). We propose the use of a non-gelling thin top layer—prepared by diluting Geltrex to 2% v/v in culture medium—which is applied daily during media changes. There are two main reasons for selecting this dilution: (i) to remain below the gelation threshold, thereby preventing the formation of a thick network that could impede the diffusion of nutrients and signaling molecules, and (ii) to create a physically stable and reproducible ECM coating on the culture surface. The physical behavior of the Geltrex solution depends on the density and solubility of its constituent proteins which typically range between 1.32 and 1.43 g/cm^3 (Quillin and Matthews 2000; Fischer et al. 2004)—slightly higher than that of water (1.0 g/cm^3) due to their compact folded structure (Erickson 2009). Geltrex is supplied at 12–18 mg/mL; thus, the 2% (v/v) dilution used here yields an effective protein concentration of approximately 0.3 mg/mL. At this concentration, sedimentation is negligible as predicted by Stokes' law (Stokes 1850), and the proteins remain stably distributed in medium.

Consequently, the diluted Geltrex does not precipitate or form a bulk gel but instead adsorbs as a thin, non-gelling layer that enables integrin-mediated attachment through its laminin, collagen IV, and entactin content (Cukierman et al. 2001). Daily supplementation with fresh medium replenishes these ECM components and prevents degradation, maintaining a stable biochemical environment without diffusion limitations typical of thick collagen gels. This layer, enriched with physiologically relevant ECM components, is expected to form a few microns thick above the cell layer, supporting cell adhesion and functional differentiation while facilitating the diffusion of nutrients and signaling molecules within the culture microenvironment. This daily supplementation of Geltrex is also expected to provide a dynamic environment that may help maintain cell viability and functionality over extended periods. To validate this method, we evaluated hepatocyte viability and functionality under four distinct culture conditions, namely monolayer (ML), monolayer + Geltrex (ML + GT), sandwich (SW), sandwich + Geltrex (SW + GT).

This Geltrex-supplemented 2D culture approach could be integrated into multi-organ microphysiological systems, such as gut-liver-on-a-chip platforms, to facilitate the study of interorgan communication and dynamic cellular interaction (Kim and Sung 2024). By providing a physiologically relevant ECM microenvironment, the method lays the foundation for investigating not only hepatocyte function but also intercellular and interorgan signaling in vitro, which makes it possible to better understand organ communication in health and disease (Zhao et al. 2023).

Our results demonstrate that daily Geltrex supplementation in a monolayer format provides a practical and effective alternative

to sandwich methods while preserving key liver functions such as protein synthesis, detoxification, and drug metabolism, thereby offering a robust foundation for future integration into advanced interorgan recapitulation platforms aimed at modeling complex interorgan interactions.

2 | Materials and Methods

2.1 | Materials

PRHs culture medium C + H, was prepared using Dulbecco's modified Eagle's medium (DMEM; Life Technologies, CA, USA), supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, USA), 2% penicillin–streptomycin, 7.5 μg/mL hydrocortisone, 20 ng/mL epidermal growth factor (EGF), and 14 ng/mL glucagon. Twelve-well tissue culture plates (Celltreat Scientific Products, Pepperell, MA, USA) were coated with Rat Tail Collagen Coating Solution (Sigma Aldrich, USA). Dulbecco's phosphate-buffered saline (DPBS) was obtained from GenClone (Genesee Scientific, Research Triangle Park, NC, USA). Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413202) was purchased from Thermo Fisher Scientific. Trypan Blue was purchased from Sigma Aldrich, USA. Cell Counting Kit-8 (CCK-8) was obtained from ApexBio (Boston, MA, USA). P450-Glo CYP3A4 Enzyme Assay was sourced from Promega Corporation (Madison, WI, USA). The urea assay kit was purchased from Stanbio Laboratory (Cat. No. 0580-250). Additional reagents, including Tween 20, albumin from rat serum (A6414), *o*-phenylenediamine dihydrochloride tablets (10 mg substrate per tablet), and hydrogen peroxide solution, were supplied by Sigma Aldrich (USA). Sheep anti-rat albumin HRP conjugate (1 mL at 1 mg/mL) was purchased from Fortis Life Sciences (Waltham, MA, USA). For live/dead staining assay live cell imaging solution (1×), ethidium homodimer-1 (EthD-1), Calcein AM and Hoechst 33342, trihydrochloride, trihydrate were purchased from Invitrogen by Thermo Fisher Scientific (Carlsbad, CA, USA).

2.2 | Primary Rat Hepatocyte Isolation

PRHs were freshly isolated from 10 to 12-week-old adult female Lewis rats (180–200 g) obtained from Charles River Laboratories, USA. The isolation was performed by the Cell Resource Core (CRC) following protocol #2011N000111, which was approved by the Institutional Animal Care and Use Committee (IACUC) at Massachusetts General Hospital (MGH). Approximately 300–400 million PRHs with 90%–95% viability, as assessed by Trypan Blue exclusion using the Cellometer K2 (Nexcelom, USA), were provided.

2.3 | Primary Rat Hepatocyte Culture

2.3.1 | Bottom Collagen Coating and Cell Seeding

All wells in 12-well plates were coated with Type I rat tail collagen prior to cell seeding. To initiate gel formation, 450 μL of collagen solution was added to each well and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 2 h. Following incubation, the remaining collagen solution was aspirated, and the wells were washed twice with 1 mL of sterile PBS.

Freshly isolated PRHs were suspended in complete hepatocyte culture medium C + H, and the suspension was adjusted to a final concentration of 560,000 cells/mL. Under sterile conditions, 1 mL of this suspension was added dropwise into each well to prevent shear stress and osmotic imbalance. The cell suspension was gently agitated before each seeding to ensure homogeneity. To promote even cell distribution, the plate was gently rocked in a cross pattern. The seeded plates were incubated at 37°C, 5% CO_2 for 40 min to allow for cell attachment. After this initial incubation, the supernatant containing unattached and dead cells was aspirated. For no-Geltrex groups, 500 μL of prewarmed C + H medium was added to each well. For Geltrex-treated groups, 2% Geltrex (v/v) was diluted in prewarmed C + H medium, and 500 μL of this solution was added to each well. All plates were then incubated under standard culture conditions overnight.

2.3.2 | Top Gel Coating for Sandwich Culture

The day after cell seeding, on day 1, supernatants were carefully collected from each well and stored at −80°C for later biochemical analysis. Wells were washed once with 1 mL PBS. A top gel solution was prepared by diluting Type I collagen to a final concentration of 1.25 mg/mL in high-glucose DMEM buffered with sodium bicarbonate (pH 7.4). Then, 200 μL of this collagen mixture was gently added on top of the cell monolayer in the relevant groups. Plates were incubated for 1 h at 37°C to allow collagen polymerization. After gelation, 500 μL of either C + H (for non-Geltrex) or 2% (v/v) Geltrex-supplemented C + H medium was added based on group assignment. Cells were incubated overnight under standard conditions to allow stabilization.

2.3.3 | Maintenance and Imaging

From day 1 to 15, the culture medium was refreshed daily. A day-old media were collected from each well and stored at −80°C. Subsequently, 500 μL of fresh, prewarmed C + H or Geltrex-supplemented C + H medium was added to each well according to group designation. Phase-contrast images were captured every other day using an EVOS M5000 imaging system at 10× magnification to monitor cell morphology and attachment.

2.4 | Confluency Analysis

The cell attachment area for each culture condition was quantified using phase-contrast microscopy images analyzed in ImageJ. All images were converted to 8-bit format, and the total well surface area was defined as 100% coverage. Regions of cell attachment were manually delineated using freehand selection tools, and their areas were measured. Confluency was calculated by expressing the total cell-attached area as a percentage of the total surface area.

2.5 | CCK-8 Viability Assay

A CCK-8 assay was used to assess the activity of cellular dehydrogenases as a measure of cellular viability. Briefly, 50 μL of CCK-8 solution was added to each well containing 500 μL of fresh C + H medium. The plate was incubated at 37°C in a 5% CO_2 atmosphere for 3 h. Subsequently, 60 μL aliquots were collected from each well, and absorbance was measured at 450 nm using a plate reader. To calculate the percent change in activity, the absorbance values of the ML and SW groups on day 2 were set as 100%. The ML + GT and SW + GT group values were normalized to the respective ML and SW groups on day 2.

2.6 | Live Dead Staining

The staining solution was prepared by mixing 5 mL live cell imaging solution (1×), 2.5 μL Calcein AM reagent, 1 μL Hoechst 33342, and 10 μL ethidium homodimer-1. Supernatants were aspirated, and the wells were washed with 1 mL of live cell imaging solution (1×). 0.5 mL of staining solution was added to wells and the plate was incubated at 37°C, 5% CO_2 for 20 min. Later, the staining solution was replaced with 1 mL of the cell imaging solution (1×) and cells were imaged with the microscope.

2.7 | Functional Assessment

2.7.1 | Urea Analysis

Urea concentration in the supernatant of PRHs was analyzed using a Stanbio Urea BUN assay kit. The manufacturer's protocol was strictly followed to ensure accurate results. Urea assay solution was prepared by mixing one part BUN color reagent with two parts BUN acid reagent. Ten microliters of both samples and standards (in triplicates) were pipetted into a 96-well plate. One hundred and fifty microliters of urea reagent was added to each well using a multichannel pipette. The plate was sealed tightly and incubated at 60°C for 90 min. After incubation, the plate was removed from the incubator and cooled at room temperature for 10 min. Absorbance was measured at 540 nm using a microplate reader.

2.7.2 | Albumin Analysis

The albumin concentration in the supernatant was quantified using an in-house enzyme-linked immunosorbent assay (ELISA) protocol. A 96-well high-binding plate was coated with 100 μL of 50 μg/mL rat albumin solution in each well. The plate was sealed and incubated overnight at 4°C. After incubation, the plate was washed four times with PBS containing 0.05% Tween-20 (PBS-T). Next, 50 μL of samples and standards were added in triplicate to the wells. Antibody solution (1:10,000 dilution in PBS-T) was prepared, and 50 μL was added to each well. The plate was sealed and incubated at 37°C for 1.5 h. After incubation, the wells were washed four times with PBS-T and developed using o-phenylenediamine dihydrochloride (OPD) solution. The reaction was stopped using 8 N H_2SO_4. Absorbance was measured at 490 nm and 650 nm using a Spectramax ID3 microplate reader. Standard curves were generated to calculate albumin concentrations.

2.7.3 | Enzyme Activity Assay

Cytochrome P450 family 3 subfamily A member 4 (CYP3A4) activity in cells was measured using the CYP450-Glo assay. The Luciferin-IPA substrate, provided in the kit at a 3 mM concentration, was diluted 1:1,000 in the cell culture medium. The supernatants were collected, and cells were washed twice with 0.5 mL C + H media. Then, 500 μL of the diluted Luciferin-IPA solution was added to each well. To determine background luminescence, the same solution was added to an empty well. The plate was incubated at 37°C for 45 min. After incubation, 25 μL of the supernatant was transferred from each well to an opaque white luminometer plate in triplicates. Subsequently, 25 μL of Luciferin Detection Reagent was added to each sample. The plate was incubated at room temperature for 20 min before measuring luminescence using a luminometer. The luminescence values of the ML and SW groups on day 2 were set as 100%. The ML + GT and SW + GT group values were normalized to the respective ML and SW groups on day 2.

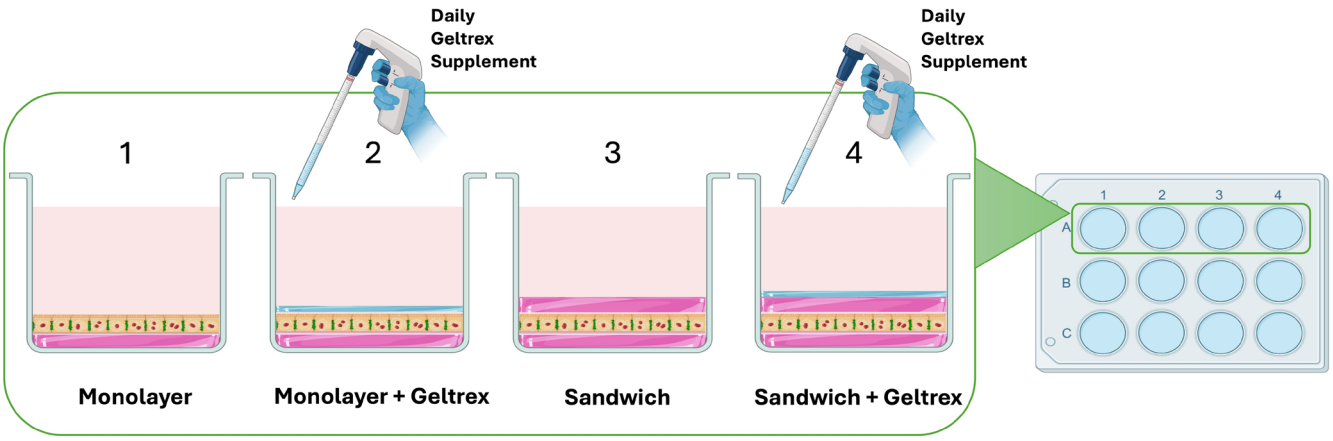
2.8 | Statistical Analysis

Hepatocytes were isolated from three different rats, representing three independent biological replicates ($N = 3$). From each isolation, cells were plated into three separate wells to serve as technical replicates ($n = 3$). Data presented in the figures are expressed as mean ± standard error of the mean (SEM). Two-way ANOVA followed by Tukey's post hoc test was used to assess statistical significance, with time and groups as the two factors in the analysis.

3 | Results

We evaluated PRH viability and function under different culture conditions, including standard monolayer (ML), monolayer + Geltrex (ML + GT), sandwich (SW), and sandwich + Geltrex (SW + GT) (Figure 1). Phase-contrast images were captured from the center of each well at 10× magnification, beginning on day 1 and continuing every other day through day 15 (Figure 2). In the ML group, cells gradually lost viability; most detached from the culture surface, while some remained as cellular debris. The surviving cells showed morphological signs of dedifferentiation. In contrast, cells in the ML + GT group maintained viability, similar to that observed in the SW group and both the ML + GT and SW groups maintained cell–cell interactions throughout the 15-day period. Similarly, cells in the SW + GT group displayed a morphology comparable to those cultured in the ML + GT group. In all groups except ML, cells gradually adopted a more cuboidal shape. Additionally, small canalicular networks were observed between adjacent cells, consistent with bile canaliculi formation and indicative of functional polarization.

We visually assessed microscopic images from day 1 and 15 to evaluate changes in cell confluency across all experimental



Experimental condition	Number of samples	ECM on bottom	ECM on top	Day of top ECM addition
Monolayer (ML)	9	Collagen	-	-
Monolayer + Geltrex (ML+GT)	9	Collagen	Geltrex	0,1,2,3,..15
Sandwich (SW)	9	Collagen	Collagen	1
Sandwich + Geltrex (SW+GT)	9	Collagen	Collagen and Geltrex	1 0,1,2,3,..15

FIGURE 1 | Experimental design of hepatocyte cultures. Cells were seeded in collagen type I-coated 12-well plates on day 0 under four different conditions: monolayer (ML), monolayer with Geltrex supplementation (ML + GT), sandwich culture (SW), and sandwich culture with Geltrex supplementation (SW + GT). Geltrex supplementation was added daily from day 0 at 2% (v/v) dilution in culture medium for the respective groups forming a thin non-gelling layer above the cells is on the order of several hundred nanometers to a few microns at most. In sandwich culture groups, a collagen type I top gel layer, approximately 0.5 mm thick was applied only on day 1 and remained throughout the culture period. The culture medium was refreshed daily, and all cultures were monitored for 15 days. Experiments were conducted with $N = 3$ biological replicates and $n = 3$ technical replicates for each condition. In the schematic, the collagen gel layer is shown in pink, while the Geltrex non-gelling layer is shown symbolically in blue.

groups. On day 1, cell confluency appeared comparable among all conditions. By day 15, the ML group showed a marked reduction in confluency, while the ML + GT, SW, and SW + GT groups maintained their coverage levels (Figure 3a). According to the results obtained from microscopic images analyzed using ImageJ software, the initial cell confluency of all groups was calculated to be ~97%. By day 15, confluency in the ML group had declined to 10%, whereas it remained around 90% in the SW and SW + GT groups. The ML + GT group showed no significant change, maintaining confluency at approximately 97% (Figure 3b). Results from visual inspection and software-based quantification were consistent.

For the calculation of CCK-8 activity (%), the day 1 values of the ML and SW groups—used as reference conditions in previous studies—were set to 100%. The results of the ML + GT group were normalized to the ML group, while those of the SW + GT group were normalized to the SW group. CCK-8 activity in the ML group declined significantly on days 7 and 15 compared to all other groups (Figure 3c). In contrast, the ML + GT group maintained activity on day 7, showing no significant difference from the SW group. By day 15, activity in the ML + GT group decreased to approximately 70%, while the SW group retained activity at around 90%. On day 7, the SW + GT group showed activity near 70%, with no statistically significant difference from either the SW or ML + GT groups. This trend persisted on day 15, with no significant change from day 7 and no difference between the ML + GT and SW + GT groups.

Representative live/dead staining images were obtained on day 2 for both Geltrex-treated and untreated groups (Figure 4a), and on day 15 for all groups (Figure 4b). Live cells were stained with calcein AM, while dead cells were labeled with ethidium homodimer. On day 15, the SW group exhibited greater viability than the ML group. Both Geltrex-treated groups (ML + GT and SW + GT) also showed improved viability relative to the ML group, comparable to that observed in the sandwich cultures.

Urea levels, a marker of hepatocyte functionality, were measured from the culture supernatant collected on days 1, and every other day thereafter (Figure 5a). In the ML group, urea levels were $163.5 \pm 17.5\,\mu g/mL$ on day 1 and declined to $9.5 \pm 2.2\,\mu g/mL$ by day 15. In the ML + GT group, urea levels were $162.2 \pm 20.6\,\mu g/mL$ on day 1 and declined to $14.5 \pm 2.8\,\mu g/mL$ by day 15. Similarly, in the SW group, urea levels dropped from $164.0 \pm 17.7\,\mu g/mL$ on day 1 to $29.8 \pm 7.2\,\mu g/mL$ on day 15. Finally, in the SW + GT group, urea levels were $162.3 \pm 18.2\,\mu g/mL$ on day 1 and $17.7 \pm 2.9\,\mu g/mL$ on day 15.

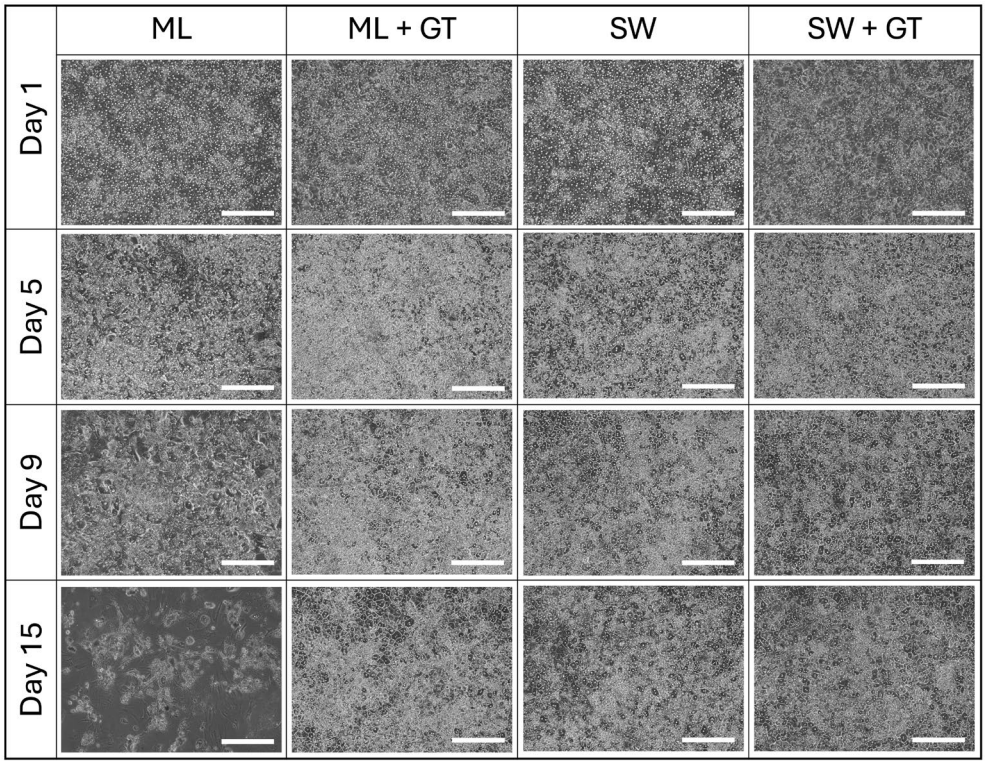


FIGURE 2 | Representative phase-contrast microscopy images of hepatocyte cultures under four different conditions (ML, ML + GT, SW, and SW + GT) on days 1, 5, 9, and 15. Imaging was performed every other day to monitor morphological changes over time; selected time points are shown to illustrate the progression of cellular morphology under each culture condition. Scale bars: 300 μm.

Albumin, a marker of liver synthetic function, levels were also assessed to evaluate cell functionality (Figure 5b). In the ML group, albumin levels were 14.1 ± 3.0 μg/mL on day 1 and 0.3 ± 0.2 μg/mL on day 15. In the ML + GT group, albumin levels increased from 23.2 ± 6.0 μg/mL on day 1 to 54.4 ± 14.8 μg/mL on day 15. In the SW group, albumin levels were 15.3 ± 4.0 μg/mL on day 1 and 9.4 ± 3.8 μg/mL on day 15. Lastly, in the SW + GT group, albumin levels were 20.5 ± 3.6 μg/mL on day 1 and 41.5 ± 17.4 μg/mL on day 15.

CYP3A4 enzyme activity was calculated by setting day 2 values of the ML and SW groups to 100%, following the same normalization approach used for CCK-8 activity. All other measurements were normalized to these reference values. In the ML group, CYP3A4 activity declined to 0.3% by day 15 (Figure 5c) indicating a near-complete loss of function. The ML + GT group began at approximately 98% on day 2 and increased to around 380% by day 15. In the SW group, activity reached 580% on day 15. A slight difference was observed between the ML + GT and SW groups at this time point. The SW + GT group exhibited enzyme activity of approximately 600% on day 15, comparable to the SW group but significantly higher than the ML + GT group.

4 | Discussion

The collagen sandwich culture method was developed as a gold standard, maintaining hepatocyte polarity and structural organization (Baker and Chen 2012; Dunn et al. 1989). However, the dense top gel layer forms a diffusion barrier (Fan et al. 2023; Lang et al. 2011), its permeability is influenced by fiber density, cross-linking degree, and gelation conditions (Cukierman et al. 2001; Gjorevski and Nelson 2012; Antoine et al. 2014). Consequently, the diffusion of larger molecules, including growth factors, lipoproteins, and antibodies (> 10 kDa), is often impeded (Netti et al. 2000; Zaman et al. 2006; Schultz et al. 2011). This barrier may delay or reduce cellular responses to stimuli and, progressive structural changes in the ECM may further exacerbate diffusion heterogeneity over time (Jain 1987; Provenzano and Keely 2011; Stylianopoulos and Jain 2013). These limitations underscore the need for a culture technique that supports a physiologically relevant 3D environment without introducing a diffusion barrier, while preserving the simplicity, accessibility, and imaging compatibility of 2D systems.

Thin, daily Geltrex supplementation in the monolayer format in the ML + GT group addresses these limitations maintaining culture confluency while preserving apicobasal polarization and hepatocyte-specific morphology (Figure 3a). The presence of well-developed bile canaliculi in the ML + GT group indicated not only sustained cell viability but also the establishment of coordinated and functional tissue architecture (Figure 2) (Turncliff et al. 2006; Tocan et al. 2021). These characteristics are essential for sustaining metabolic activity and intercellular transport (Dunn et al. 1989). CCK-8 enzyme activity in the ML + GT group was comparable to both the SW and SW + GT groups (Figure 3b), and live/dead staining further confirmed high hepatocyte viability across these groups (Figure 4b). Collectively, these findings suggest that Geltrex supplementation enhances both structural integrity and PRH function by supporting cell adhesion and organization.

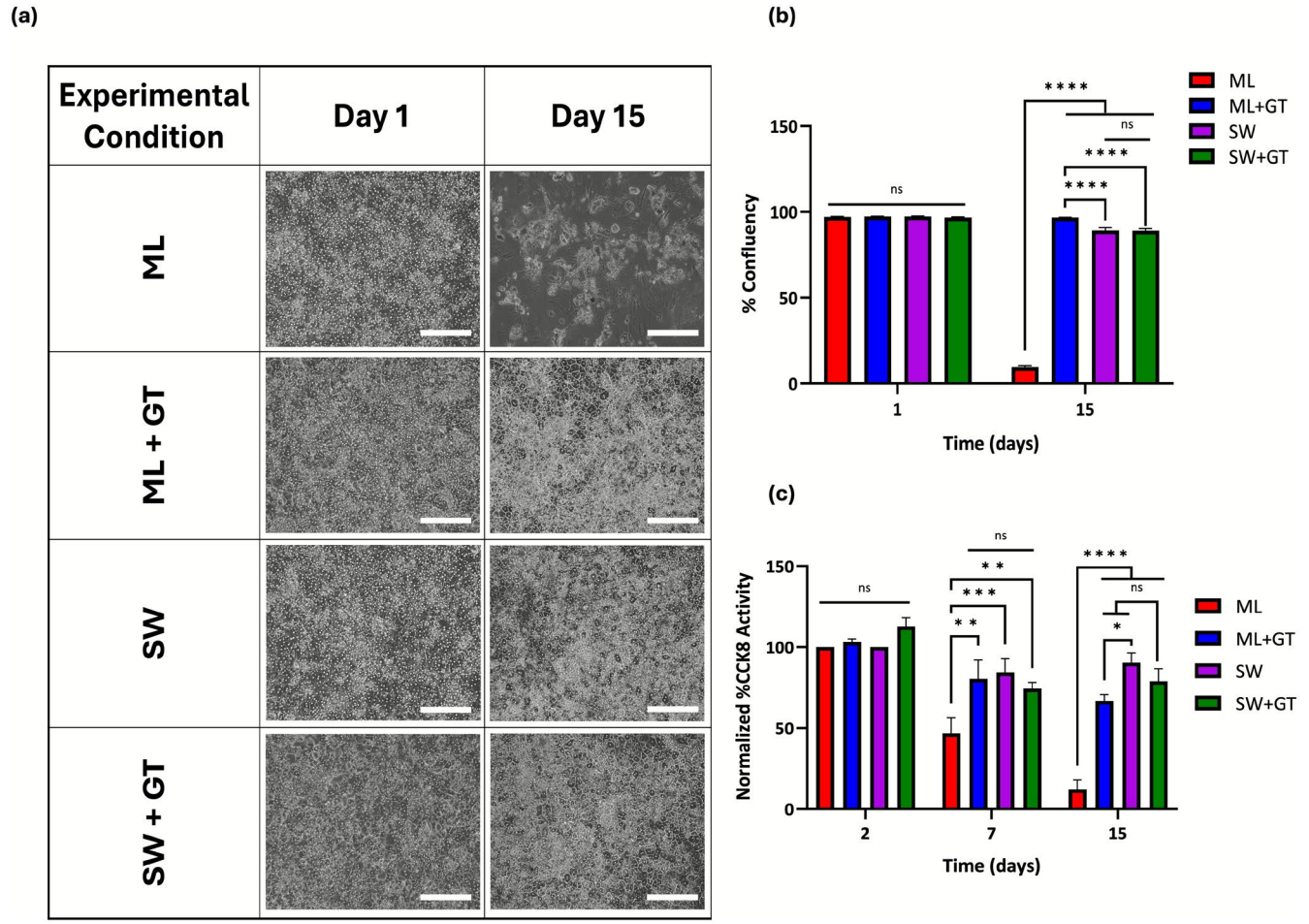


FIGURE 3 | (a) Representative phase-contrast microscopy images of hepatocyte cultures under four different conditions (ML, ML + GT, SW, and SW + GT) on day 1 and 15. Side-by-side comparisons highlight changes in cell confluency from the beginning to the end of the culture period. (b) Quantification of cell confluency based on microscopy images processed using ImageJ software. Confluency values were compared across time points (day 1 and 15) and culture conditions. (c) Percentage CCK-8 activity over time in hepatocyte cultures under four different conditions. CCK-8 values from day 2 of ML and SW groups were set as 100%, and the values from ML + GT and SW + GT groups were normalized accordingly. All data are presented as mean ± SEM. Significance levels: ns, non-significant, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$. Scale bar: 300 μm.

Functional biomarkers, albumin and urea secretion, further corroborate these observations (Buyl et al. 2014; Paulusma et al. 2022). In the ML + GT group, albumin production progressively increased (Figure 5b), consistent with prior studies showing that basement membrane-like matrices derived from EHS tumors enhance albumin gene expression and mRNA levels, primarily via laminin content (Orkin et al. 1977). Given that Geltrex is EHS-derived and rich in laminin, the elevated albumin production in the ML + GT group suggests that laminin-rich ECMs promote hepatocyte function. A similar increase was observed in the SW + GT group, whereas albumin levels in the SW group remained relatively unchanged. Laminin-rich structures have been shown to increase both albumin mRNA and protein levels in primary hepatocytes (Caron 1990; Bissell et al. 1987; Kowalski-Saunders et al. 1992). Therefore, the observed functional increase is consistent with the physiologically relevant microenvironment and biochemical support provided by Geltrex, and is not only due to medium replacement. In contrast, urea secretion remained consistent across all groups except the ML group (Figure 5a).

Enhanced hepatocyte functionality was also evident at the molecular level, as reflected by CYP3A4, a key drug metabolic enzyme responsible for the metabolism of approximately 50%–60% of clinically used drugs (Mulder et al. 2021). CYP3A4 activity is thus routinely used to assess liver cell functionality in vitro (Guillouzo and Guguen-Guillouzo 2008). In the ML + GT group, enzyme activity increased, approaching the levels observed in the SW group, the gold standard. Although the SW + GT group exhibited slightly higher activity than the SW group, this effect was small and did not reach statistical significance (Figure 5c). These results suggest that Geltrex not only provides structural support to the cells but also enhances hepatocyte functionality at the molecular level.

5 | Conclusion

The ML + GT approach may offer a stable and sustainable 2D culture platform, representing a reliable alternative to conventional PRH-based sandwich culture models. Our study may provide a comparison between the ML + GT and SW groups. Both groups maintained high cell viability and preserved cell–cell interactions over 15 days, with cells adopting a cuboidal morphology and forming canalicular networks. The ML + GT

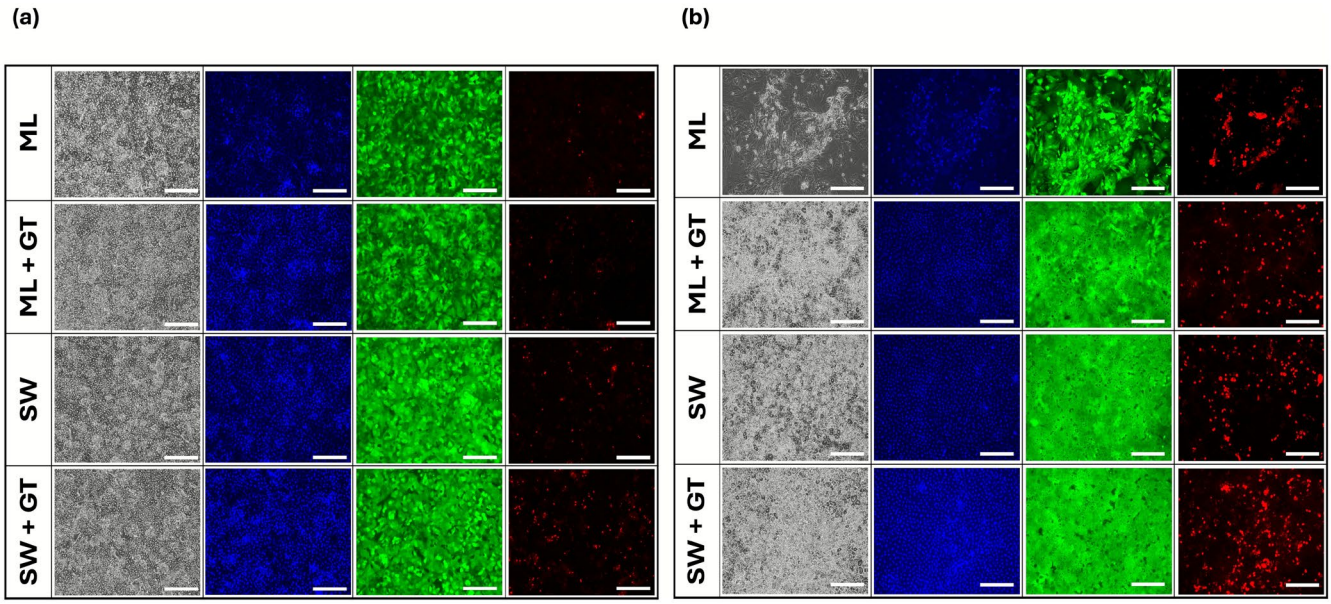


FIGURE 4 | Live/dead staining of hepatocyte cultures under four different conditions (ML, ML + GT, SW, and SW + GT). (a) Fluorescence and phase-contrast microscopy images from day 2 showing nuclear staining (Hoechst), live cells (Calcein-AM), and dead cells (Ethidium Homodimer-1), alongside corresponding phase-contrast images. (b) Live/dead staining of the same culture groups on day 15, using the same labeling agents. Scale bars: 300 μm.

group showed greater albumin synthesis, whereas the SW group exhibited slightly higher performance in other functional parameters, including urea production and CYP3A4 activity. These findings demonstrate that both culture models may support long-term cell viability and functional maintenance, while highlighting specific differences in their performance.

Overall, our results demonstrate that low-dose, daily Geltrex supplementation creates a supportive microenvironment that maintains cellular polarity and hepatocyte function over extended periods in vitro, which could serve as a practical platform for drug screening and metabolic assays. However, this study has several limitations. Although the Geltrex layer formed over the cells is expected to be only a few micrometers thick, our study does not include direct experimental evidence confirming its thickness. Future studies could address this limitation by visualizing the layer using scanning electron microscopy or by fluorescently labeling key components of Geltrex, such as laminin and collagen IV, to confirm their presence and distribution over the hepatocyte monolayer (Rüdiger et al. 2020; Passaniti et al. 2022). Image-based quantitative analyses could then be performed to assess layer thickness, density and homogeneity (Brown et al. 2006). The short experimental duration restricts conclusions about long-term cellular stability and function, and small sample sizes reduce statistical power. Additionally, only a small subset of prototypical enzymatic and metabolic liver functions was assessed, leaving other aspects of hepatocyte physiology unexamined.

Interorgan recapitulation is essential for understanding human physiology, disease mechanisms, and drug safety and efficacy. This is particularly critical in systemic conditions such as metabolic dysfunction-associated steatotic liver disease (MASLD), inflammatory bowel disease, and drug-induced toxicity, where organ cross-talk can drive pathogenesis and adverse effects. Single-organ models provide valuable insights but fail to capture these complex interorgan interactions. Emerging multi-tissue platforms are thus increasingly important for generating more predictive and comprehensive data by recapitulating such interorgan effects. These platforms align with regulatory shifts toward new approach methodologies (NAMs) designed to reduce reliance on animal testing.

The hepatic ECM is not a passive scaffold but a dynamic functional unit that interacts bidirectionally with hepatocytes to maintain homeostasis (Arteel 2024). It actively responds to cellular stress and environmental changes and under physiological conditions, these adaptive responses help preserve tissue stability. In vivo, matrix components undergo continuous cycles of degradation and renewal (Mayorca-Guiliani et al. 2025). Daily low-concentration Geltrex supplementation enables controlled renewal of matrix proteins that are naturally degraded by cellular metabolism (Kim et al. 1997), providing in vitro systems with a more dynamic ECM support that better resembles in vivo conditions This addition may help maintain structural integrity, preserve hepatocyte phenotype and stabilize communication pathways relevant for interorgan signaling (Loneker et al. 2016), thereby promoting prolonged functional stability of primary hepatocytes and enabling more reliable modeling of interorgan interactions.

The need for routine Geltrex replenishment may represent a limitation when adapting this approach to novel methodologies (NAMs) such as microphysiological systems aimed at achieving authentic organ-level remodeling. Nevertheless, automated microfluidic platforms can mitigate this challenge by enabling precise, reproducible Geltrex delivery through programmable pumps, microvalves and dosing modules. These systems may reduce operator variability, maintain stable flow and support

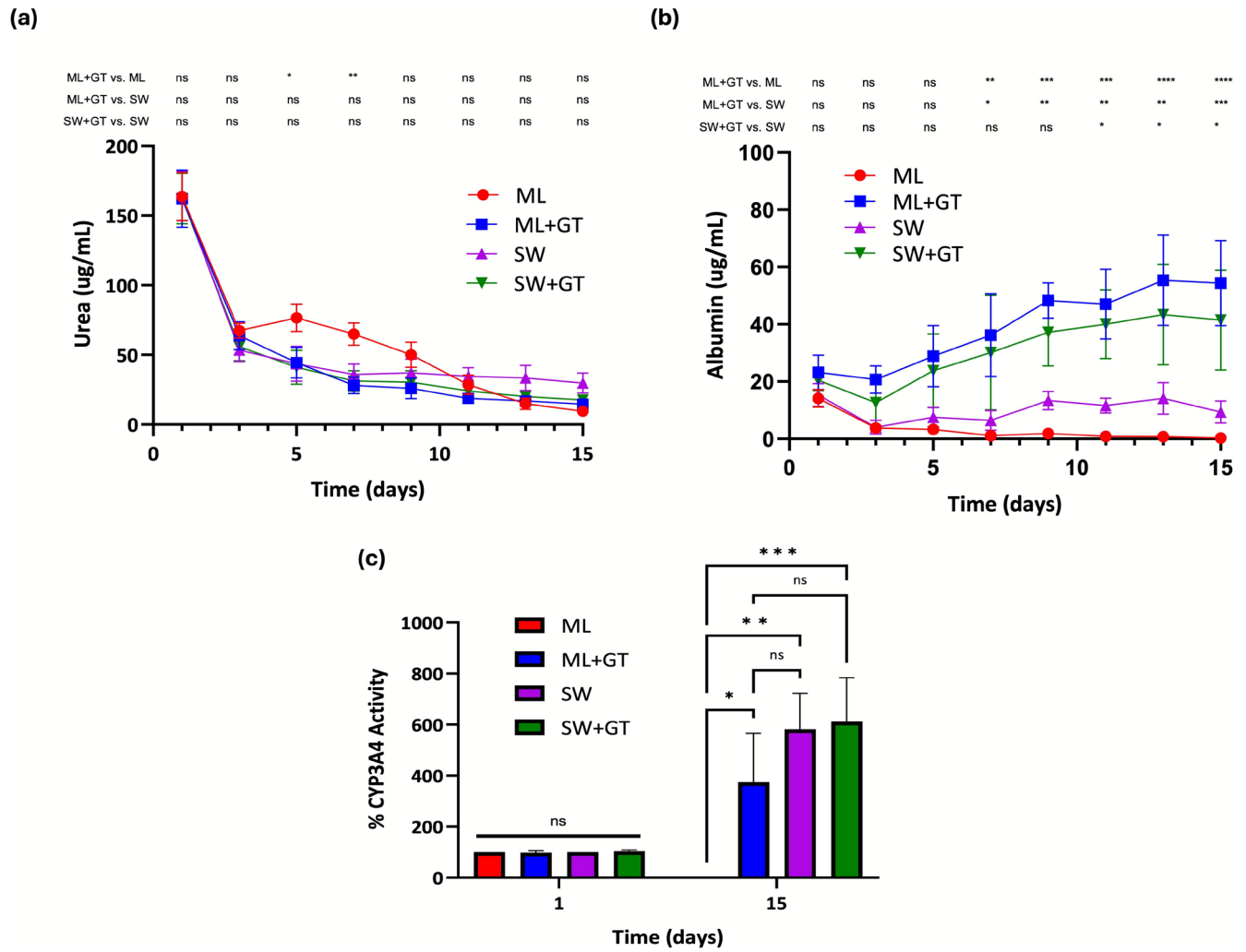


FIGURE 5 | (a) Urea and (b) albumin concentrations synthesized over 15 days by hepatocyte cultures under four conditions: ML, ML + GT, SW, and SW + GT. (c) Percentage CYP3A4 enzyme activity over time. Values on day 2 for ML and SW groups were set at 100%, and values from ML + GT and SW + GT groups were normalized accordingly. Data are presented as mean ± SEM. Significance levels: ns, non-significant, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

functionally stable long-term cultures. Our previous work with perfuse microphysiological liver models suggests that such platforms might sustain hepatocyte phenotype more effectively than static cultures, with potentially higher albumin and urea secretion and improved enzymatic activity (Prodanov et al. 2016), likely driven by ECM remodeling within the hepatic niche (Hegde et al. 2014). Automated platforms may also facilitate parallel multi-organ experiments, improving reproducibility and enabling systematic investigation of interorgan interactions (Meyvantsson et al. 2008; Kurniawan et al. 2024). Integrating Geltrex supplementation into these systems could offer controlled ECM renewal and scalability for multi-organ models. While continuous supplementation was used here, shorter-term dosing might suffice; future studies will compare stabilization durations in static versus perfused systems.

In this study, we show that daily Geltrex supplementation in monolayer primary rat hepatocyte cultures preserves key liver functions and offers a practical alternative to traditional sandwich methods. Unlike traditional gels that can clog microchannels, the Geltrex strategy is readily translatable to microfluidic organ-on-a-chip systems, where dilute supplementation in media sustains tissue functionality. These systems can connect liver tissues to gut, cardiac, or renal models developed using similar Geltrex-based ECM environments. Combined with microfluidic features such as controlled gradients (Kang et al. 2018), automated liquid handling (Gopinathan et al. 2023) and dosing (Vollertsen et al. 2020), Geltrex-based integrated organ-on-chip platforms may hold strong potential for modeling systemic physiology and efficiently assessing off-target effects in health and disease.

Author Contributions

Eda Beldek: conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing – original draft (lead), writing – review and editing. **Matilda Holtz:** visualization, formal analysis, writing – original draft: supporting, writing – review and editing. **Adil Denizli:** supervision, project administration, writing – review and editing. **Osman Berk Usta:** conceptualization, funding acquisition, project administration, resources, supervision, writing – original draft: supporting, writing – review and editing.

Acknowledgments

The authors would like to express their gratitude for the assistance and utilization of the Morphology and Imaging Shared Facility and Regenerative Medicine Shared Facility at Shriners Children's, Boston.

The authors acknowledge the use of AI tools (ChatGPT) to identify and correct typos and similar language-related issues. All final wording and scientific content were produced by the authors.

Funding

This work was supported by the National Institutes of Health (NIH 1R21GM141683, NIH 1R21GM136002, NIH 5R21GM140656, and NIH 5R01HL145031), National Science Foundation (EEC-1941543), Massachusetts General Hospital Executive Committee on Research Interim Support Fund, and Shriners Hospital Research Grant (SHC 85700).

Ethics Statement

The animal study was approved by the Institutional Animal Care and Use Committee (IACUC) at Massachusetts General Hospital (MGH) (protocol #2011 N000111). The study was conducted in accordance with the local legislation and institutional requirements.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data presented in this study is available on request from the corresponding author.

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