FABRICATION AND ATOMIC FORCE MICROSCOPY-BASED MECHANICAL ANALYSIS OF HEPATIC ORGANOIDS

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Abstract. The liver is a vital organ involved in a wide range of processes such as detoxification, protein synthesis, metabolism, and hormone production. Liver diseases, both inherited and viral hepatitis, liver cancer and fatty degeneration are among the leading causes of death in the world. Recent advances in 3D cell culture technology include the use of pluripotent stem cells and adult stem cells that are cultured in vitro to form self-organizing systems. Organoids are self-organizing multicellular structures that reproduce the structure and function of organs and can be used to model the development, maintenance and repair of organs ex vivo. That is why the search for new methods for the formation of hepatic organoids seen as an urgent task. For the fabrication of 3D organoids we isolated hepatic duct cells from rat liver. The obtained cellular structures were analyzed using atomic force microscopy to estimate their mechanical properties and demonstrated increasing of Young Modulus in comparison with normal liver sections. Morphometric evaluation showed that extracellular matrix fibers occupy up to 60% of the organoid, while cell agglomerations up to 40% of it. We observed the spontaneously formed fibrotic liver tissue-like constructs within 21 days. So, obtained hepatic organoids characterized by a tissue-like structure with a predominance of extracellular matrix fibers in its composition similar to liver tissue affected by fibrosis.

Keywords: hepatic organoids; Atomic Force Microscopy; fibrosis; extracellular matrix; mechanical properties.

List of Abbreviations

2D – two-dimensional
3D – three- dimensional
ESCs – embryonic stem cells
iPSCs – induced pluripotent stem cells
PEG – Polyethylene glycol
HSCs – hepatic stellate cells
TGFβ – Transforming growth factor beta
CHF – congenital hepatic fibrosis
PKHD1 – Polycystic Kidney And Hepatic Disease 1
IL-8 – Interleukin-8
AFM – Atomic Force Microscopy
DMEM – Dulbecco's Modified Eagle's Medium
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
DMT – Derjaguin–Mueller–Toporov

Introduction

Flat 2D monolayer cell cultures do not adequately reproduce the physiological conditions of the human body, and therefore, various technologies for creating 3D cell cultures are currently being developed (Paradiso *et al.*, 2023). Self-organized 3D cell assemblies, commonly referred to as organoids, are miniature three-dimensional structures grown from stem cells in a culture dish. These organoids can replicate the organization and function of specific tissues or organs in the body, making them valuable tools for studying human biology and disease. Organoids offer several advantages over traditional cell culture models. Firstly, they can recapitulate the cellular diversity and spatial organization found in human tissues, providing a more realistic representation of in vivo conditions. Additionally, organoids can mimic disease processes and drug responses, making them useful for personalized medicine and drug screening applications. Organoids can be obtained from differentiated embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), or adult stem cells (Lee *et al.*, 2022). In the

presence of a certain set of signals, cell aggregates differentiate to mimic a miniaturized organ of interest (Sullivan *et al.*, 2022). To obtain a given type of organoid, it is necessary to select and strictly control a certain set of parameters, including cell concentration, cultivation conditions, the presence/absence of an extracellular matrix, substrate geometry, etc. (Brassard *et al.*, 2019). For example, intestinal and hepato-organoids are obtained by fine-tuning the concentrations of growth factors common to both processes of organogenesis, and an organoid can change its specialization if conditions change during its formation (Messina *et al.*, 2020). Hepatoorganoids or liver organoids are a convenient tool for modeling congenital and metabolic liver diseases (Shiota et al, 2021) drug toxicity and therapeutic efficacy (Brooks *et al.*, 2021), and can also potentially be transplanted to restore liver function (Kuse *et al.*, 2019). 3D cultures of hepatocytes are more relevant for liver research compared to 2D hepatocyte cultures, as the expression of some specific transporter proteins can be lost in 2D cultures as a result of reduced cell polarization and impaired cell signaling due to limited interactions between hepatocytes (Thompson & Takebe, 2021).

One of the most important fields of hepato-organiods applications is to model hepatic fibrosis, which is a characteristic feature of many liver diseases and manifests itself as the increased accumulation of extracellular matrix, eventually resulting in organ dysfunction (Bao *et al.*, 2021). 3D cell cultures allow including several cell types into one system and thus to better reproduce the complex cell-to-cell interactions existent in real organs. To create an in vitro culture model containing hepaticytotes together with non-parenchymal cells (hepatic stellate cells, liver sinusoidal endothelial cells and liver macrophages) a synthetic PEG-based hydrogel, functionalized with a combination of extracellular matrix components and cell-adhesion molecule peptides was developed (Kumar *et al.*, 2021). When the cultures were treated with either TGFβ or fatty acid to induce fibrosis, the 3D co-cultures demonstrated higher increase in pro-fibrogenic and pro-inflammatory genes than monocultures, supporting the idea that liver fibrosis is dependent on the interaction between hepatocytes and non-parenchymal cells. To study the role of hepatic stellate cells in the development of liver fibrosis, a 3D spheroid co-culture of hepatic stellate cells (HSCs) differentiated from human pluripotent stem cells (iPSC-HSCs) and the human hepatocyte line HepaRG was used (Coll *et al.*, 2018). In contrast to pure HepaRG spheroids, the 3D-co-culture demonstrated high expression of fibrogenic markers and pro-collagen type I secretion as a response to fibrogenic stimuli and thus was capable of recapitulating liver fibrosis. iPSC-HSC spheroids efficiently supported the drug metabolization activity of HepaRG cells allowing the use of this system in anti-fibrotic drug testing.

The crucial role of stellate cells in fibrosis progression was also shown in a liver-on a-chip platform composed of a 3D spheroid containing four types of liver cells (hepatocytes, endothelial cells, Kupffer cells, and stellate cells) that resembled the architecture as well as the nutrients and oxygen gradients in the hepatic lobules (Cho *et al.*, 2021).

3D in vitro cultures are also advantageous because specific gene mutations can be introduced to study inherited diseases. The mechanism of congenital hepatic fibrosis (CHF) was partly understood using genetically engineered human induced pluripotent stem iPS cell line, differentiated into cholangiocyte-like cells in a 3D-culture system (Tsunoda *et al.*, 2019). Animal models of CHF have some phenotypic differences from human CHF, and thus, the disease models based on human cells are needed. To model CHF, the PKHD1 gene responsible for CHF was edited in iPS cells and it was found that pathogenesis of CHF is associated with increased production of IL-8 and CTGF and the enhanced cholangiocyte proliferation by IL-8 stimulation in an autocrine manner (Tsunoda *et al.*, 2019). Thus, the close similarity of 3D liver cell cultures to the liver allows safe and efficient studies of the mechanisms underlying fibrosis development and monitoring different stages of fibrosis progression as well as to screen for new anti-fibrotic drugs. Atomic force

microscopy (AFM) is a cutting-edge imaging technique that allows researchers to visualize and manipulate surfaces at the nanoscale. By using a sharp tip mounted on a cantilever, AFM can detect the forces between the tip and the sample surface, providing high-resolution images and precise measurements of mechanical properties.

Here we demonstrate the one-stage fabrication of 3D liver organoids with fibrotic structure obtained from rat hepatic duct cells using Matrigel dome technique and their mechanical evaluation using Atomic Force Microscopy.

Materials and Methods

Isolation of fragments of the rat hepatic duct

For the experiments, two male Wistar rats were used for isolation of hepatic duct cells. The liver was removed from euthanized animals, cut into 3–5 mm pieces, and incubated with digestion solution (Dulbecco's Modified Eagle's Medium (DMEM)/F12 supplemented with 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), collagenase IV (1 mg/ml) and dispase (1 U/ml)) at 37 °C. Fresh digestion solution was added every 20 min and the previous supernatant was removed and left on ice until the liver was completely digested. The combined mixture was passed through a 70 μm filter and then through a 37 μm reversible filter, and the cell suspension was discarded. The filter was inverted, and the duct fragments captured by the strainer were eluted with ice-cold DMEM/F12. Then, the fragments were precipitated at 300×g at 4 °C for 5 minutes. All animal experiments were maintained according to the protocol of study was approved by the Biomedicine Ethic Expert Committee of Kazan Federal University (protocol 3; 05.05.2015) under institutional and international ethical guidelines.

Cultivation of hepatic organoids

Ice thawed Growth Factor Reduced Matrigel (356231, Corning, MA, USA) was added to the precipitated duct fragments and carefully resuspended with a pipette. 40 μl of the mixture was pipetted into the middle of the well of a pre-warmed 24-well plate. The plate was incubated at 37 °C for 15 minutes to allow the Matrigel suspension to solidify into a dome. HepatiCult™ Organoid Growth Medium (STEMCELL Technology, Vancouver, Canada) was prepared by adding of 5 ml of Supplement to 94 mL of Basal Medium. Aliquots of 700 μl of medium were added per well and organoids were incubated at 37 °C in a humidified atmosphere with 5% CO2. 50 organoids were obtained from each liver. Organoids were monitored daily using inverted microscope AxioObserver A1 (Carl Zeiss, Oberkochen, Germany) in phase contrast mode. The images were obtained using an AxioImager microscope (Carl Zeiss, Oberkochen, Germany) and processed with ZEN software 2.0 (blue edition). Medium was changed every 2–3 days.

Histological study of hepatic organoids

Prior the study the histological structure of three-dimensional organoids, they were fixed in 10% buffered formalin for 48 h and then frozen sections (5 μm) were prepared using a Tissue-Tek Cryo3 cryotome (Sakura, Japan). The sections were stained according to the standard histological method with hematoxylin and eosin to study the general morphology of the hepatic organoid and according to the Van Gieson method to study the presence of collagen fibers. For hematoxylin/eosin staining, frozen sections were washed with distilled water, then stained with Harris' hematoxylin (7 min), nuclei were differentiated in a solution of 0.5% ammonia, then washed with distilled water and stained in eosin (1 min). For the staining according to the Van Gieson method, frozen sections were washed with distilled water, then stained with Weigert's hematoxylin (10 min), washed in running water (10 min), stained with picrofuchsin (5 min), and washed with distilled water. After staining, all sections were dehydrated in alcohols and cleared in xylene. Sections were then mounted in BioMount (Bio Optica, Italy) and covered with coverslips. Histological analysis and morphometry of the obtained sections were performed using a Nikon H550S light microscope (Nikon, Japan) with NIS-Elements Basic Research software.

Atomic Force Microscopy (AFM)

AFM images were taken using a Dimension Icon microscope (Bruker, USA) operating in PeakForceTapping mode. To get the images probes ScanAsyst-air (Bruker) were used (nominal length 115 μm, tip radius 2 nm, spring rate 0.4 Nm-1). Images were taken at 512-1024 lines/scans at a scan rate of 0.8-0.9Hz to ensure high image resolution and display of mechanical properties. The Derjaguin–Mueller–Toporov (DMT) model was chosen to determine mechanical characteristics of a thin sections surface (Young's modulus). The measurements were carried out on a sample area of 10x10 μm in the DMT Modulus channel. To ensure the reliability of the results obtained, in each experiment, measurements were made of 10-20 liver sections and spheroids.

Statistical analysis

Statistical differences between different groups of cells were examined using a one-way ANOVA test in GraphPad Prism 8. Significance of differences was calculated using a two-sided Dunnett test with a 95% confidence interval. Differences were considered statistically significant when $p < 0.05$.

Results

Morphological changes during the cultivation of liver organoids from ductal cells consisted in the gradual compaction of cells inside the Matrigel dome, while at the stage of 21 days, the appearance of lateral outgrowths was observed (Fig. 1 C-E). At the same time, their adhesion to the substrate and the proliferation of cells of the organoid surface were observed (Fig. 1B), which indicates its normal metabolic activity and the ability of cells to proliferate throughout the entire cultivation period.

Histological evaluation of a three-dimensional liver organoids (Fig. 1 F-H) showed that they commonly have an oval cyst-like structure. Organized heterogeneity of this three-dimensional formation is also observed. In the composition of the organoid, the outer coating is easily distinguishable, which is represented by endothelial cells exhibited flat structure and flattened nuclei. The architecture of the fabricated hepatic organoid is based on extracellular matrix (ECM) fibers (including collagen fibers, as shown by Van Gieson staining (Fig. 1 G)), as well as cell agglomerates scattered throughout the organoid. Morphometric evaluation showed that ECM fibers can occupy up to 60% of the organoids, and cell agglomerations up to 40% of the organoids.

Cellular agglomerations within the hepatic organoid are characterized by the presence of cells of a presumably hepatocyte-like structure (parenchymal cells), which have a multifaceted shape with a rounded nucleus occupying a central position. In addition, it is worth assuming the presence of stellate cells (HSC), which are involved in the formation of collagen fibers. It is also important to note the presence of duct-like structures (Fig. 1H) in the volume of the hepatic organoid, the walls of which are formed by endothelial-like cells. Thus, these three-dimensional liver organoids are characterized by a tissue-like structure with a predominance of ECM fibers and can be used as a models of liver fibrosis for the screening of antifibrotic drugs contributing to the discovery of effective treatments. However, the observed spontaneous collagen secretion and rapid formation of a dense collagen matrix will be the subject of further detailed study.

Morphological differences between sections of native liver tissue and organoid from ductal cells at 21 days of formation consisted in the fact that cell clusters present in organoids had weak intercellular contacts and formed looser agglomerations (Fig. 2B) compared to the liver parenchyma (Fig. 2C). In the models described earlier, authors observed the cavities of the internal contents due to autophagy (Elkasaby *et al.*, 1991) which can be associated with the activation of hepatic stellate cells (Yu *et al.*, 2022), however, in the organoids obtained by us, we observed the filling of the entire internal content of the cell-free organoid with collagen fibers, which was shown using histological analysis with Van Gieson staining (Fig. 1G).

We employed Atomic Force Microscopy to quantify the stiffness of thin section. The Derjaguin–Mueller–Toporov (DMT) model was

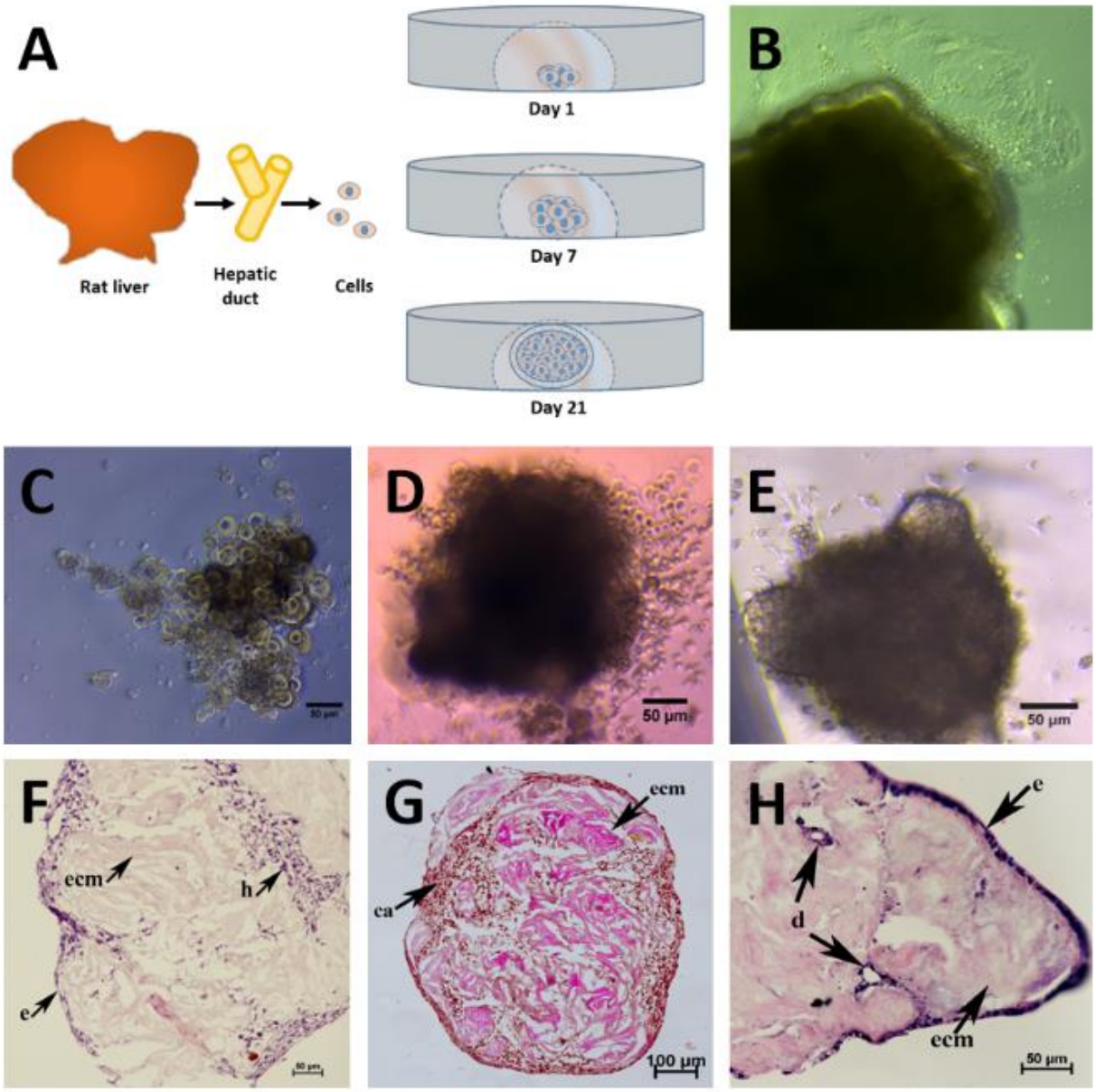
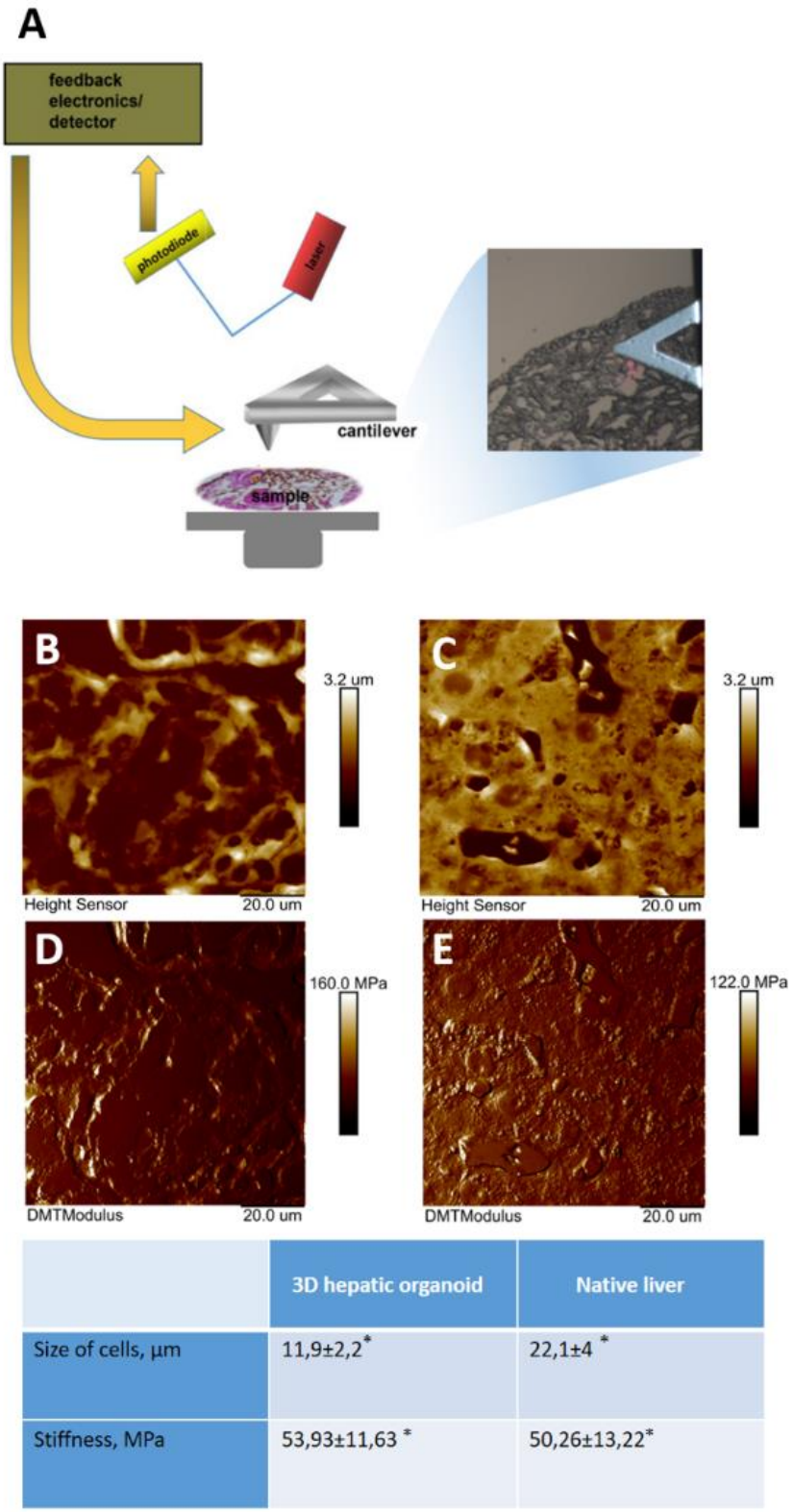


Fig. 1. Fabrication and morphological evaluation of 3D rat liver organoids. (A) – scheme demonstrated the overall process of ductal cells isolation and cultivation of 3D tissue-like structures; (B) – proliferation of cells of the organoid surface after incubation on adhesive plates; (C–E) – light microscopy of organoid in Matrigel dome after 24 h, 7 d and 21 d, respectively, (F–H) – histological profile of 3D liver organoids: (F, H) – hematoxylin-eosin staining, magnification 200x; (G) – Van Gieson staining, magnification 100x. Abbreviations: ecm – extracellular matrix fibers, h – hepatocyte-like cells (parenchymal cells), e – endothelial cells, d – duct-like structures, ca – cellular agglomerations



	3D hepatic organoid	Native liver
Size of cells, µm	11,9±2,2*	22,1±4 *
Stiffness, MPa	53,93±11,63 *	50,26±13,22*

Fig. 2. Scheme of formation of atomic force images (A) and analysis of topography and Young's Modulus of 3D hepatic organoids (B, D, respectively) in comparison with native rat liver sections (C, E). Numerical values in table below AFM images represent the average value of cell size and stiffness determined from the analysis of 10 sections of 3D organoids and native rat liver using AFM. Significance $p < 0.05$ (*)

chosen to determine mechanical characteristics of a thin sections surface (Young's modulus). This model refines the simplest model (Hertz), taking into account adhesion outside the contact area, and is more applicable to small cantilevers (Heinz & Hoh, 1999) which we used here. Previously, we showed the possibilities of AFM for analyzing changes in stiffness during stem cell differentiation (Naumenko *et al.*, 2021).

Samples of liver organoids' thin sections exhibited slight enhancement in stiffness (Fig. 2) in comparison with normal rat liver sections. We measured the Young's modulus of normal liver tissue and hepatic organelles characterized by a high content of collagen fibers and showed some increase in stiffness, which can be used in the complex diagnosis of fibrotic changes.

Discussion

In our study, we demonstrated the possibility of one-stage formation of liver organoids with spontaneously occurred signs of fibrotic changes by cultivation of ductal cells in Matrigel. In the study of Ouchi *et al.* pluripotent stem cell lines we used to develop a reproducible method to derive multi-cellular human liver organoids composed of hepatocyte-, stellate-, and Kupffer-like cells (Ouchi *et al.*, 2019). A number of studies have shown that the liver contains stem cells that are associated with ducts (Vessey & Hall, 2001), and in this regard, we used the protocol of STEMCELL Technology as the basis for the formation of organoids. The resulting three-dimensional structures contained various cell types and connective tissue fibers distinguishable by histological staining and it was revealed that 3D structures have pronounced signs of fibrosis. Previously the model of spontaneous liver fibrosis in mice were induced by natural aging were established (Qiu *et al.*, 2023). The presence of viable cells in the organoids was clearly demonstrated by the presence of a proliferation zone when the organoid is placed on an adhesive surface. This method has been previously used to demonstrate cell viability within a 3D multicellular spheroid (Dzamukova *et al.*, 2015).

According to a number of studies, damage to hepatocytes leads to an increase in liver stiffness (Georges *et al.*, 2007; Wells, 2008) which directly affects the function of hepatocytes and activates hepatic stellate cells. Then, portal fibroblasts differentiate into collagen-depositing myofibroblasts (Olsen, 2011; Sandrin *et al.*, 2003). The deposition of fibrous extracellular matrix further contributes to a further increase in liver stiffness, which in turn contributes to an even greater increase in liver stiffness and activation of matrix-producing cells (self-reinforcing feedback loop). However, at present there are few studies on the mechanical properties of the liver and *in vitro* fibrosis models using AFM.

The study of the mechanical properties of liver organoids in comparison with native liver was carried out using atomic force microscopy, which allows obtaining high-resolution images of tissue topography and simultaneously measuring elasticity and rigidity. Ojha *et al.* demonstrated that liver sections of mice subjected to fibrosis induction had greater rigidity than control samples (Ojha *et al.*, 2022). In this work, the studies were carried out in an aqueous medium, which imposes more serious requirements on sample preparation. In our study, we demonstrated the possibility of obtaining data on the hardness of samples in dry samples in air.

Further research will be aimed at analyzing using atomic force microscopy the mechanical properties of organoids at earlier stages of their formation and molecular mechanism of fibrotic changes in hepatic organoids.

Conclusions

Overall, liver organoids provide a valuable platform for studying liver diseases, including fibrosis, under controlled laboratory conditions. Organoids can provide important insight into the mechanisms of fibrosis assessment, but it is important to remember that they are not exact replicas of the native liver because organoids are composed of a limited number of cell types. We obtained organoids with some structures similar to native liver, including a duct-like structure, but it was demonstrated that in the

case of organoids the cell size was reduced. Our pilot study shows that fibrotic changes in 3D liver organoids from ductal cells can develop without any chemical or physical influence in 21 days, which requires further study of the molecular mechanisms of such changes. In conclusion, our studies evaluating the histological structure and mechanical characteristics are relevant from the point of view of global trends in the development of tissue engineering and pharmacology.

Author Contributions

EN and IG contributed toward the conception and design of the study. All authors contributed toward data acquisition, analysis, and/or interpretation of data and drafted the work or revised it critically for intellectual content, and approved the submitted version.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

BAO Y.L., WANG L., PAN H.T., ZHANG T.R., CHEN Y.H., XU S.J., MAO X.L. & LI S.W. (2021): Animal and organoid models of liver fibrosis. *Front. Physiol*. **12**, 666138.

BRASSARD J.A. & LUTOLF M.P. (2019): Engineering stem cell self-organization to build better organoids. *Cell Stem Cell* **24**, 860–876.

BROOKS A., LIANG X., ZHANG Y., ZHAO C.X., ROBERTS M.S., WANG H., ZHANG L. & CRAWFORD D.H. (2021): Liver organoid as a 3D in vitro model for drug validation and toxicity assessment. *Pharmacol. Res.* **169**, 105608.

CHO H.J., KIM H.J., LEE K., LASLI S., UNG A., HOFFMAN T., NASIRI R., BANDARU P., AHADIAN S., DOKMECI M.R. & LEE J. (2021): Bioengineered Multicellular Liver Microtissues for Modeling Advanced Hepatic Fibrosis Driven Through Non-Alcoholic Fatty Liver Disease. *Small* **17**(14), 2007425.

COLL M., PEREA L., BOON R., LEITE S.B., VALLVERDÚ J., MANNAERTS I., SMOUT A., EL TAGHDOUINI A., BLAYA D., RODRIGO-TORRES D. & GRAUPERA I. (2018): Generation of hepatic stellate cells from human pluripotent stem cells enables in vitro modeling of liver fibrosis. *Cell Stem Cell* **23**(1), 101–113.

DZAMUKOVA M.R., NAUMENKO E.A., ROZHINA E.V., TRIFONOV A.A. & FAKHRULLIN R.F. (2015): Cell surface engineering with polyelectrolyte-stabilized magnetic nanoparticles: A facile approach for fabrication of artificial multicellular tissue-mimicking clusters. *Nano Research* **8**, 2515–2532.

ELKASABY A., XU D., SCHRÖTER-KERMANI C. & MERKER H.J. (1991): Morphology, differentiation and matrix production of liver cells in organoid cultures (high density cultures) of fetal rat livers. *Histol Histopathol*. **6**(2), 217–28.

GEORGES P.C., HUI J.J., GOMBOS Z., MCCORMICK M.E., WANG A.Y., UEMURA M., MICK R., JANMEY P.A., FURTH E.E. & WELLS R.G. (2007): Increased stiffness of the rat liver precedes matrix deposition: Implications for fibrosis. American Journal of Physiology. Gastrointestinal and Liver *Physiology* **293** (6), 1147–1154.

HEINZ W.F. & HOH J.H. (1999): Spatially resolved force spectroscopy of biological surfaces using the atomic force microscope. *Trends Biotechnol.* **17**(4), 143–150.

KUSE Y. & TANIGUCHI H. (2019): Present and future perspectives of using human-induced pluripotent stem cells and organoid against liver failure. *Cell Transplant*. **28**(1S), 160S–165S.

KUMAR M., TOPRAKHISAR B., VAN HAELE M., ANTORANZ A., BOON R., CHESNAIS F., DE SMEDT J., TRICOT T., IDOYPE T.I., CANELLA M. & TILLIOLE P. (2021): A fully defined matrix to support a pluripotent stem cell derived multi-cell-liver steatohepatitis and fibrosis model. *Biomaterials* **276**, 121006.

LEE J., MUN S.J., SHIN Y., LEE S. & SON M.J. (2022): Advances in liver organoids: model systems for liver disease. *Arch. Pharm. Res*. **45**, 390–400.

MESSINA A., LUCE E., HUSSEIN M. & DUBART-KUPPERSCHMITT A. (2020): Pluripotent-stem-cell-derived hepatic cells: hepatocytes and organoids for liver therapy and regeneration. *Cells* **9**(2), 420.

NAUMENKO E., GURYANOV I., ZAKIROVA E. & FAKHRULLIN R. (2021): Forskolin-Loaded Halloysite Nanotubes as Osteoconductive Additive for the Biopolymer Tissue Engineering Scaffolds. *Polymers* **13**, 3949.

OJHA S., PRIBYL J., KLIMOVIC S., HADRABA D., JIROUSKOVA M. & GREGOR M. (2022): Measurement of Liver Stiffness Using Atomic Force Microscopy Coupled with Polarization Microscopy. *J. Vis. Exp*. **185**, e63974.

OLSEN A.L. (2011): Hepatic stellate cells require a stiff environment for myofibroblastic differentiation. *American Journal of Physiology. Gastrointestinal and Liver Physiology* **301**(1), 110–118.

OUCHI R., TOGO S., KIMURA M., SHINOZAWA T., KOIDO M., KOIKE H., THOMPSON W., KARNS R.A., MAYHEW C.N., MCGRATH P.S., MCCAULEY H.A., ZHANG R.R., LEWIS K., HAKOZAKI S., FERGUSON A., SAIKI N., YONEYAMA Y., TAKEUCHI I., MABUCHI Y., AKAZAWA C., YOSHIKAWA H.Y., WELLS J.M. & TAKEBE T. (2019): Modeling Steatohepatitis in Humans with Pluripotent Stem Cell-Derived Organoids. *Cell Metab.* **6;30**(2), 374–384.

PARADISO A., VOLPI M., RINOLDI C., CELIKKIN N., NEGRINI N.C., BILGEN M., DALLERA G., PIERINI F., COSTANTINI M., ŚWISZKOWSKI W. & FARE S. (2023): In vitro functional models for human liver diseases and drug screening: beyond animal testing. *Biomater. Sci.* **11**, 2988–3015

QIU L., MA Z., SUN J., WU Z., WANG M., WANG S., ZHAO Y., LIANG S., HU M. & LI Y. (2023): Establishment of a Spontaneous Liver Fibrosis Model in NOD/SCID Mice Induced by Natural Aging. *Biology* **12**, 1493.

SANDRIN L., FOURQUET B., HASQUENOPH J.M., YON S., FOURNIER C., MAL F., CHRISTIDIS C., ZIOL M., POULET B., KAZEMI F. & BEAUGRAND M. (2003): Transient elastography: A new non-invasive method for assessment of hepatic fibrosis. *Ultrasound in Medicine & Biology* **29** (12), 1705–1713.

SHIOTA J.J, SAMUELSON L.C. & RAZUMILAVA N. (2021): Hepatobiliary organoids and their applications for studies of liver health and disease: are we there yet? *Hepatology* **74**(4), 2251–2263.

SULLIVAN K.M., KO E., KIM E.M., BALANCE W.C., ITO J.D., CHALIFOUX M., KIM Y.J., BASHIR R. & KONG H. (2022): Extracellular microenvironmental control for organoid assembly. *Tissue Engineering Part B* **28**, 1209–1222.

THOMPSON W.L. & TAKEBE T. (2021): Human liver model systems in a dish. *Develop. Growth. Differ*. **63**, 47–58.

TSUNODA T., KAKINUMA S., MIYOSHI M., KAMIYA A., KANEKO S., SATO A., TSUCHIYA J., NITTA S., KAWAI-KITAHATA F., MURAKAWA M. & ITSUI Y. (2019): Loss of fibrocystin promotes interleukin-8-dependent proliferation and CTGF production of biliary epithelium. *J. Hepatol*. **71**(1), 143–152.

VESSEY C.J. & HALL P.D.L.M. (2001): Hepatic stem cells: a review. *Pathology* **33**, 130–141.

WELLS R.G. (2008): The role of matrix stiffness in regulating cell behavior. *Hepatology* **47** (5), 1394–1400.

YU X., ELFIMOVA N., MÜLLER M., BACHURSKI D., KOITZSCH U., DREBBER U., MAHABIR E., HANSEN H.P., FRIEDMAN S.L., KLEIN S., DIENES H.P., HÖSEL M., BUETTNER R., TREBICKA J., KONDYLIS V., MANNAERTS I. & ODENTHAL M. (2022): Autophagy-related activation of hepatic stellate cells reduces cellular miR-29a by promoting its vesicular secretion. *Cell Mol Gastroenterol Hepatol*. **13**(6), 1701–1716.