A pre-clinical model combined cryopreservation technique with precision-cut slice culture method to assess in vitro drug response of hepatocellular carcinoma

Yuan Zhang[1], Zhen-yu Wang[1], Hong-shu Jing[1], Hong-dan Zhang[2], He-xin Yan[1], Jian-xia Fan[3], and Bo Zhai[1]

[1]Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital
[2]Celliver Biotechnology Co. Ltd, Shanghai, China.
[3]International Peace Maternity and Child Health Hospital

March 07, 2024

Abstract

Background The aim of this study was to create a patient-derived slice model by combining cryopreservation technique with precision-cut slice culture method and explore its effectivity of predicting anti-cancer drug sensitivity in vitro. Methods We prepared 0.3 mm thick tissue slices by a microtome and maintain its cell viability by cryopreservation technique. Slices were cultured individually in the presence or absence of regorafenib (REG) for 72 hours. Alterations in morphology and gene expression was assessed by histological and genetic analysis. Overall viability was also analyzed in tissue slices by CCK-8 quantification assay and fluorescent staining. Tissue morphology and cell viability could be evaluated to quantify drug effects. Results Histological and genetic analysis showed that no significant alterations in morphology and gene expression were induced by vitrification-based cryopreservation. The viability of warmed HCC tissues was up to 90% of the fresh tissues. The viability and proliferation could be retained for at least four days in filter culture system. The positive drug responses in precision-cut slice culture in vitro were evaluated by tissue morphology and cell viability. Conclusions In summary, the successful application of precision-cut HCC slice culture combining cryopreservation technique in a systematic drug screen demonstrates the feasibility and utility of slice culture method for drug response.

A pre-clinical model combined cryopreservation technique with precision-cut slice culture method to assess in vitro drug response of hepatocellular carcinoma

Yuan Zhang[1,2,3], Zhen-yu Wang[1,4], Hong-shu Jing[1,4], Hong-dan Zhang[6], He-xin Yan[1,5], Jian-xia Fan[2,3*], Bo Zhai[1,4*]

[1]Department of Interventional Oncology, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China;

[2]International Peace Maternity and Child Health Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China;

[3]Shanghai Key Laboratory of Embryo Original Diseases, Shanghai, China

[4]State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai;

[5]Department of Anesthesiology and Critical Care Medicine, Renji Hospital, Jiao Tong University School of Medicine, Shanghai, China.

Posted on 31 Jan 2024 — The copyright holder is the author/funder. All rights reserved. No reuse without permission. — https://doi.org/10.22541/au.170668421.10975879/v1 — This is a preprint and has not been peer-reviewed. Data may be preliminary.

Posted on 31 Jan 2024 — The copyright holder is the author/funder. All rights reserved. No reuse without permission. — https://doi.org/10.22541/au.170668421.10975879/v1 — This is a preprint and has not been peer-reviewed. Data may be preliminary.

[6]Celliver Biotechnology Co. Ltd, Shanghai, China.

Correspondence to:

[*]Bo Zhai, Department of Interventional Oncology, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, 160 Pujian Road, Shanghai, China.

E-mail: *zhaiboshi@sina.com*; Postal address: 200127.

[*]Jian-xia Fan, International Peace Maternity and Child Health Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China.

E-mail: fanjianxia122@126.com; Postal address: 200030.

[*]Contributed equally

Key words

Pre-clinical model; Cryopreservation; Slice culture; Drug response; Hepatocellular carcinoma.

List of Abbreviations: Hepatocellular carcinoma, HCC; Hematoxylin and eosin, HE; Immunohistochemistry, IHC; Lactate dehydrogenase, LDH; Regorafenib, REG; Cell Counting Kit-8, CCK 8.

Abstract

Background

The aim of this study was to create a patient-derived slice model by combining cryopreservation technique with precision-cut slice culture method and explore its effectivity of predicting anti-cancer drug sensitivity in vitro.

Methods

We prepared 0.3 mm thick tissue slices by a microtome and maintain its cell viability by cryopreservation technique. Slices were cultured individually in the presence or absence of regorafenib (REG) for 72 hours. Alterations in morphology and gene expression was assessed by histological and genetic analysis. Overall viability was also analyzed in tissue slices by CCK-8 quantification assay and fluorescent staining. Tissue morphology and cell viability could be evaluated to quantify drug effects.

Results

Histological and genetic analysis showed that no significant alterations in morphology and gene expression were induced by vitrification-based cryopreservation. The viability of warmed HCC tissues was up to 90% of the fresh tissues. The viability and proliferation could be retained for at least four days in filter culture system. The positive drug responses in precision-cut slice culture in vitro were evaluated by tissue morphology and cell viability.

Conclusions

In summary, the successful application of precision-cut HCC slice culture combining cryopreservation technique in a systematic drug screen demonstrates the feasibility and utility of slice culture method for drug response.

Introduction

Liver cancer is one of the most common type of malignant cancer and there are approximately 850,000 new cases yearly worldwide[1]. The high incidence of HCC has induced the development of novel targeted and personalized therapies[2]. Personalization of cancer treatment requires reliable prediction of chemotherapy responses in individual patients. Various strategies have been applied to generate primary cultures from individual tumors which include 2D-cell culture of dissociated tumor cells, 3D spheroid cultures and patient

Posted on 31 Jan 2024 — The copyright holder is the author/funder. All rights reserved. No reuse without permission. — https://doi.org/10.22541/au.170668421.10975879/v1 — This is a preprint and has not been peer-reviewed. Data may be preliminary.

derived mouse xenograft cultures[3-7]. However, the difficulties in replicating the heterogeneous microenvironment in primary tumor reduce their efficiency in drug experiments[8]. It was estimated that over 90% of novel anticancer drugs fail in clinical trials because these models could not simulate complete tissue structure and maintain the biological heterogenicity of primary tumor[9]. For these reasons, it is crucial for us to create a novel model that are more predictive of in vivo efficacy.

Precision-cut slice is a new method of tissue culture in vitro, which derived directly from primary tumor[10]. However, there is no preservation method applied to maintain living fresh tissue. Conventional preservation of fresh tumor tissue like formalin-fixed paraffin embedded samples and flash freezing in liquid nitrogen always leads to the absolute inactivation of the fresh tissue. Therefore, a reliable and efficient cryopreservation method for living tissue is indispensable. Vitrification-based cryopreservation method can be developed to preserve fresh tissue, by which the biological characteristics of the original tumor can be retained and the utilization of specimens may be markedly improved[11].

Here, we explore a precision-cut slice culture method combining with cryopreservation technique to establish a preclinical model, which is derived from fresh tissues of HCC patients. Besides, we show systematic optimization of HCC slices ex vivo by comparing different culture conditions. What's more, this culture system allowed detection in tumor responses to REG chemotherapy.

Materials and methods

Collection of HCC specimens

Surgically resected specimens were obtained from 30 cases of HCC patients at the Renji Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (Shanghai, China). Samples were maintained at 4@C on ice and transported in preservation medium (Tissue Mate; Celliver Biotechnology Co. Ltd). This investigation was approved by the ethics committee of Renji Hospital. The pathological diagnosis of all patients was HCC and none had received any prior treatment. Details were illustrated in Figure 1.

Cryopreservation and warming procedures

All specimens were cut into 1mm-thick slices in a metal mold before cryopreservation (Fig. 2C). Cryopreservation solutions (LT2601; Tissue Mate) and warming solutions (LT2602; Tissue Mate) were provided by Celliver Biotechnology Co. Ltd (Fig. 2B). For tissue cryopreservation, vitrification solution 1 (V1), vitrification solution 2 (V2) and vitrification solution 3 (V3) were pre-warmed in a 2˜8@C water bath. Fresh HCC tissues were cleaned twice with sterile PBS and transferred into 10 ml V1, 10 ml V2 and 10 ml V3 for 8, 8, 10 mins, respectively. Tissues were then placed onto a thin metal strip and submerged into liquid nitrogen for at least 5 minutes. Finally, the strips with tissue were placed into frozen storage tubes and preserved in the nitrogen canister. The tissue samples were stored in the liquid nitrogen. For tissue warming, the frozen storage tubes were removed from the nitrogen canister and the strips with the cryopreserved biopsy tissues were quickly transferred into 30 ml warming solution 1 (T1), and incubated for 3 mins in a 37@C water bath. The tissues were then transferred into 10 ml warming solution 2 (T2) and 10 ml warming solution 3 (T3) for 5 and 10 minutes, respectively, at room temperature. Warmed tissues were cleaned twice with sterile PBS and kept on ice (Fig. 2D). The timeline of cryopreservation and warming procedures were depicted in Figure 2A.

Tissue slice preparation and cultivation

Surgically resected specimens were cut into 300µM-thick precision-cut slices using a microtome for slice preparation (Bio-Gene Technology Ltd.) (Fig. 2E). 300-µM was considered the most suitable thickness for HCC after several early slicing pre-experiments (Fig. 2F). Parameter settings, such as the frequency and amplitude of vibration slicing, were determined by the diverse cirrhosis degree and tumor stage. Tissue slices (diameter, 2 mm) were then prepared using a hand-held coring tool (Fig. 2G), and all the procedures were performed under sterile conditions. One-third of the precision-cut slices were maintained on transwell inserts (pore size, 0.4 µm; Corning Inc.) (Fig. 2I). One-third of the precision-cut slices were individually submerged in medium (Fig. 2H) and incubation was performed on a shaking platform (TYZD-III, QiQian Technology

Posted on 31 Jan 2024 — The copyright holder is the author/funder. All rights reserved. No reuse without permission. — https://doi.org/10.22541/au.170668421.10975879/v1 — This is a preprint and has not been peer-reviewed. Data may be preliminary.

Ltd.). The rest precision-cut slices were cultured statically in medium as control (Fig. 2H). Cultivation was performed in 12-well plates containing 450 µl DMEM medium (Gibco) with 10% fetal bovine serum (Gibco), penicillin and streptomycin (100 U/ml; Gibco), and kept at 37@C in a humidified incubator with 5% CO2.

Cell Counting Kit-8 (CCK-8) assay

A CCK-8 assay (Dojindo Molecular Technologies, Inc.) was used to evaluate the viability of tissue slices at each time point (24h, 48h, 72h, 96h). DMEM medium (90 µl/well) and CCK-8 solution (10 µl/well) were added into 96-well plates. The tissue slices were added one slice/well. The plates were kept at 37@C in a humidified incubator with 5% CO2 for 2 h. The slices were removed from the 96-well plates and the plates were transferred to microplate reader (Multiskan GO; Thermo Fisher Scientific Inc.). The absorbance at 450 nm was measured and three wells were tested for each sample at each time point.

Calcein-AM cell viability assay and Hoechst 33342 staining

The Live/Dead® Viability Assay kit (Nanjing KeyGen Biotech Co.) and Hoechst 33342 (Beyotime Institute of Biotechnology Co.) were stored at -20@C and allowed to warm to room temperature prior to experimentation. The viability assay stock reagents (calcein-AM, 4 mM) were diluted to 1 µM in physiological solution and mixed with 2 µg/ml Hoechst 33342 stock reagents at room temperature for 30 min. Live cells are characterized by a bright green fluorescent and cell nucleus are blue. Representative images were captured with the Leica TCS SP8 confocal microscope (Leica Microsystems GmbH). The ratio of living cells in the calcein-AM cell viability assay/Hoechst 33342 staining were calculated based on manual counting within ten random microscopic fields.

HE/IHC staining

Tumor slices were formalin-fixed, embedded in paraffin and cut to 4µm thick sections. Paraffin sections (4 µm) were stained with HE at room temperature. IHC staining was carried out by standard protocols. Briefly, sections were de-waxed in xylene and rehydrated in graded ethanol, heat mediated antigen retrieval of tissue sections was carried out before being allowed to cool. Endogenous peroxidases were blocked using 0.9–3% hydrogen peroxide for 10 minutes, and non-specific antibody binding blocked by incubation with serum free blocking solution or 10% normal serum block for 30 minutes. Tissue sections were then incubated with primary antibodies (Ab15580; Abcam; 1:1,000 dilution), before being probed with secondary antibodies (Ab150077; Abcam; 1:1,000 dilution). Antibodies were visualized using 3,3'-diaminobenzidine chromogen and counterstained with Meyer´s Hematoxylin for 2 minutes. Sections were then dehydrated through graded alcohols, cleared in xylene and mounted. Confocal laser scanning microscopy was performed using an Olympus Corporation BX51 instrument. The ratio of proliferative cells in the Ki67 staining were calculated based on manual counting within ten random microscopic fields.

Real-time polymerase chain reaction (PCR) analysis

Quantitative PCR was performed using SYBR Green PCR Kit (Applied Biosystems, Foster City, CA). The messenger RNA (mRNA) level of specific genes was normalized against β-actin.

Experimental methods for mRNA sequencing

RNA purity was checked using the kaiaoK5500® Spectrophotometer (Beijing Kaiao Technology Development Co. Ltd.). RNA integrity and concentration was assessed using the RNA Nano 6000 Assay kit and the Bioanalyzer 2100 system (Agilent Technologies Inc.). A total amount of 2µg RNA/sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra RNA Library Prep kit for Illumina(r) (E7530L; New England BioLabs Inc.), following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from the total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and RNase H. Second strand cDNA synthesis was subsequently performed using buffer, dNTPs, DNA polymerase I and RNase H. The library

Posted on 31 Jan 2024 — The copyright holder is the author/funder. All rights reserved. No reuse without permission. — https://doi.org/10.22541/au.170668421.10975879/v1 — This is a preprint and has not been peer-reviewed. Data may be preliminary.

fragments were purified with QiaQuick PCR kits (Qiagen Inc.) and elution with EB buffer, then terminal repair, A-tailing and adapter adding were implemented. The products were retrieved and PCR was performed, then the library was completed. The RNA concentration of the library was measured using a Qubit(r) RNA Assay kit in Qubit(r) 3.0 (Thermo Fisher Scientific Inc.)for preliminary quantification, and then diluted to 1 ng/µl. Insert size was assessed using the Agilent Bioanalyzer 2100 system (Agilent Technologies Inc.), and qualified insert size was accurately quantified using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Inc.; library valid concentration, >10 nM). The clustering of the index-coded samples was performed on a cBot cluster generation system using a HiSeq PE Cluster kit v4-cBot-HS (Illumina Inc.) according to the manufacturer's instructions. After cluster generation, the libraries were sequenced on an Illumina, Inc. platform and 150 bp paired-end reads were generated. The variations in gene expression could be detected by different colors in the heat map.

Metabolic Activity of PH/Glucose/LDH

For the testing of potential of hydrogen (PH), we extract 15µl culture medium from the slice culture system by detecting instrument (InLab Ultra Micro-ISM, Mettler Toledo); Glucose was tested by detecting instrument (GlucCell^{TM}, Brookfield), using 3µl culture medium; For LDH (lactate dehydrogenase), we tested by detection kit (G1780, Promega) in 96-well plate. All the process were conducted by the operation manual.

Drug sensitivity test in vitro

Drug testing commenced after 24 h of slice culture and was performed for an additional 72 h. For drug testing of slices in vitro, Regorafenib (REG; MedChemExpress LLC) was used and tested at a concentration of 5µM, 10µM, 20 µM, respectively. To investigate cell proliferation and tissue morphology, slices were incubated with CCK-8 solution and stained with HE/fluorescent dyes.

Statistical analysis

Statistical evaluations were performed using a Student's t-test and one-way ANOVA with post hoc least significant difference test by IBM SPSS Statistics 22.0 (IBM Corp). $P<0.05$ was considered to indicate a statistically significant difference. Three repeats were performed.

Results

Biological characteristics of HCC tissues are maintained by vitrification-based cryopreservation and precision-cut slice method

All the fresh HCC specimens were obtained from Renji Hospital Affiliated to Shanghai Jiao Tong University. The workflow was strictly performed by standard procedures, as depicted in Figure 1. The specific explanation is given in the section of methods. Human liver tissues were found to be very well sliceable and showed a good reproducibility as well as tissue viability. The 1mm-thick HCC slices were cryopreserved and warmed according to the timeline in Fig. 2A, using cryopreservation solutions (Fig. 2B, left) and warming solutions (Fig. 2B, right). 30 fresh specimens were derived from 30 HCC patients. Half of each specimen were processed by cryopreservation and warming procedures, and the rest tissues were control group. 300µM-thick precision-cut slices were made and cultured successfully (Fig. 2C-I). HE staining of fresh tissue slices revealed no obvious differences in the morphology when compared to warmed tissue slices. From the fluorescent staining and immunohistochemistry staining, we found that the living cell ratio was 93% in fresh tissues and 90% in warmed tissues, which indicated that no obvious difference was detectable between fresh HCC and warmed HCC tissues (Fig. 3A-B). Gene expression analysis of cancer-associated genes of three HCC cases showed that no significant alterations in gene expression were introduced by this cryopreservation method (Fig. 3D). The heat map indicated that the color of left column was mostly consistent with the right column (Fig. 3E). Only a small part of differential gene expression was detected from the volcano plot (Fig. 3F) and distribution of sample expression (Fig. 3G). According to the GO analysis, it was found that differential genes were closely related to cell metabolism (Fig. 3C). Therefore, the variation in gene expression between fresh and warmed tissues were limited. These results confirmed that vitrification-based

Posted on 31 Jan 2024 — The copyright holder is the author/funder. All rights reserved. No reuse without permission. — https://doi.org/10.22541/au.170668421.10975879/v1 — This is a preprint and has not been peer-reviewed. Data may be preliminary.

cryopreservation method was able to largely maintain the biological activity and histological features of the HCC tissues.

Medium composition and culture mode is critical to tissue viability

In order to find best slice culture methods, we optimized the slicing process with different culture method and selected the optimal culture medium. Our results showed that Medium I (DMEM with high glucose+10% FBS) could obviously maintain cell viability; Medium II (1640+10%FBS) and Medium III (DMEM/F12) were not suitable for slice culture (Fig. 4A). Filter culture were viable for up to 4 days and could receive a higher cell viability than floating and rotating culture (Fig. 4B). Subsequently, we conducted slice culture on transwell insert with DMEM added 10%FBS for 72 hours. From the fluorescent staining and immunohistochemistry staining, we found that the living cell ratio was decreased slightly and a little change in tissue morphological feature was detected. Besides, these changes were observed both in fresh and warmed tissues (Fig. 4C-D). What's more, we detected that the level of PH and glucose decreased while LDH increased obviously (Fig. 4E).

Positive drug responses could be detected in slice culture model

Drug testing commenced after 24 h of slice culture and was performed for an additional 72 h. To study the activity of anticancer drug REG in this tissue culture model, we treated HCC slices with different concentrations of REG (5 µM; 10 µM; 20 µM) for another 72 hours as depicted in Figure 5A. Our results revealed that 20 µM is the most obvious concentration to decrease the cell viability. Morphological staining and viability assay both indicated that no obvious differences were detectable after 24h of slice culturing. However, compared with control group, both fresh and warmed tissue slices in drug treatment group cultured for 72h showed a remarkable decrease in cell viability. Besides, tissue slices in drug treatment group evidently lost the morphological structure of original tumor (Fig. 5B-E).

Discussion

Several researches indicated that tissue slices culture system can be applied to perform preclinical and clinical studies for medical research[12-15]. In our research, we describe precision-cut slice cultures as a novel model to perform ex vivo experiments on tumors of HCC, which preserves the three-dimensional structure of the tumor and provides an alternative to in vivo experiments. Our study was performed using standard procedures, as depicted in Figure 1. Some studies have shown that there may be a drastic difference between a drugs effect on cancer cells in a normal monolayer cell culture vs 3D cell culture[13, 16, 17]. These evidences indicated the importance of normal tissue architecture and cell–cell communications that clearly exist in vivo.

One way to maintain these features is the tissue slice method, which was originally described for culture of breast and colon tumors[18-20]. It has several important advantages. Firstly, slice culture system provides the possibility to investigate the relationship between tumor cells and specific tumor microenvironments, which was suitable for the evaluation of drug effects and many other biological studies[21]. Secondly, they may reduce the need for animal testing, since they provide a biologically relevant platform for screening compounds. Normally, exact control of thickness will be beneficial for full diffusion of nutrients and oxygen. Optimal thickness of slices was related to the different type of tissue[10, 15, 22]. In order to find optimal thickness of slicing and culturing, we optimized the slicing process with slicer and found that 300 µM was the most suitable thickness for HCC slice after pre-experiments (Fig. 2). Some previous studies reported that viability and proliferation could be retained for three to seven days[10, 23-25]. Our results showed that slices (300 µm) cultured on filter inserts are viable for up to 4 days (Fig. 4B). We did not characterize later time points, but there are no significant signs of tissue deterioration after four days, suggesting that extended incubations may be possible if required for a specific functional assay.

In order to maintain the viability of tissue and improve the utilization of specimens, we developed a standardized vitrification-based cryopreservation method. The cryopreservation and warming procedure should be implemented strictly in accordance with the time schedule (Fig. 2A). In fact, several types of cells, such as embryo and stem cells have been successfully vitrified[26, 27]. The results of our research showed that no

Posted on 31 Jan 2024 — The copyright holder is the author/funder. All rights reserved. No reuse without permission. — https://doi.org/10.22541/au.170668421.10975879/v1 — This is a preprint and has not been peer-reviewed. Data may be preliminary.

obvious difference was detected in the cell viability and morphological characteristics of the original tumor before and after cryopreservation (Fig. 3A-B). Gene expression analysis also showed that no significant alterations in gene expression were introduced by this cryopreservation method, except a little part alteration associated with cell metabolism (Fig. 3C-G). By pre-experiments, we found that no difference was induced by different lengths of preservation time in liquid nitrogen after cryopreservation. These findings further support the conclusion that vitrification is less damage to cell viability and function due to the minimal ice crystallization in the process of cryopreservation[28].

To test and optimize the culture condition, we compared the different composition of medium and different growth support (Fig. 2H-I). We adapted the culture medium for long-term expansion of slice (Fig. 4A), because composition of the culture medium is highly important to maintain tumor slice viability. Similar as observed with other slices[24], filter culture was superior to rotating culture and floating culture (Fig. 4B). The reason may be attributed to a better oxygen supply of the tissue in filter cultures. In our research, tissue slices processed by microtome all showed evident responses to anticancer drugs. Slice model therefore has tremendous potential in selecting the sensitive anticancer drugs via examining morphology and proliferation rate (fig. 5).

We have demonstrated that HCC tissue slices could be effectively cryopreserved, and the tumor biological characteristics were well retained. Tissue slice model provides us better predictability of cancer drug and improves the efficiency of precision or personalized treatment. Similar assays can be developed to investigate other drugs. At present, human tissue slice culture have their limitations in vitro cultivation time and low throughput. Accordingly, further development is required to allow for high throughput analysis which is not possible in the current experiments.

Acknowledgements

Yuan Zhang: Conceptualization, Investigation, Writing - Original Draft. Zhen-yu Wang: Investigation. Hong-shu Jing: Validation. Hong-dan Zhang: Data Curation, Formal analysis. He-xin Yan: Writing - Review & Editing, Methodology. Jian-xia Fan: Resources, Supervision. Bo Zhai: Conceptualization, Supervision, Project administration.

This work was supported by National Natural Science Foundation of China [82070619].

Conflicts of interest statement

The authors declare no commercial or financial conflict of interest.

Ethics approval and consent to participate

Patient-derived specimens were used in the research. The manuscript does not contain experiments using animals. The investigation was approved by ethics committee of Renji Hospital and all patients provided written informed consent.

References

[1] J. Wang, Y. Mao, Y. Liu, Z. Chen, M. Chen, X. Lao, S. Li, "Hepatocellular Carcinoma in Children and Adolescents: Clinical Characteristics and Treatment," *J Gastrointest Surg,* Apr 10 2017.

[2] A. Huang, X. R. Yang, W. Y. Chung, A. R. Dennison, J. Zhou, "Targeted therapy for hepatocellular carcinoma," *Signal Transduct Target Ther,* vol. 5, p. 146, Aug 11 2020.

[3] T. Usui, M. Sakurai, S. Enjoji, H. Kawasaki, K. Umata, T. Ohama, N. Fujiwara, R. Yabe, S. Tsuji, H. Yamawaki, S. Hazama, H. Takenouchi, M. Nakajima, R. Tsunedomi, N. Suzuki, H. Nagano, K. Sato, "Establishment of a Novel Model for Anticancer Drug Resistance in Three-Dimensional Primary Culture of Tumor Microenvironment," *Stem Cells Int,* vol. 2016, p. 7053872, 2016.

[4] Y. Yu, Y. Wang, X. Xiao, W. Cheng, L. Hu, W. Yao, Z. Qian, W. Wu, "MiR-204 inhibits hepatocellular cancer drug resistance and metastasis through targeting NUAK1," *Biochemistry and cell biology = Biochimie*

Posted on 31 Jan 2024 — The copyright holder is the author/funder. All rights reserved. No reuse without permission. — https://doi.org/10.22541/au.170668421.10975879/v1 — This is a preprint and has not been peer-reviewed. Data may be preliminary.

*et biologie cellulaire,*vol. 97, pp. 563-570, 2019.

[5] V. Kumarasamy, P. Vail, R. Nambiar, A. K. Witkiewicz, E. S. Knudsen, "Functional Determinants of Cell Cycle Plasticity and Sensitivity to CDK4/6 Inhibition," *Cancer Res,* vol. 81, pp. 1347-1360, Mar 1 2021.

[6] M. van de Wetering, H. E. Francies, J. M. Francis, G. Bounova, F. Iorio, A. Pronk, W. van Houdt, J. van Gorp, A. Taylor-Weiner, L. Kester, A. McLaren-Douglas, J. Blokker, S. Jaksani, S. Bartfeld, R. Volckman, P. van Sluis, V. S. Li, S. Seepo, C. Sekhar Pedamallu, K. Cibulskis, S. L. Carter, A. McKenna, M. S. Lawrence, L. Lichtenstein, C. Stewart, J. Koster, R. Versteeg, A. van Oudenaarden, J. Saez-Rodriguez, R. G. Vries, G. Getz, L. Wessels, M. R. Stratton, U. McDermott, M. Meyerson, M. J. Garnett, H. Clevers, "Prospective derivation of a living organoid biobank of colorectal cancer patients," *Cell,* vol. 161, pp. 933-45, May 7 2015.

[7] A. Bruna, O. M. Rueda, W. Greenwood, A. S. Batra, M. Callari, R. N. Batra, K. Pogrebniak, J. Sandoval, J. W. Cassidy, A. Tufegdzic-Vidakovic, S. J. Sammut, L. Jones, E. Provenzano, R. Baird, P. Eirew, J. Hadfield, M. Eldridge, A. McLaren-Douglas, A. Barthorpe, H. Lightfoot, M. J. O'Connor, J. Gray, J. Cortes, J. Baselga, E. Marangoni, A. L. Welm, S. Aparicio, V. Serra, M. J. Garnett, C. Caldas, "A Biobank of Breast Cancer Explants with Preserved Intra-tumor Heterogeneity to Screen Anticancer Compounds,"*Cell,* vol. 167, pp. 260-274 e22, Sep 22 2016.

[8] K. A. Naipal, N. S. Verkaik, H. Sanchez, C. H. van Deurzen, M. A. den Bakker, J. H. Hoeijmakers, R. Kanaar, M. P. Vreeswijk, A. Jager, D. C. van Gent, "Tumor slice culture system to assess drug response of primary breast cancer," *BMC Cancer,* vol. 16, p. 78, Feb 9 2016.

[9] J. A. Hickman, R. Graeser, R. de Hoogt, S. Vidic, C. Brito, M. Gutekunst, H. van der Kuip, I. P. Consortium, "Three-dimensional models of cancer for pharmacology and cancer cell biology: capturing tumor complexity in vitro/ex vivo,"*Biotechnol J,* vol. 9, pp. 1115-28, Sep 2014.

[10] D. L. Holliday, M. A. Moss, S. Pollock, S. Lane, A. M. Shaaban, R. Millican-Slater, C. Nash, A. M. Hanby, V. Speirs, "The practicalities of using tissue slices as preclinical organotypic breast cancer models," *J Clin Pathol,*vol. 66, pp. 253-5, Mar 2013.

[11] M. Zeng, Q.-R. Yang, G.-B. Fu, Y. Zhang, X. Zhou, W.-J. Huang, H.-D. Zhang, W.-J. Li, Z.-Y. Wang, H.-X. Yan, B. Zhai, "Maintaining viability and characteristics of cholangiocarcinoma tissue by vitrification-based cryopreservation,"*Cryobiology,* vol. 78, pp. 41-46, 2017.

[12] E. J. Chadwick, D. P. Yang, M. G. Filbin, E. Mazzola, Y. Sun, O. Behar, M. F. Pazyra-Murphy, L. Goumnerova, K. L. Ligon, C. D. Stiles, R. A. Segal, "A Brain Tumor/Organotypic Slice Co-culture System for Studying Tumor Microenvironment and Targeted Drug Therapies," *Journal of Visualized Experiments,* vol. 105 p. e53304, 2015.

[13] H. A. Kenny, M. Lal-Nag, E. A. White, M. Shen, C. Y. Chiang, A. K. Mitra, Y. Zhang, M. Curtis, E. M. Schryver, S. Bettis, A. Jadhav, M. B. Boxer, Z. Li, M. Ferrer, E. Lengyel, "Quantitative high throughput screening using a primary human three-dimensional organotypic culture predicts in vivo efficacy,"*Nat Commun,* vol. 6, p. 6220, Feb 5 2015.

[14] N. Parajuli and W. Doppler, "Precision-cut slice cultures of tumors from MMTV-neu mice for the study of the ex vivo response to cytokines and cytotoxic drugs," *In Vitro Cell Dev Biol Anim,* vol. 45, pp. 442-50, Sep 2009.

[15] M. M. Gerlach, F. Merz, G. Wichmann, C. Kubick, C. Wittekind, F. Lordick, A. Dietz, I. Bechmann, "Slice cultures from head and neck squamous cell carcinoma: a novel test system for drug susceptibility and mechanisms of resistance," *Br J Cancer,* vol. 110, pp. 479-88, Jan 21 2014.

[16] S. E. Burdall, A. M. Hanby, M. R. Lansdown, V. Speirs, "Breast cancer cell lines: friend or foe?,"*Breast Cancer Res,* vol. 5, pp. 89-95, 2003.

[17] S. F. Boj, C. I. Hwang, L. A. Baker, Chio, II, D. D. Engle, V. Corbo, M. Jager, M. Ponz-Sarvise, H. Tiriac, M. S. Spector, A. Gracanin, T. Oni, K. H. Yu, R. van Boxtel, M. Huch, K. D. Rivera, J. P. Wilson,

Posted on 31 Jan 2024 — The copyright holder is the author/funder. All rights reserved. No reuse without permission. — https://doi.org/10.22541/au.170668421.10975879/v1 — This is a preprint and has not been peer-reviewed. Data may be preliminary.

M. E. Feigin, D. Ohlund, A. Handly-Santana, C. M. Ardito-Abraham, M. Ludwig, E. Elyada, B. Alagesan, G. Biffi, G. N. Yordanov, B. Delcuze, B. Creighton, K. Wright, Y. Park, F. H. Morsink, I. Q. Molenaar, I. H. Borel Rinkes, E. Cuppen, Y. Hao, Y. Jin, I. J. Nijman, C. Iacobuzio-Donahue, S. D. Leach, D. J. Pappin, M. Hammell, D. S. Klimstra, O. Basturk, R. H. Hruban, G. J. Offerhaus, R. G. Vries, H. Clevers, D. A. Tuveson, "Organoid models of human and mouse ductal pancreatic cancer," *Cell,* vol. 160, pp. 324-38, Jan 15 2015.

[18] H. van der Kuip, T. E. Murdter, M. Sonnenberg, M. McClellan, S. Gutzeit, A. Gerteis, W. Simon, P. Fritz, W. E. Aulitzky, "Short term culture of breast cancer tissues to study the activity of the anticancer drug taxol in an intact tumor environment," *BMC Cancer,* vol. 6, p. 86, Apr 7 2006.

[19] L. A. Schwerdtfeger, N. J. Nealon, E. P. Ryan, S. A. Tobet, "Human colon function ex vivo: Dependence on oxygen and sensitivity to antibiotic," *PLoS One,*vol. 14, p. e0217170, 2019.

[20] V. Vaira, G. Fedele, S. Pyne, E. Fasoli, G. Zadra, D. Bailey, E. Snyder, A. Faversani, G. Coggi, R. Flavin, S. Bosari, M. Loda, "Preclinical model of organotypic culture for pharmacodynamic profiling of human tumors," *Proc Natl Acad Sci U S A,* vol. 107, pp. 8352-6, May 4 2010.

[21] L. Vesci, V. Carollo, G. Roscilli, L. Aurisicchio, F. F. Ferrara, L. Spagnoli, R. De Santis, "Trastuzumab and docetaxel in a preclinical organotypic breast cancer model using tissue slices from mammary fat pad: Translational relevance," *Oncol Rep,* vol. 34, pp. 1146-52, Sep 2015.

[22] S. H. Grosso, M. L. Katayama, R. A. Roela, S. Nonogaki, F. A. Soares, H. Brentani, L. Lima, M. A. Folgueira, A. F. Waitzberg, F. S. Pasini, J. C. Goes, M. M. Brentani, "Breast cancer tissue slices as a model for evaluation of response to rapamycin," *Cell Tissue Res,* vol. 352, pp. 671-84, Jun 2013.

[23] F. Unger, S. Bentz, J. Kruger, C. Rosenbrock, J. Schaller, K. Pursche, A. Sprussel, H. Juhl, K. David, "Precision Cut Cancer Tissue Slices in Anti-Cancer Drug Testing,"*Journal of Molecular Pathophysiology,* vol. 4, p. 108, 2015.

[24] E. J. Davies, M. Dong, M. Gutekunst, K. Narhi, H. J. van Zoggel, S. Blom, A. Nagaraj, T. Metsalu, E. Oswald, S. Erkens-Schulze, J. A. Delgado San Martin, R. Turkki, S. R. Wedge, T. M. af Hallstrom, J. Schueler, W. M. van Weerden, E. W. Verschuren, S. T. Barry, H. van der Kuip, J. A. Hickman, "Capturing complex tumour biology in vitro: histological and molecular characterisation of precision cut slices," *Sci Rep,* vol. 5, p. 17187, Dec 9 2015.

[25] S. L. Maund, R. Nolley, D. M. Peehl, "Optimization and comprehensive characterization of a faithful tissue culture model of the benign and malignant human prostate,"*Lab Invest,* vol. 94, pp. 208-21, Feb 2014.

[26] F. Karimi-Busheri, A. Rasouli-Nia, M. Weinfeld, "Key Issues Related to Cryopreservation and Storage of Stem Cells and Cancer Stem Cells: Protecting Biological Integrity," *Adv Exp Med Biol,* vol. 951, pp. 1-12, 2016.

[27] M. Ochota, B. Wojtasik, W. Nizanski, "Survival rate after vitrification of various stages of cat embryos and blastocyst with and without artificially collapsed blastocoel cavity," *Reprod Domest Anim,* vol. 52 Suppl 2, pp. 281-287, Apr 2017.

[28] E. Elder, Z. Chen, A. Ensley, R. Nerem, K. Brockbank, Y. Song, "Enhanced tissue strength in cryopreserved, collagen-based blood vessel constructs," *Transplant Proc,* vol. 37, pp. 4625-9, Dec 2005.

Figure legends

Figure 1. Workflow of cryopreserved slice culture and drug testing. HCC tissues were obtained and transported to the laboratory in preservation medium at 4@C within 2 h. Tissues were cryopreserved and stored in a nitrogen canister. Warmed tissues were used when required. 1mm-thick tissue slices were embedded in agarose and cut into 300-µm-thick slices using a VF-300 microtome. Tissue slices (2 mm in diameter) were maintained on different culture support. Slices were maintained on transwell insert and treated with REG

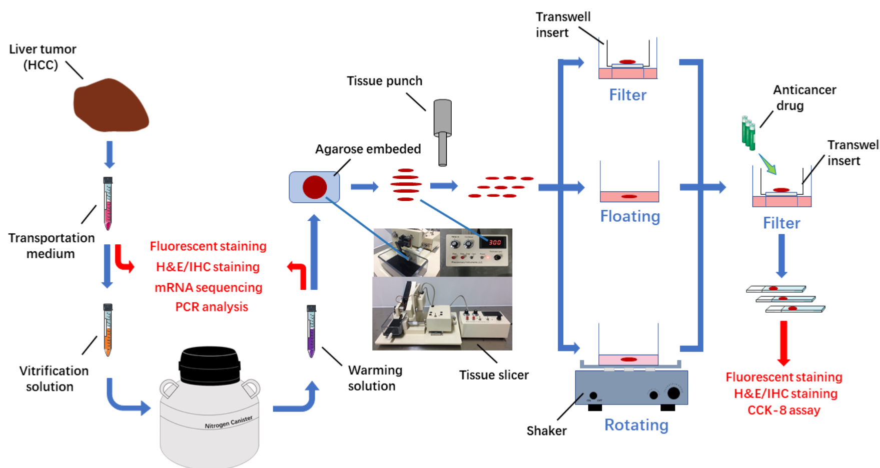
at s concentration of 20 μM. Experiments were performed to evaluate the efficacy of cryopreservation combined with slice cultivation in the assessment of anticancer drug responses by viability assay and fluorescent staining.

Figure 2. Cryopreservation technology and precision-cut tissue slice culture method. (A) The standardized time schedule of the cryopreservation and warming procedures. (B) Cryopreservation solutions and warming solutions. (C) Tissues were cut into 1mm-thick slices by a stainless slicer in a metal mould. (D) Cryopreserved tissues and warmed tissues. (E) VF 300 microtome. (F) 300μm-thick slices. (G) 2mm-diameter slices. (H) floating culture. (I) Filter culture (Transwell insert).

Figure 3. The effects of cryopreservation on maintaining the biological characteristics of tissues. (A) Calcein-AM/Hoechst staining and HE/IHC staining. Blue nuclei comprise both living and dead nuclei, while green sections represent the cytoplasm of living cells. IHC staining indicated the percentage of cells expressing the proliferation marker Ki67. (B) The viability of fresh HCC slice was more than 10% of the warmed tissues, which was determined by CCK-8 assay (n = 7). (C) The GO analysis indicated that differential genes were closely related to cell metabolism. (D) PCR assays of fresh and warmed HCC tissues (n = 3). (E) Heat map of mRNA sequencing. The color change in the heat map is defined as the difference in gene expression between fresh and warmed tissues. The deeper the red, the more greatly increased the gene expression. The deeper the blue, the lesser the gene expression. (F-G) Volcano plot and distribution of sample expression showed the differential gene expression between fresh and warmed tissues.

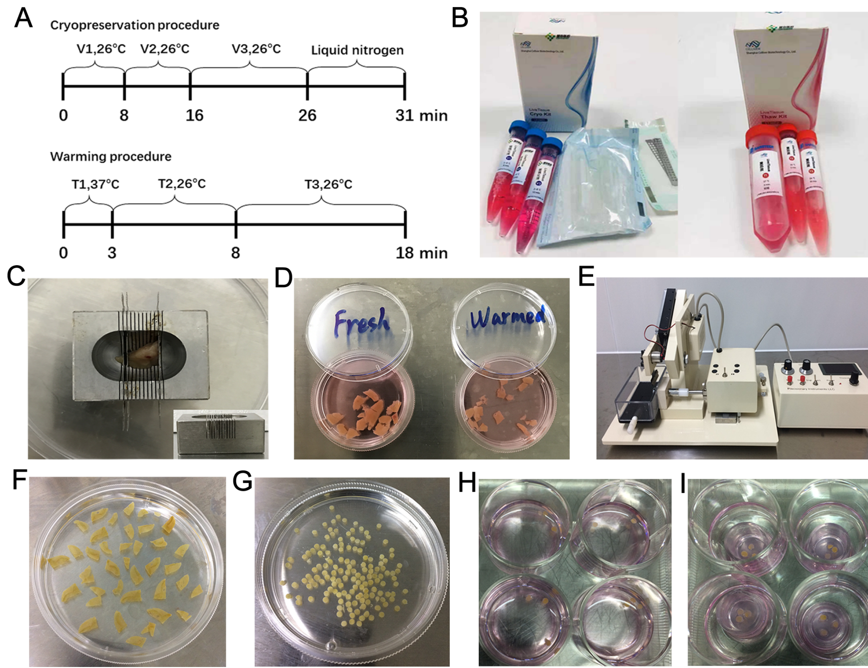
Figure 4. Optimization of medium composition and culture mode. (A) The adaption of Medium I (DMEM with high glucose added 10% FBS) could provide a higher cell viability and a longer culture time than Medium II (1640+10%FBS) or Medium III (DMEM/F12). (B) Filter culture were viable for up to 4 days and could receive a higher cell viability than floating and rotating culture. (C) Calcein-AM/Hoechst staining and HE/IHC staining. (D) The ratio of live cells in fresh and warmed slices after 96h. (E) The variation of PH, glucose and LDH during the slice culture.

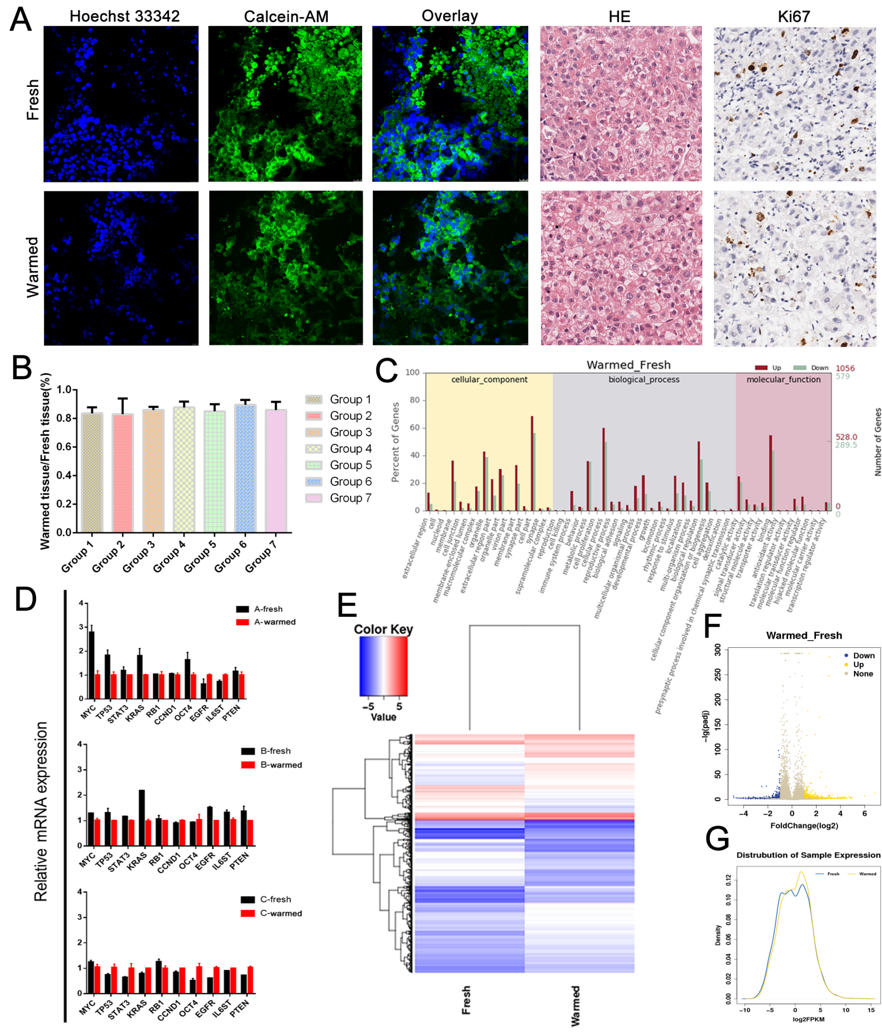
Figure 5. Positive drug responses in slice culture model. (A) HCC slices were treated with different concentrations of REG (5 μM; 10 μM; 20 μM) for 72 hours. (B) CCK-8 cell viability assay for 72 hours. (C) The quantification of Ki67 positive cells, the proliferation rate before and after cryopreservation was not statistically different by Student's t-test (p=0.71). (D) The quantification of live cells, the number of live cells before and after cryopreservation was not statistically different by Student's t-test (p = 0.54). (E) Morphological staining and fluorescent staining in fresh and warmed tissues during drug testing.



Posted on 31 Jan 2024 — The copyright holder is the author/funder. All rights reserved. No reuse without permission. — https://doi.org/10.22541/au.170668421.10975879/v1 — This is a preprint and has not been peer-reviewed. Data may be preliminary.

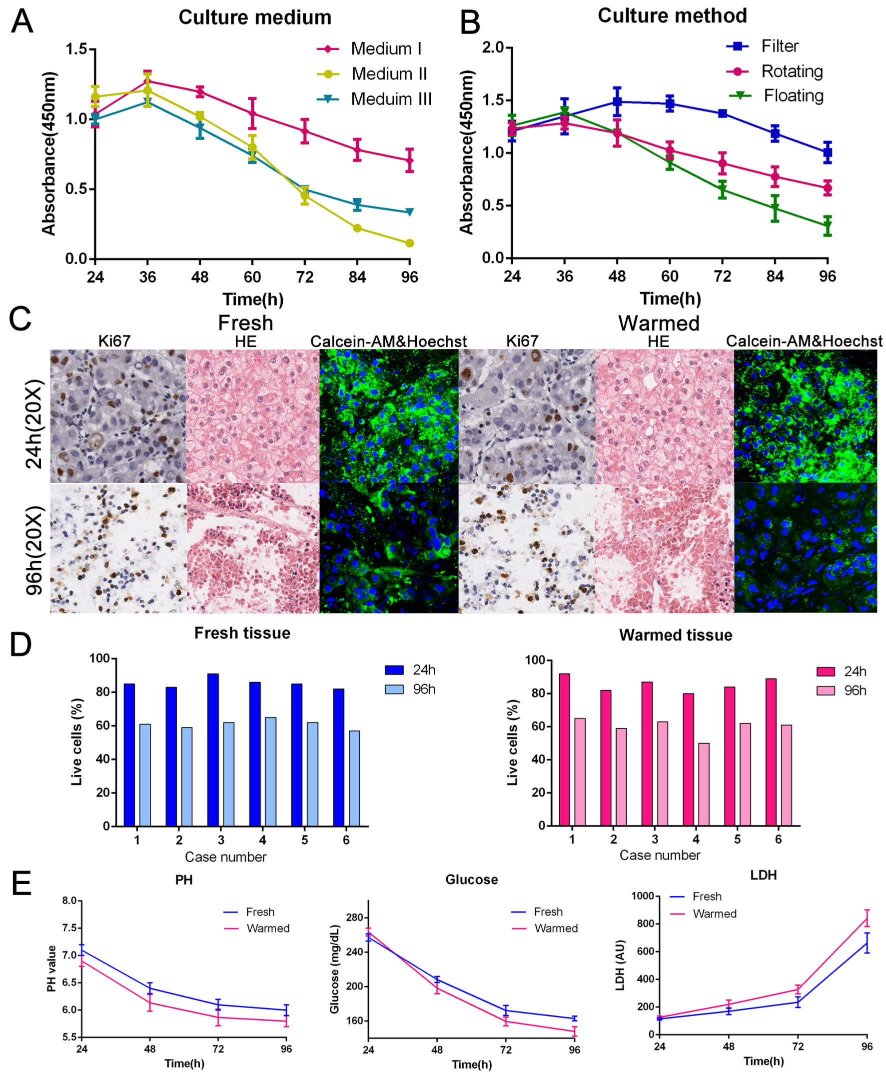
Posted on 31 Jan 2024 — The copyright holder is the author/funder. All rights reserved. No reuse without permission. — https://doi.org/10.22541/au.170668421.10975879/v1 — This is a preprint and has not been peer-reviewed. Data may be preliminary.





Posted on 31 Jan 2024 — The copyright holder is the author/funder. All rights reserved. No reuse without permission. — https://doi.org/10.22541/au.170668421.10975879/v1 — This is a preprint and has not been peer-reviewed. Data may be preliminary.

Posted on 31 Jan 2024 — The copyright holder is the author/funder. All rights reserved. No reuse without permission. — https://doi.org/10.22541/au.170668421.10975879/v1 — This is a preprint and has not been peer-reviewed. Data may be preliminary.



Posted on 31 Jan 2024 — The copyright holder is the author/funder. All rights reserved. No reuse without permission. — https://doi.org/10.22541/au.170668421.10975879/v1 — This is a preprint and has not been peer-reviewed. Data may be preliminary.