PATROLS
Advanced Tools for NanoSafety Testing

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PATROLS Standard Operating Procedures (SOP)

Evaluation of nanomaterial-induced hepatotoxicity in a primary human multi-cellular microtissue model with emphasis on physiologically meaningful toxicological end-points

This is a SOP recommended for external use by PATROLS

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Document History:

Version	Approval Date	Description of the change	Author(s) change
1.0	12/05/2020	Initial Document	Ali Kermanizadeh
2.0	6/06/2020	Amended Protocol	Ali Kermanizadeh
3.0	15/04/2021	Amended Protocol	Ali Kermanizadeh

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1 Introduction:

It is essential to establish more advanced, physiologically relevant *in vitro* assessment tools for improved prediction of the adverse effects caused by chronic nanomaterial (NM) exposure in humans. The utilization of human primary hepatic cells is the closest representative *in vitro* model for the human liver. However, these cells are phenotypically unstable in 2D cultures and have an extremely limited life span (typically no longer than 7 days - with continued reduced viability, functional and metabolic activity). Moreover, in most traditional 2D hepatic models, non-parenchymal cell (NPC) populations are not included or considered. In an attempt to address this issue, the use of scaffold free 3D liver microtissue (MT) model composed of primary human hepatocytes and primary human liver-derived NPC could be beneficial.

1.1 Limits of the protocol:

- Microtissues are rather cost-intensive owned to their complexity and long-term performance capability
- Cell culture is technically challenging and time-consuming
- Limited volumes of supernatant and cells
- There are complications in dosimetry in the model due to the shape of the well and unknowns parameters concerning the rotation of the MT in the well over time.
- As endothelial cells deteriorate over time, no proper liver sinusoidal structure is formed, thus spheroids lack an overall liver architecture. However, canalicular configuration can be detected and conjugated bile salts identified in the medium demonstrate intact bile metabolism and drainage.

1.2 *Validation state of protocol*

Level of advancement towards standardization	**Level reached** (please mark only one with "X")
Stage 1: Internal laboratory method under development	
Stage 2: Validated internal laboratory method	X
Stage 3: Interlaboratory tested method	
Stage 4: Method validated by Round Robin testing	
Standardisation plans	
Is the method considered for standardisation (OECD SPSF or similar)?	Maybe
Has the method been submitted for standardisation (to OECD, CEN, ISO,…) in its own right or as part of another standardisation project?	N
Is the method included in an existing standard (or ongoing standardisation work)	N

2. Terms and definitions:

Nanomaterial: Material with any external dimension in the *nanoscale* or having internal structure or surface structure in the nanoscale.

3 Abbreviations:

AK - Adenylate kinase

AST - Aspartate aminotransferase

ELISA - Enzyme linked immunosorbent assay

KC - Kupffer cell

NMs - Nanomaterials

MT - Microtissue

NPC - Non-parenchymal cell

PBS - phosphate buffered saline

4. Principals of methods:

Of particular importance and unique for the liver, is the inability of in vitro models to emulate the liver's incredible regeneration capability. The livers ability to regenerate is critical in disease recovery, and so is important in distinguishing between the ability of different NMs to induce acute versus longer-term harm to the human liver. This consideration of liver recovery is therefore imperative for *in vitro* NM hazard assessment.

The following protocol was developed solely for toxicological assessment of NMs at very low exposure relevant doses relevant doses and end-points assessed on a commercially available 3D liver microtissues (3DInSight™ human liver MT) composed of primary human hepatocytes and non-parenchymal cells, i.e. Kupffer cells and sinusoidal endothelial cells

5 Description of the method:

5.1 Biological setting & test system used:

This SOP should be carried out under laboratory based conditions, with all work performed under sterile conditions and in a Class 2 Laminar Tissue Culture Hood.

5.2 Chemicals and reagents used:

3D liver microtissues (3DInSight™ human liver MT) composed of primary human hepatocytes and non-parenchymal cells - InSphero AG - MT-02-302-04
3D InSight™ human liver maintenance medium - AF - InSphero AG - CS-07-001a-01

40% formaldehyde solution - Sigma - 128775
Agarose - Sigma – A9539
10x phosphate buffered saline (PBS) - Sigma - P5493
Hematoxylin and eosin stains - Sigma - E4009 and H9627
10 x Tris buffered saline (TBS) - Sigma- T5912
Triton-X-100 - Sigma - T8787
Albumin ELISA - e.g. Bethyl laboratories (E88-129) or R and D Systems (DY1455)
AST ELISA Kit - Novus Biologicals - AK1625
ToxiLight™ bioassay kit - Lonza - LT07
Bond™ Epitope Retrieval Solution 2 – Leica (AR9640)

5.3 Apparatus and equipment used:

1.5 ml eppendorf tube - preferably with flat bottom - e.g. Eppendorf - 0030120086
Paraffin embedding cassettes e.g. Fischer Scientific - 10364511
Luminescence compatible 96 well plate
8 well microscopy chambers - Ibidi – 80826
Micropipettes
Centrifuge
Microcentrifuge
Plate shaker
-80°C freezer
Humidified incubator (37°C and 5% CO_2)
Inverted light microscope - e.g. ZEISS Axiovert 40 C light microscope
Inverted wide field fluorescence microscope - i.e. Leica AF6000
Tissue processor - e.g. Leica HistoCore PELORIS 3
Plate-reading luminometer
Bio-Rad® Bio-Plex® MAGPIX multiplex reader
Digital microscope – e.g. Olympus BX 43 microscope

5.4 Reporting of protected elements:

This SOP are generated utilizing commercially available MT and cell culture medium.

5.5 Health and safety precautions:

Standard health and safety precautions associated with working within a laboratory environment and performing mammalian cell culture, as described by the European Agency for Safety and Health at Work (https://osha.europa.eu/en/safety-and-health-legislation/european-guidelines), should be adopted when conducting this SOP. In addition, all health and safety precautions outlined in the MSDS data sheets associated with the specific chemicals required must also be followed.

5.6 Nanomaterials used/handling procedures:

1. NMs to be prepared following the NANOGENOTOX dispersion protocol.
2. Following the sonication step, transfer all samples to ice before being diluted in human liver maintenance medium just prior to the experiments.
3. Remove cell culture medium.
4. Add the NM to the wells at a total volume of 50 µl.
5. Include negative (cell culture medium) and positive (1% Triton-X) controls (or other appropriate controls for different toxicological end-points).

Note: For each treatment, five wells are used, and all experiments are repeated on three separate occasions.

5.7 Procedure:

5.7.1 Liver MT maintenance:

1. Maintain the human liver in human liver maintenance medium AF at 37°C, 5% CO_2 and 95%.humidity.
2. Exchange old medium (70 µl per well) with new preheated medium (37°C) on the day of arrival.

3. The medium is exchanged every 2 days thereafter (50 µl per well) until the experiments are commenced.

5.7.2 Morphological characterisation of 3D human liver MT:

5.7.2.1 Haematoxylin and eosin staining:

1. The MT are harvested at the required time-points by placing the pipette tip close to the bottom of the well using a 1 ml pipette tip and aspirating 50 µl of medium supernatant. Alternatively, a multi-channel pipette can be used for parallel harvesting of multiple MTs (aspirate 1.2-1.5x sample volume at medium aspiration speed)
2. Transfer the MT into a 1.5 ml Eppendorf tube
3. Allow the MT to settle to the bottom of the tube by gravity (5 min).
4. Optional: centrifuge for 10 seconds at minimum speed of 200 RCF. Use a centrifuge with fixed angle horizontal rotor, if available. Otherwise spin twice (10 s for each spin), with turning the tube by 180° in between spins.
5. Carefully aspirate the supernatant and discard it.
6. Fix the MT by the addition of 500 µl of 4% formaldehyde (diluted in distilled water) solution into the reaction tube for at least 1 hr at room temperature or overnight at 4°C.
7. Wash 2 times with 1 ml PBS (centrifugation step between washes - 200 RCF, 2 min).
8. Prepare 2% agarose solution in PBS, boil and allow to cool to approximately 50-60°C with constant stirring.
9. Carefully remove PBS from the MT.
10. Carefully pipette 600 µl agarose solution into the tube containing the MT.
11. Immediately spin the sample for 4 sec using a centrifuge (200 RCF) with a horizontal rotor. If no horizontal centrifuge is available, centrifuge twice for 4 sec, with turning the tube by 180° in between spins.
12. For easier removal of the agarose plug from the tube, insert a short string with a single knot at one end, and insert knot into the still liquid agarose

immediately after the centrifugation, before the agarose is completely solidified.

13. Let the agarose plug solidify on ice for 5 min.
14. Add 500 µl PBS and place at 4°C for intermediate storage (stable for a minimum of a week).
15. Remove the agarose plug with a disposable plastic pipette by generating gentle suction (or by pulling the thread).
16. Transfer the agarose plug with the MT pellet into a conventional paraffin embedding cassette.
17. Dehydrate the tissue sample in a tissue processor using a standard dehydration program.
18. Embed the agarose plug into a paraffin embedding mould with the MT pellet facing the bottom of the mould. Make sure the agarose plug stays upright until the paraffin starts to solidify from the bottom.
19. Section and stain paraffin sections with H&E

5.7.2.2 Brightfield and enhanced darkfield imaging of MT:

Cytoviva enhanced darkfield hyperspectral system (USA) was used for detection of NMs in the cross section of the MT (prepared in section 3.7.2.1).

1. Prepare enhanced darkfield images by the acquisition of multiple overlapping images at 100x magnification i.e. using an Olympus BX 43 microscope with a Qimaging Retiga 4000R camera.
2. Assemble overlapping images via ImageJ using the MosaicJ plugin
3. Acquire brightfield images at 100x i.e. via an Olympus BX 43 microscope with a Nikon DS-Fi2 camera.

5.7.3 Toxicological analysis:

5.7.3.1 AK assay:

The cell membrane integrity can be evaluated utilising a Lonza ToxiLight™ bioassay kit.

1. Transfer 20 µl of cell supernatant from liver MT to a luminescence compatible plate.
2. Transfer 80 µl of AK detection buffer to all wells.
3. Incubate plates for 5 min at room temperature in the dark.
4. Measure luminescence using a standard plate-reading luminometer.

5.7.3.2 AST quantification:

1. Centrifuge the supernatant (minimum 100 µl) from MT at 1000 x g.
2. Transfer supernatant to a new tube to remove NMs and cell debris.
3. AST levels were determined in the supernatant by ELISA according to the manufacturer's instructions.

5.7.3.3 Cytokine secretion:

After exposure, the supernatants (from both the control and treated cells as described above) are collected and stored at -80ºC.

1. Centrifuge the supernatant at x 1000 g
2. Transfer supernatant to new tube to remove NMs and cell debris.
3. The levels of human cytokines/chemokines are determined in the cell supernatant using R&D Systems magnetic Luminex® Performance Assay multiplex kits (bead based immunoassay; Bio-techne, USA) according to the manufacturer's instructions.
4. The protein concentrations were evaluated via a Bio-Rad® Bio-Plex® MAGPIX multiplex reader.

5.7.3.4 Albumin production:

After exposure, the supernatants (from both the control and treated cells as described above) are collected and stored at -80ºC.

1. Centrifuge the supernatant at 1000 x g
2. Transfer supernatant to new tube to remove NMs and cell debris.
3. Dilute the supernatant two fold in 1xPBS
4. Albumin levels are determined by ELISA according to the manufacturer's instructions

6 Data analysis and reporting of data:

Not applicable for this SOP.

7 Publications:

Kermanizadeh A, Berthing T, Guzniczak E, Wheeldon M, Whyte G, Vogel U, Moritz W, Stone V. 2019. Assessment of nanomaterial-induced hepatotoxicity using a 3D human primary multi-cellular microtissue exposed repeatedly over 21 days - the suitability of the *in vitro* system as an *in vivo* surrogate. Particle and Fibre Toxicology 16: 42.

Kermanizadeh A, Powell LG, Stone V. 2020. A review of hepatic nanotoxicology - summation of recent findings and considerations for next generation of study designs. Journal of Environmental Health B: Critical reviews 23: 137-176.