Development of an animal-free methodology for mechanical performance assessment of engineered skin substitutes

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Abstract

Functional substitutes for the treatment of skin wounds are nowadays widely available thanks to the progress in skin tissue engineering. However, the performance of the existing substitutes in terms of mechanical behavior, which is a determinant for their success, still does not match the native tissue. Since the mechanical behavior of skin is governed by dermis, an in-depth investigation of human dermis mechanics would be pivotal for integrating the design and validation of skin substitutes. In the perspective of reducing and replacing animal experiments through validated alternative tools, an accurate *in silico* constitutive model describing the human dermis mechanics is presented. Biaxial tests were performed on human dermis samples, and resulting data were used for setting the constitutive parameters of the model, able to reliably describing the mechanical properties of the native tissue, and to be compared with the mechanical behavior of skin substitutes attainable through the same procedure.

Introduction

Progress in skin tissue engineering has led to the development of functional substitutes for the treatment of acute and chronic skin wounds. However, the performance of the existing substitutes in terms of mechanical behavior still does not match native human skin.[1] Since the mechanical behavior of skin strongly depends on dermis, an in-depth investigation of the human dermis mechanics would be essential for supporting the design and the validation of skin substitutes.[2] In the perspective of reducing and replacing animal experiments thanks to validated alternative tools, here we present an accurate *in silico* constitutive model describing the human dermis mechanics. Biaxial tests were performed on human dermis samples, and resulting data were used for setting the constitutive parameters of the model adopted for faithfully describing the mechanical behavior of dermis under load.

Materials and Methods

Dermis samples were harvested from the lower back of a human donor, coherently with the anatomical orientations[3] (cranio-caudal-CC and mediolateral-ML). Planar equi-biaxial tests were performed using a purpose-built fixture[4] mounted on a uniaxial testing machine (loading rate = 0.16 mm/s) (Figure 1a). Results were reported in terms of Cauchy stresses *vs* engineering strains, computed by tracking the average distance along the CC and ML directions of four central markers drawn on the sample surface.[4] Since dermis exhibits a highly nonlinear behavior, with anisotropic and heterogeneous responses during loading,[3] the Gasser-Ogden-Holzapfel (GOH) model (Eq. 1, for parameters details see Aldieri *et al.*[5]) was selected. Its constitutive parameters, describing the anisotropic hyperelastic response of dermis, were extracted from experimental data through a minimization procedure.

$$W = \frac{c}{2}(\bar{I}_1 - 3) + \frac{k_1}{2k_2}\left[e^{k_2[\kappa\bar{I}_1+(1-3\kappa)\bar{I}_4-1]^2} - 1\right] + \frac{k_3}{2k_4}\left[e^{k_4[\kappa\bar{I}_1+(1-3\kappa)\bar{I}_6-1]^2} - 1\right] \quad (1)$$

The GOH model was validated by repli-

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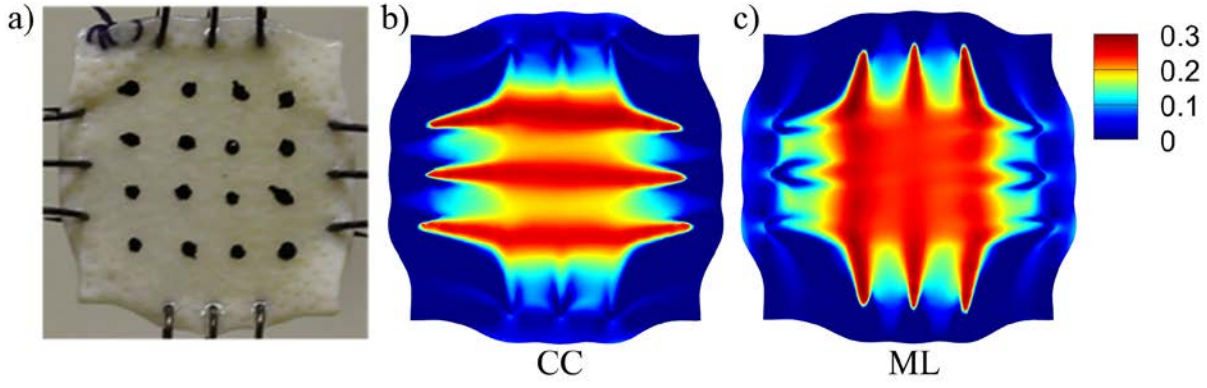

Figure 1. Human dermis sample during equi-biaxial test (a), and contour plots of engineering strains along the CC (b) and ML (c) loading directions.

cating experimental tests through Finite Element (FE) analyses and computing the normalized mean square error (NMSE) between numerical and experimental displacement magnitudes of the central markers.

Results

The obtained GOH constitutive parameters (k_1=90.3 *kPa*, k_3=50.6 kPa, k_2=11.0, k_4=8.0, c=1.2 *kPa*, κ=0.0005) were implemented in the FE model. Figure 1b and c shows the contour plots of engineering strains along the CC and ML directions, respectively. The numerical displacement magnitudes of the central markers were in good agreement with the experimental ones (average NMSE = 0.908), and, in accordance with experimental evidences,[3,4] a higher degree of deformation was achieved along the ML direction. This confirmed that the FE model outcomes accurately described the sample biaxial response, mirroring the experimental anisotropy.

Conclusions

In this work biaxial characterization and FE analyses on human dermis samples were combined to identify the GOH constitutive parameters for dermis mechanical description. The proposed animal-free methodology enables to investigate the mechanical performance of engineered skin substitutes with a combined *in vitro/in silico* approach, representing a powerful tool for selecting the optimal engineered substitute to be *in vivo* tested, with consequent reduction of animal testing.

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Real-time cellular impedance monitoring and imaging in a dual-flow bioreactor

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Abstract

The generation of physiologically relevant *in vitro* models of biological barriers can play a key role in understanding human diseases and in the development of more predictive methods for assessing toxicity and drug or nutrient absorption. Here, we present an advanced cell culture system able to mimic the dynamic environment of biological barriers, while monitoring cell behaviour through real-time impedance measurements and imaging. Caco-2 cells were cultured in the Trans Epithelial Electric Impedance (TEEI) bioreactor under both flow and static conditions. The cells in dynamic conditions developed higher impedance values at low frequencies and showed a typical RC behaviour, while the controls showed minimal capacitive behaviour. These results highlighted the differences between flow and static conditions and the ability of the TEEI measurements to provide a more precise indication of monolayer formation.

Introduction

Biological barriers allow the separation between different compartments of the human body or between the body and the external environment. They have a fundamental role in controlling the absorption of exogenous substances such as nutrients and xenobiotics, as well as in the maintenance of homeostasis in different body compartments. The integrity of the barrier is usually characterised by measuring the Trans Epithelial Electric Resistance (TEER). However, Impedance spectroscopy, *i.e.* the application of a frequency sweep of current, can provide additional information on the capacitive component of the cellular barrier (Figure 1A).[1] Here, we present a new millifluidic double-flow bioreactor, which integrates a TEEI measuring system.

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Materials and Methods

The TEEI bioreactor is an adaptation of the modular, dual flow commercial Live Box 2 bioreactor (LB2, IVTech S.r.l. - Massarosa, Italy). Silver circular electrodes were integrated on the internal surface of the glass slides placed at the top and bottom of the bioreactor. Spring contacts were inte-

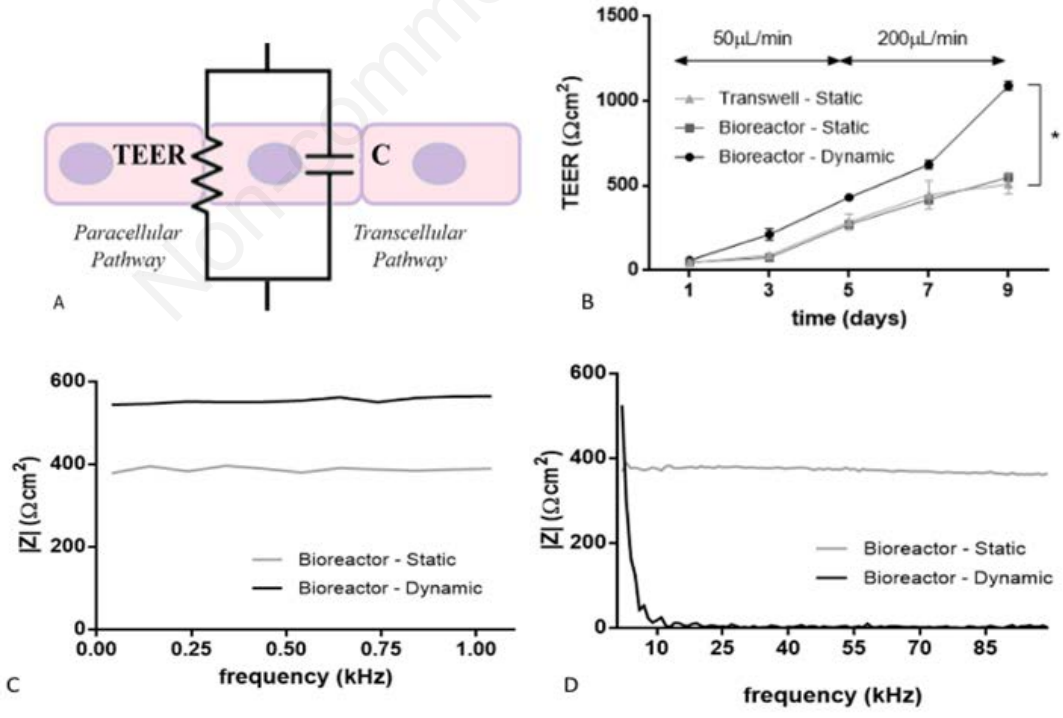

Figure 1. A) Electric equivalent of an epithelial or endothelial cell layer, B) TEER measurements during cell culture performed with the impedance-meter (f<1 kHz) in the bioreactors and with the EVOM in the transwells (*=P<0.05 between static and dynamic conditions). TEEI measurement 5 days after seeding in the bioreactor in static and dynamic conditions: C) low frequencies (0.40- 1 kHz) and D) high frequencies (2-100 kHz).

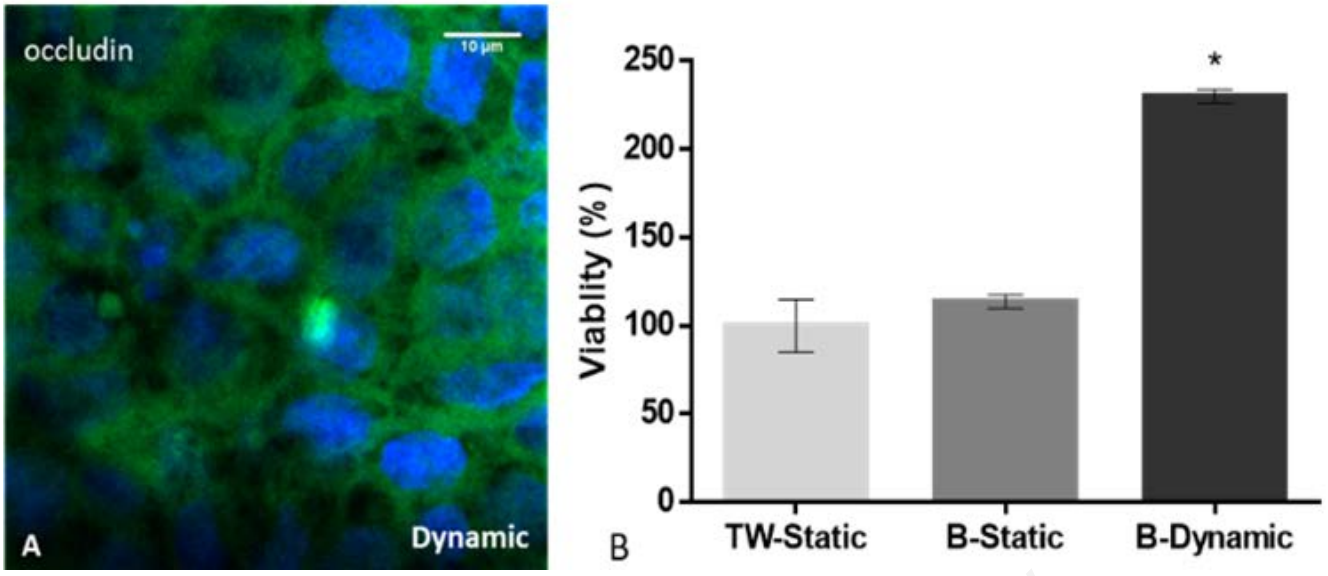

Figure 2. A) Confocal Imaging at day 9 in dynamic conditions (40X magnification); B) Viability at day 9 in the transwell and in the bioreactors (data are normalised with respect to static controls in transwells) *=P<0.05.

grated in the bioreactor clamp system allowing to interface the bioreactor with the measuring circuit, based on the AD5933 and an analog front-end that adapt the chip to biological measurements.[2] Caco 2 cells ($1x10^5$ cells/cm^2) were seeded both in the tranwell and cultured for 9 days in static and dynamic conditions. At the end of the culture, cell viability was assessed with a resazurin based in metabolic assay (Sigma Aldrich). Then, the cells were fixed and stained with DAPI and occludin monoclonal antibody (OC-3F10) directly conjugated with Alexa Flour 488 (Thermo-Fisher, Massachusetts, USA) and images were acquired with a confocal microscope (Nikon A1, Tokyo, Japan).

Results

Dynamic conditions were able not only to improve barrier tightness, as shown by the higher TEER values (Figure 1B), but also cell viability (Figure 2B). Moreover, as it possible to observe in Figure 1 C-D, impedance measurements provided a more precise indication regarding monolayer formation. FInally, the presence of tight junctions was assessed with immunostaining for occluding (Figure 2A).

Conclusions

In this work a new dual-flow bioreactor with integrated TEEI monitoring was developed and tested with an intestinal *in vitro* model, demonstrating the importance of dynamic conditions for barrier-forming cells. Thus, this sensorized system can be used to improve the relevance of further *in vitro* studies.

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In vitro microfluidic modelling of the human blood-brain-barrier microvasculature and testing of nanocarrier transport

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Abstract

ADD THE ABSTRACT

Introduction

The blood-brain-barrier (BBB) is a selective barrier that help to maintain brain homeostasis, however it also constitutes a nearly-impenetrable obstacle against therapeutic delivery to the central nervous system. Since only small compounds can cross the BBB, this reduces the treatments available for neurodegenerative diseases and cancer.[1] Polymer-based nanoparticles (NPs), due to their small size and surface functionalization potential, have emerged as a solution to deliver therapeutic cargo across the BBB. Although these techniques are considered the gold standard, current models, such as transwell or mouse models, fail to reproduce the anatomical complexity of the human BBB. Indeed, 80% of drug candidates that were successful tested *in vitro* and in animals later failed in clinical trials.[2] Moreover, as transport across the BBB represents the first evidence of NP delivery capabilities, the development of a *in vitro* testing platform and method for quantifying NP transport behavior provides an invaluable tool to assess therapeutic efficacy.

For these reasons, a cost-effective and reliable *in vitro* BBB model and method that adequately reflects human *in vivo* conditions is required.

Materials and Methods

PDMS microfluidic devices were designed (Autocad) and fabricated by soft lithography. To develop our 3D BBB model, human induced pluripotent stem cell-derived endothelial cells (iPSC-ECs, CDI), primary astrocytes and pericytes (ScienCell) were suspended in fibrin gel (3 mg/mL), injected into the device, incubate to allow the gel polymerization and supported with culture medium (Vasculife) (Figure 1a).[3] RT-PCR, vascular permeability and immunocytochemistry assays were performed. Polystyrene NPs (PS-NPs) (FluoSpheres) and synthesized polyurethane NPs (PU-NPs) transport was preliminary evaluated across the human BBB model. 3D permeability was quantified by the increase in fluorescence intensity in the region outside the BBB vasculature in confocal z-stack images (Figure 1b-d).

Results

This *in vitro* microfluidic model of the BBB containing human induced pluripotent stem cell-derived endothelial cells, brain pericytes, and astrocytes as self-assembled microvasculature supported in fibrin gel matrix. Gene expression of tight junctions (ZO-1, occludin, and claudin-5), extra-cellular matrix proteins (Laminin and Collagen IV), and membrane transporters (PG-P, LAT1, LRP1) was higher in tri-culture condition consistently with quantitative immunocytochemistry analysis indicating BBB-like maturation. Laser confocal microscopy validated microvessel-pericytes/astrocytes contact-interactions. Characterization of microvascular network parameters as vascular diameter, branches length and vascular network area coverage were lower when including pericytes and astrocytes. This revealed that morphological changes were induced by not only the secretion of pro-angiogenic and vasculogenic growth factors but also contact signaling between cells. The BBB model exhibited perfusable and selective microvasculature created within 5-7 days (Figure 1d), showing permeability coefficient comparable to previous models and similar to *in vivo* measurements in rat brain.[3]

This established 3D *in vitro* model of the human BBB might be exploited to evaluate nanocarrier permeability such as nanoparticles. Indeed, ongoing experiments are showing that the 3D BBB model might be capable to elucidating differences in 3D NP transport between PS-NPs and PU-NPs compared to Transwell assays.

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Conclusions

The BBB microfluidic model has the main advantage over other BBB-on a chip models, that all three cell types are seeded simultaneously into a single 3D gel region, producing a perfusable vascular network with permeabilities lower than those of other published models. The contribution of co-culture with pericytes and astrocytes also improved BBB formation and maturation. It also helped to regulate the upregulation of tight junction proteins and membrane transporters by the iPS endothelial cells, highlighted as potential targets to enhance the penetration of drugs into the brain.

The work also presents an innovative translational application of the 3D BBB microvascular model to assess nanocarrier transport and quantify the permeability of different nanocarriers that cross the human BBB.[4] A permeability method is being optimized in order to perform pre-clinical screening of drug candidates within a physiologically-relevant BBB microvasculature, reducing the use of animal models.

This robust 3D BBB microvascular model could be potentially applied to

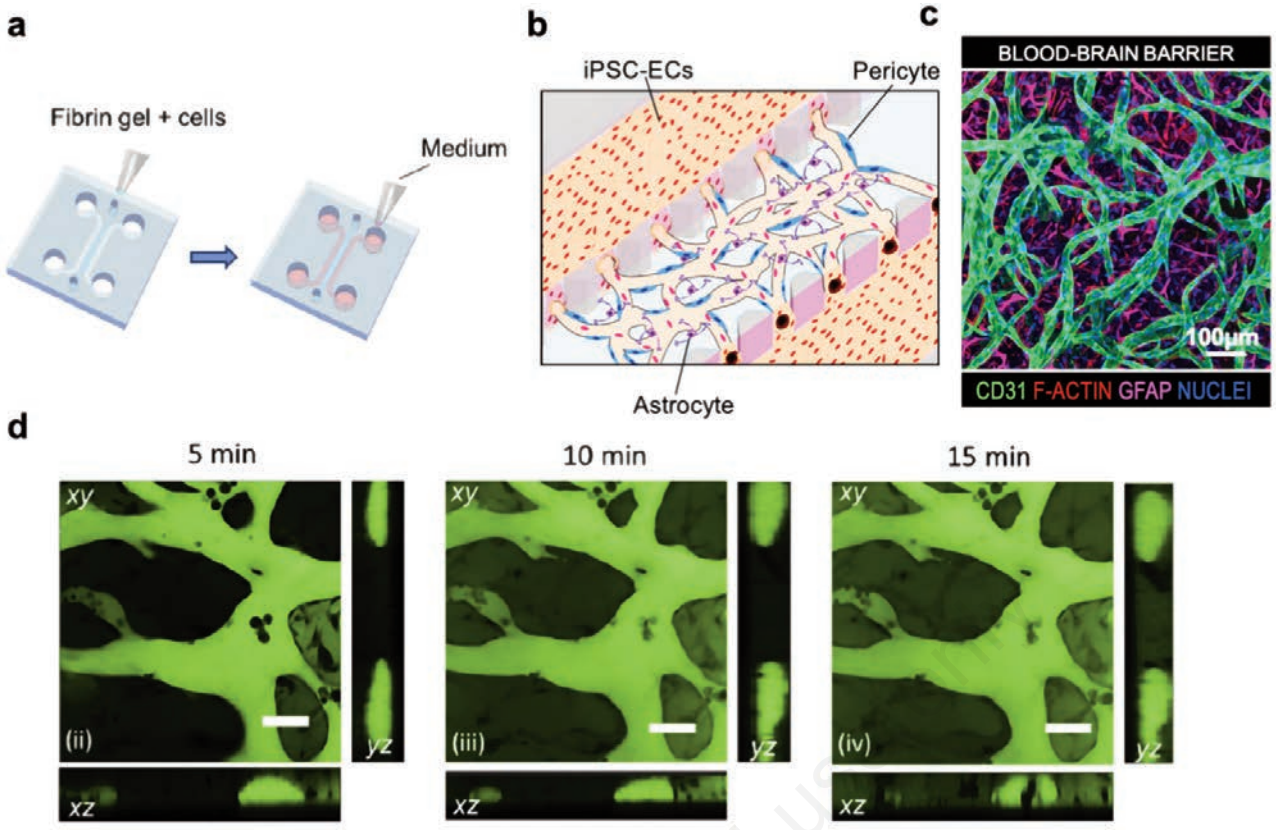

Figure 1. Representative images of the BBB model: (a) microfluidic device seeding; (b) the microvasculature obtained by triculture; (c) immunohistochemistry analysis of the BBB microvasculature; (d) confocal microscope analysis of the BBB microvasculature after perfusion with fluorescent dextran. Reproduced with permission from Campisi *et al.*[3] PLEASE MAKE SURE THAT THE FIGURE CAN BE REPRODUCED: https://www.sciencedirect.com/science/article/pii/S0142961218304915?via%3Dihub

patient-specific and neurodegenerative diseases modelling, offering a novel platform to study both drug transport for preclinical screening as well as neurovascular functions within a physiologically-relevant BBB microvasculature.

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Quantification of the foreign body reaction by means of a miniaturized imaging window for intravital nonlinear microscopy

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Abstract

Brand new biomaterials, intended to be used on humans, must undergo *in vivo* quantification standardized, expensive and unethical procedures mainly based on histopathological analysis, from dissections, as defined by the ISO 10993 normative set. The aim is to prove the biomaterials biocompatibility. There exist no methods based on intravital microscopy able to satisfy the normative quantification requirements both reducing the number of employed animals and related costs. We developed a miniaturized imaging window, the Microatlas, which allows subcutaneous repeated observations *in vivo* of the foreign body reactions, for example to the implantation of a biomaterial. Confocal and two-photon microscopy inspections at Microatlas implantation sites demonstrated growth of the recipient tissue inside the microgrids both with micro vascularization formation and collagen generation. In conclusion, the Microatlas guided *in vivo* a quantifiable localized reaction inside its microscaffold, both in terms of cell repopulation, collagen and capillary formation as a probable foreign body reaction.

Introduction

To gain the authorization of being used on humans, brand new biomaterials must undergo *in vivo* quantification standardized, expensive and unethical procedures (defined by the ISO 10993) to prove their biocompatibility. These ones are mainly based on histopathological analyses in large number of mammalians, subcutaneously implanted, through different timepoints. Currently there exist no methods based on intravital microscopy able to satisfy the normative quantification requirements, with the aim to reduce the number of employed animals and related costs. We developed a miniaturized imaging window, the Microatlas, which can be implanted subcutaneously and allows repeated observation *in vivo* of the foreign body reactions, for example to the implantation of a biomaterial. The device hosts a miniaturized scaffold able to guide cell migration close to the desired target. By applying two-photon fluorescence microscopy to the Microatlas, once implanted *in vivo* and repopulated by cells and blood vessels, it is possible to observe and quantify the foreign body reactions in the same animal and tissue district, at different time points. Thus, we can reduce the number of employed animals in subcutaneous validation protocols, refine and boost the conducted validation analyses and replace old and outdated quantification processes in term of cellular density, blood vessels sprouting, collagen and fatty infiltrate generation. Here, we grafted the Microatlas in living chicken embryos to conduct *in vivo* validation assays.

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Key words: Imaging window; nonlinear microscopy; two-photon polymerization.

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Materials and Methods

The Microatlas micro scaffolds were fabricated by two-photon laser polymerization on circular glass coverslips (Ø:5-12 mm), with a biocompatible photoresist, SZ2080. The micro scaffolds consist in several micro grids (500 μm x 500 μm x 100 μm). Reference structures were integrated to allow the microscope field-of-view repositioning at different time-points (Figure 1). The chicken embryo *ex ovo* culture was optimized and the optimal implantation time points were selected. The Microatlas was implanted and it was inspected by two-photon fluorescence and confocal microscopy. At each time point, the embryo was two-photon imaged first, then formalin-fixed, labelled with DRAQ5™ and imaged in confocal microscopy.

Results

Confocal and two-photon inspections at Microatlas implantation sites demonstrated growth of the recipient tissue inside the micro grids both with micro vascularization formation. Two-photon fluorescence acqui

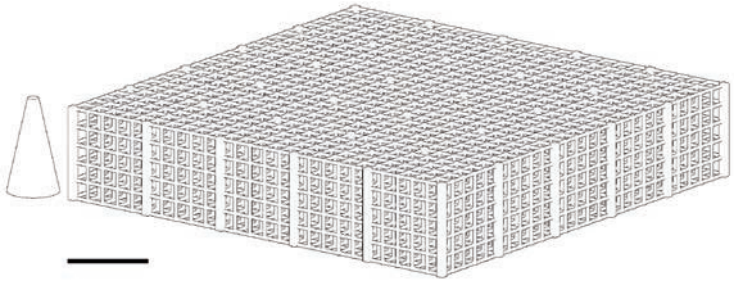

Figure 1. Representation of a Microatlas micro scaffold. Scale bar 100 μm.

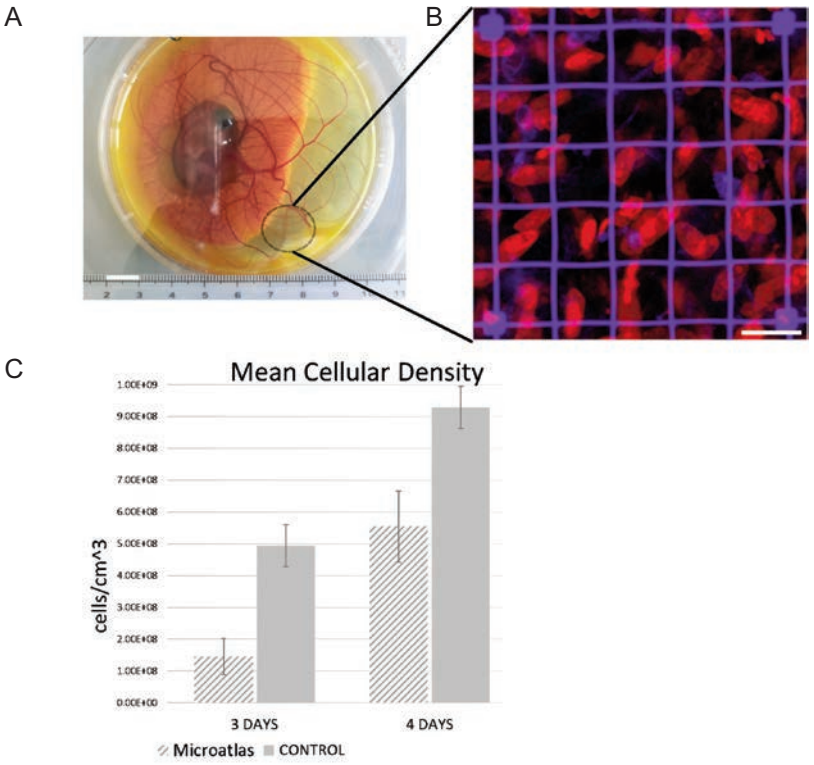

Figure 2. A) Chick embryo at incubation day 11, scalebar 1 cm. B) cells imaged inside the Microatlas, scalebar 20 µm. C) cellular density trend inside the Microatlas microgrids.

sitions of label-free specimens specifically showed the presence of a layer of collagen type I, localized mainly around and inside the implanted Microatlas. Microscope images allowed quantification of cell density, collagen formation and neo-vascularization rate (Figure 2) inside the Microatlas as required by the ISO10993-6.

Conclusions

The Microatlas guided *in vivo* a quantifiable localized reaction inside its microscaffold, both in terms of cell repopulation, collagen generation and capillary formation as a probable foreign body reaction. Thus, our device can be used as a powerful imaging window for intravital fluorescence microscopy with the capability to quantify *in vivo* the reaction to biomaterial implantation.

Chitosan biopolymer: Alternative adhesion factor and scaffold matrix for 2D and 3D neuronal cultures

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Abstract

The increase of different types of cell cultures, which can be used for the *in vitro* studies of physiological and/or pathological processes, has introduced the need to improve culture techniques through the use of materials and culture media that promote growth, recreating a cellular micro-environment that can be asserted in *in vivo* condition. The standard methods for the functionalization of supports used for cell cultures are based on the use of synthetic or natural biopolymers, which generally have high costs, such as poly-lysine and poly-ornithine. The aim of this work is to demonstrate the alternative use of the polysaccharide chitosan as adhesion factor and structural component for 2D/3D neuronal cultures. Thanks to its versatility, it could be easily functionalized for the fabrication of personalized of *in vitro* models.

Introduction

Cell cultures are fundamental for a wide of applications involving both research and industries. The increase of different types of cell cultures, which can be used for the *in vitro* studies of physiological and/or pathological processes, has introduced the need to improve culture techniques through the use of materials and culture media that promote growth, recreating a cellular micro-environment that can be asserted in *in vivo* condition. Therefore, it is important to design and develop new biologically sustainable methods, such as to contribute to the "closer-to-*in vivo*" condition.[1]

Related to that, in this work, we present the biopolymer Chitosan (CHI) as support for 2D and 3D neuronal cell cultures. Chitosan is a copolymer of glucosamine and N-acetyl-glucosamine, obtained by the deacetylation of chitin; it is well known for its low-cost, biocompatibility, biodegradability, muco-adhesiveness, antibacterial activity as well as its bioaffinity.[2]

Materials and Methods

CHI was dissolved in 0.1 M acetic acid at different concentrations (0.01% - 2% w/v); 2% sodium hydroxide solution. For 2D cultures only, Poly- ornithine (PORN) solution 0.15 mg/mL in water, as control.

2D: Chitosan nanometric films were obtained by dip coating.

3D: Chitosan microspheres were fabricated by a phase-inversion process using an aerodynamic encapsulator.

Chitosan films and microspheres were then used as support for the *in vitro* growth of primary neuronal cells. To validate the ability of chitosan to support neuronal adhesion, networks development and the differentiation capacity, morphological and functional characterization were carried out by confocal, transmission electronic and atomic force microscopies. A preliminary electrophysiological characterization of spontaneous activity was conducted by Micro-Electrode Arrays (Figure 1).

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Key words: Chitosan; adhesion factor; scaffold; neuronal network.

Contributions: DDL and MT contributed equally to this work.

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Results

Chitosan films showed the ability to support the adhesion and differentiation of neuronal culture. The growth of neurons plated on chitosan films is comparable with

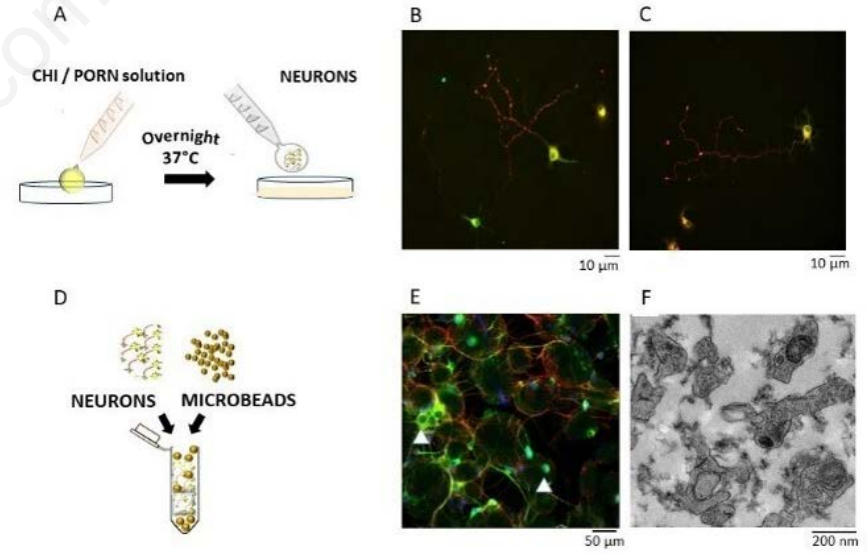

Figure 1. A) Scheme of adhesion factor deposition for 2D cultures. B-C) Hippocampal culture development on dip-coating chitosan and on poly-ornithine staining staining for MAP 2 (green) and TAU (red) at 7 *days in vitro*; D) Scheme of 3D cell cultures assembly. E) Confocal microscope images of *3D neural network* at DIV 25 on 2% CHI microbeads labeled for MAP-2 (green), Tubulin βIII (red) and DAPI (blu). F) Low-mag TEM micrograph of a portion of chitosan scaffold with the neuronal network: neuritic processes inside microbeads.

ones on standard adhesion factors (poly-ornithine). Furthermore, it is noted that 3D cultured neurons, show distinct morphologies that are more representative of the *in vivo* environment. In particular, these results have been confirmed by a preliminary electrophysiological characterization.[3]

Conclusions

We successfully demonstrate the alternative use of the polysaccharide chitosan as adhesion factor and structural component for 2D/3D neuronal cultures. Thanks to its low cost and versatility, it could be easily functionalized for the fabrication of personalized of *in vitro* models.

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European regulatory framework for the clinical translation of bioprinted scaffolds and tissues

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Abstract

Tissue Engineering and Regenerative medicine, empowered by Biofabrication technologies, hold the premises to provide solutions to unmet clinical needs, such as organ donor shortage or genetic diseases. These huge advancements are determining a changing scenario, with a quite confusing understanding about the steps toward the clinical translation of new researches and products, giving as result an overestimation or an underestimation of the required *in vitro* and *in vivo* tests for their validation. The proper definition and classification of the research products can be considered an action toward the refinement of animal experiments. An appropriate classification is crucial because the complications due to the combination of biological and non-biological materials need the application of specific rules. This paper aims at helping the academic and industrial community to clarify the identification and classification of their research products.

Introduction

Tissue Engineering and Regenerative medicine, empowered by Biofabrication technologies, hold the premises to provide solutions to unmet clinical needs, such as organ donor shortage or genetic diseases.[1] Bioprinting scaffolds or other implants are changing not only the surgical procedures, but also the organization of hospitals, which can potentially produce in-house these products.[2] In this context, new biomaterials but also bioprinters and bioreactors are reaching the market. Figure 1 shows the several areas in the bioprinting healthcare scenario.

These huge advancements are determining a changing scenario, with a quite confusing understanding about the steps toward the clinical translation of new researches and products, giving as result an overestimation or an underestimation of the required *in vitro* and *in vivo* test for their validation.

Materials and Methods

The proper definition and classification of the research products can be considered an action toward the refinement of animal experiment. For example, generic term "scaffold" is used to indicate an implantable substrate, which can be correctly classified as Medical Device (MD) or as Advanced Therapy Medicinal Product (ATMP) according to its working principles and/or components. Similarly, bioprinters are normally used to fabricate scaffolds or *in vitro* model for biological experiment, but they could potentially be used to bioprint *in situ* biomaterial inks and bionks: in this case, they have to be considered as MD, with the proper request of safety and efficacy. At European level, these requirements are defined by the EU Regulation 2017/745 and 1394/2007, which set the legislative framework for the definition and validation toward commercialization of MDs and ATMPs, respectively.[3,4]

Results

With the aim at helping the academic and industrial community to clarify the identification and classification of their research products, Figure 2 tries to clarify these differences with practical examples.

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Key words: Bioprinting; 3D printing; regulation; ATMP; medical device.

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Conclusions

Bioprinting raises questions about the exact legal nature and specific classification of bioprinted-related products. An appropriate classification is crucial, in particular, because the complications due to the combination of biological and non-biological materials need the application of specific rules. This suggests that the additive manufacturing technologies applied to bioprinting need an appropriate legal framework in particular in the domain of pharmaceutical, medical devices, advances therapies, tissues and cells where significant regulatory and socio-ethical challenges are faced.

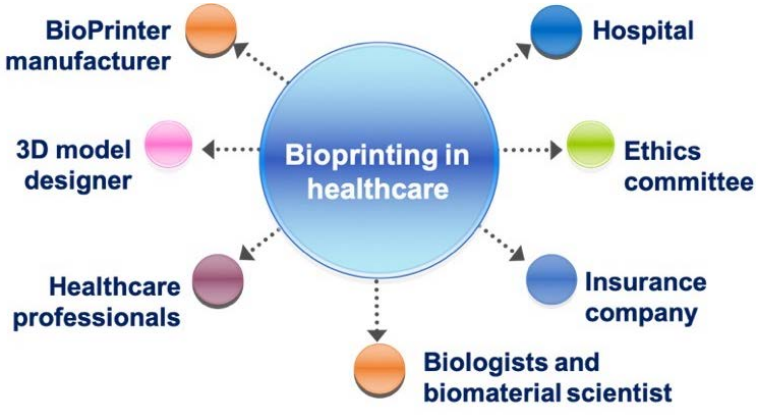

Figure 1. Bioprinting in healthcare.

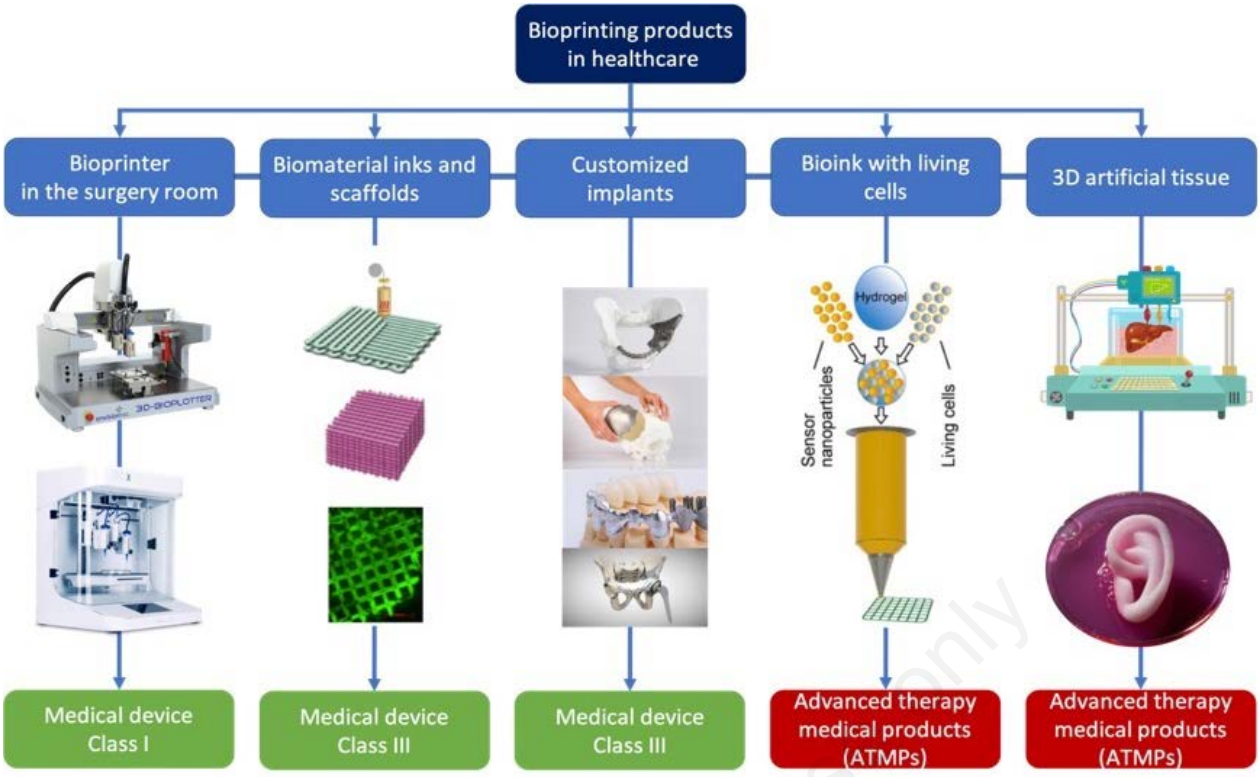

Figure 2. Bioprinting products in healthcare.

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Use of an *in vitro* model of hepatic steatosis for studying the anti-oxidant and anti-steatotic effects of fucoidan polysaccharides

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Abstract

Non Alcoholic Fatty Liver Disease (NAFLD) is characterised by fat accumulation in hepatocytes in the form of triacylglycerols (TAGs) within cytosolic lipid droplets. Fucoidans (FUs) are biologically active polysaccharides usually isolated from brown marine algae, but recently identified also in terrestrial plants. In this study, we aimed to investigate the anti-oxidant and anti-steatotic effects of FUs purified from *C. compressa*, *F. hermonis*, and *E. globulus*. To this aim, we used a validated NAFLD *in vitro* model consisting of rat hepatoma FaO cells exposed to an oleate/palmitate mixture. Such a model is suitable for rapid investigation of direct effects of natural and artificial compounds, together with satisfying the strategy of 3Rs for laboratory use of animals. Our results indicated that all FUs display anti-oxidant and anti-steatotic activities. Steatotic FaO cells may be employed to further study the biological effects of FUs.

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Key words: Non alcoholic fatty liver disease; fucoidan; anti-steatotic; anti-oxidant.

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Introduction

Non Alcoholic Fatty Liver Disease (NAFLD) is the most common cause of liver disorders with high negative impact on human health. It is characterized by fat accumulation in more than 5% of hepatocyte, stored in the form of triacylglycerols (TAGs) within cytosolic lipid droplets (LDs), as a result of hepatic lipid metabolism unbalance. Molecular mechanisms and therapeutic strategies for NAFLD can be studied using *in vivo* models, which offer the advantage to study the effects of active compounds at the level of the whole organism. However, major disadvantages are ethical concerns, requirement of skilled manpower, time consuming protocols and high costs, thus supporting the application of *in vitro* models which may satisfy the strategy

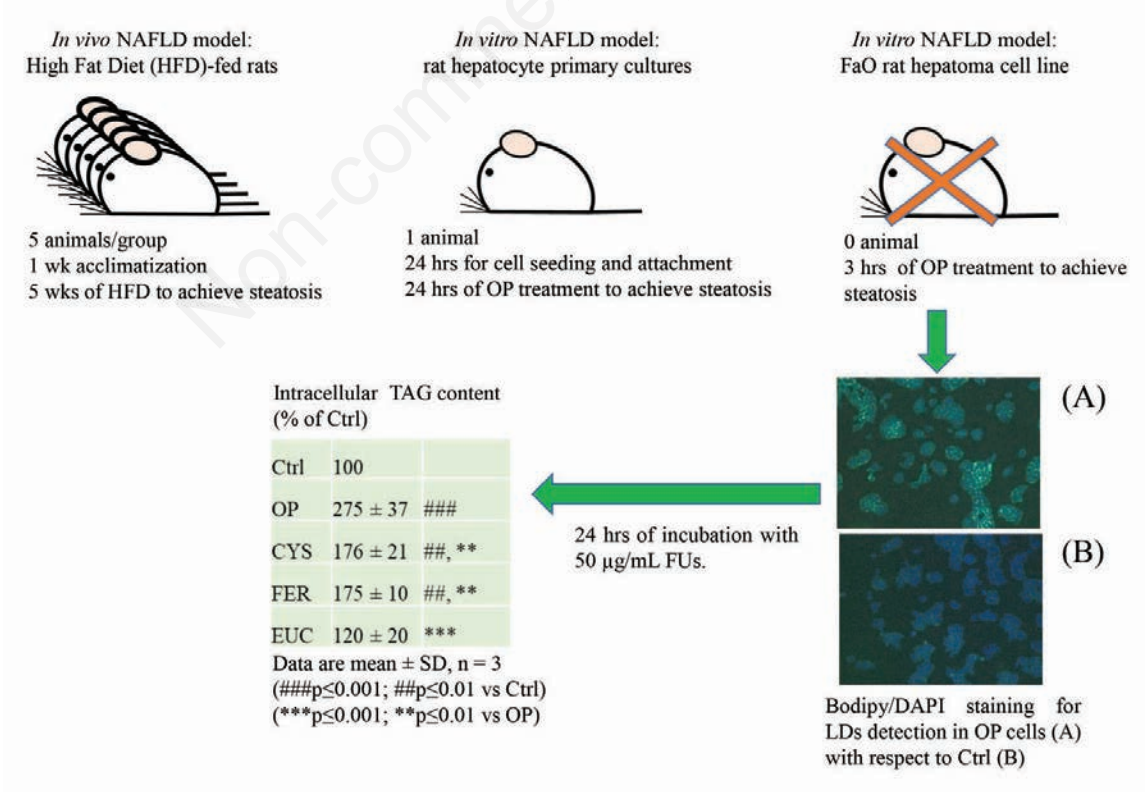

Figure 1. Schematic description of different *in vivo* and *in vitro* NAFLD experimental models with reference to the 3R strategy; the anti-steatotic effects of FUs extracted from CYS, FER and EUC is also shown.

of 3 Rs (*i.e.* reduction, refinement and replacement) for laboratory use of animals.[1] In the last years our group used both *in vivo* and *in vitro* models to study NAFLD, and demonstrated that the main markers of hepatic steatosis are maintained in all the experimental models, thus making the *in vitro* model suitable for rapid investigation of direct anti-steatotic effects of natural and artificial compounds. In the present study, we used an *in vitro* model of NAFLD to study the anti-oxidant and anti-steatotic effects of fucoidans (FUs). FUs are fucose rich polysaccharides predominantly found in the cell wall of brown algae such as *Cystoseira compressa,* but recently identified also in terrestrial plants such as *Eucalyptus globulus* and *Ferula hermonis,* growing in Lebanon.

Materials and Methods

FUs were purified from *C. compressa* (CYS), *F. hermonis* (FER), and *E. globulus* (EUC) as previously described.[2] Anti-oxidant activity was determined by DPPH test. Steatotic rat hepatoma FaO cells were obtained by 3 h exposure to an oleate/palmitate (OP) mixture and then exposed to 50 µg/mL FUs for 24 h.[1,3] The anti-steatotic effect of FUs was investigated by measuring intracellular TAG content and by detecting LD formation through fluorescence microscopy. The expression of PPAR (Peroxisome Proliferator Activated Receptor) isoforms and LD associated perilipins (PLINs) that play important roles in lipid homeostasis, was evaluated by qPCR.[3]

Results

The upper part of Figure 1 shows that a dramatic decrease in animal use and a high speeding up of the experimental procedures can be appreciated when comparing the number of animals needed and the duration of the treatments used *in vivo* and *in vitro* to obtain three different models of NAFLD. The lower part of Figure 1 shows the detection of LDs by Bodipy fluorescent staining in OP-treated FaO cells with respect to control (Ctrl) cells; the table shows that intracellular TAG content was significantly reduced in steatotic cells upon any FU treatment. This result was in line with a suppression of lipogenic genes such as PPARγ, PLIN2 and PLIN5, which was measured by qPCR. DPPH assay allowed to demonstrate a significant anti-oxidant activity, which was exerted by FUs purified from all three species (IC 50 was 158.5 ± 1.81, 152.9 ± 3.30, 4.466 ± 3.26 µg/mL for CYS, FER and EUC, respectively).

Of note, FUs purified from terrestrial plants exerted stronger anti-oxidant and anti-steatotic effects than those obtained from marine brown algae, with *E. globulus* FUs showing the highest activities.

Conclusions

Different *in vivo* and *in vitro* models have been established to resemble the most important features of NAFLD. *In vitro* approaches have the advantage of providing a simple and highly reproducible model, where the mechanisms can be studied directly at the cellular level. Our results validate the use of cell cultures as effectual tools to replace the use of laboratory animals when studying NAFLD and the effects and mechanisms of action of natural compounds with therapeutic potential, such as FUs. Steatotic FaO cells may be employed to further study the biological effects of FUs, particularly those extracted from *E. globulus,* which is confirmed as a powerful medicinal plant.

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Effects of occupational exposure to glyphosate in winegrowers

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Abstract

Glyphosate is a non-selective systemic herbicide used in agriculture. For almost half a century, the International Agency for Research on Cancer has run a Monographs program, the conclusion in March 2015 that glyphosate is "probably carcinogenic to humans" in addition to being genotoxic and carcinogenic in animals, while the regulatory European Food Safety Authority have asserted that glyphosate poses no public risk. The scientific debate is still lively. We collected detailed socio-demographic, occupational exposures and health surveillance information for 26 winegrowers as aim to investigate exposure to glyphosate and other pesticide. Exposure was assessed through biological monitoring (24-hour urine collection), immune function (IL-4, IL-5, IL-8, IL-12, IL-17, IL-33, IFN- γ), transcriptional and post transcriptional alterations (miRNA) and genotoxic effects (Comet assay). The exposure conditions in our winegrowers, as referred to the parameters so far analyzed, did not reveal a significant glyphosate absorption nor significant health concerns.

Introduction

Glyphosate is a widely used herbicide. Within occupational settings, most relevant routes of absorption are via inhalation and skin. Since 2015, the International Agency for Research on Cancer (IARC) classified glyphosate as a probable human carcinogen, referring to haemolymphopoietic system as the main target, while the European Food Safety Authority (EFSA) concluded that it is unlikely that glyphosate poses a carcinogenic hazard to humans, taking into account the CLP classification.[1-3] The scientific debate is still lively. Until now, there are no studies simultaneously evaluating, in winegrowers exposed to glyphosate and other pesticides, indicators of exposure and effect through biological monitoring, immunological indicators, genotoxicity and regulators of gene expression.

Our study aimed to investigate exposure to glyphosate and other pesticides in winegrowers as well as potential effects regarding genotoxicity, immunomodulation and gene expression. The study was carried out within the framework of the Regional Plan for Prevention 2014/18 denominated "Prevenzione degli infortuni e malattie professionali in agricoltura".

Materials and Methods

By means of questionnaires, we collected detailed socio-demographic, occupational exposures and health surveillance information for 26 winegrowers; quantities, timing and ways of glyphosate and pesticides use were also collected through official health authority registers. As for biological monitoring of exposure, 24-hour urine glyphosate was chosen as the indicator which was evaluated pre and post application. The following analyses were performed before and after the use of glyphosate during the period of pesticides treatments: we monitored general blood chemistry parameters, immune function (IL-4, IL-5, IL-8, IL-12, IL-17, IL-33, IFN-γ), possible transcriptional and post-transcriptional alterations (miRNA), potential genotoxic effects (Comet-assay on lymphocytes DNA). Statistical analysis used Wilcoxon signed-rank test and three-way variance analysis with repeated measures after normalizing the outcome variables with appropriate linearizing transformations.

Results

In our study population, glyphosate exposure was low in quantity, limited in duration and appropriate in mode. Biological monitoring did not show high absorption rates; immunologic tests seemed to show some modification (after *vs* before usage), limited to IL-4, IL-5, IL-5, IL-8 and IFN-γ. Genotoxic alterations were not evident. Further statistical analyses are in progress for the remaining indicators.

Conclusions

The exposure conditions in our winegrowers, as referred to the parameters so far analyzed, did not reveal a significant glyphosate absorption nor significant health concerns. Potential effects due to the use of glyphosate and other pesticides on immunomodulation, as well as on gene transcription and post-transcriptional regulation are currently under investigation.

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Key words: Glyphosate; winegrowers; comet assay; miRNA; immune function.

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Versatile electrical stimulator for providing cardiac-like electrical impulses *in vitro*

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Abstract

In the perspective of reliable methods alternative to *in vivo* animal testing for cardiac tissue engineering (CTE) research, the versatile electrical stimulator ELETTRA has been developed. ELETTRA delivers controlled and stable cardiac-like electrical impulses, and it can be coupled to already existing bioreactors for providing *in vitro* combined biomimetic culture conditions. Designed to be cost-effective and easy to use, this device could contribute to the reduction and replacement of *in vivo* animal experiments in CTE.

Introduction

Cardiac tissue engineering (CTE) aims to develop functional substitutes of native myocardium to be exploited as *in vitro* models for cardiac development and disease research, and ultimately for cardiac repair.[1] In the perspective of reliable methods alternative to *in vivo* animal tests,[2] bioreactors are technological devices designed to provide biomimetic culture environments *in vitro*. In CTE they are widely used as model systems to investigate the individual or combined effects of cardiac-like physico-chemical stimuli on cardiac cells and substitutes, with the advantage of excluding systemic effects existing *in vivo*.[3] Here we present the versatile electrical stimulator ELETTRA, designed to provide controlled cardiac-like electrical impulses for CTE applications and to be integrated in already existing bioreactors.

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Key words: Electrical stimulation; cardiac tissue engineering; *in vitro* models; versatility.

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Materials and Methods

ELETTRA design was guided by specific requirements: accuracy in mimicking the *in vivo* pulsatile electric field experienced by human cardiac cells (resting rate = 1.0-1.7 Hz, electric field = 0.1-10.0 V/cm, pulse duration = 1-2 ms);[3] versatility to be used with different bioreactors; ease of use; cost-effectiveness. ELETTRA's core consists of an Arduino Due board running a purpose-built software, interfaced to analog and digital peripherals. A user-friendly interface, based on a push button, a rotary encoder and an LCD display, allows the tuning of stimulation parameters. Banana sockets are used as output ports and a sensing resistor enables monitoring the current flowing between the electrodes. Control and stimulation subunits allow to separately increase stimulation amplitude and deliver monophasic or biphasic pulses in a wide range (frequency = 0.5-10.0 Hz, amplitude = 0.5-12.0 V, pulse duration= 1-10 ms). To test the system, ELETTRA was connected to two carbon rod electrodes (length = 2.5 cm, diameter = 0.3 mm) embedded in a sil-

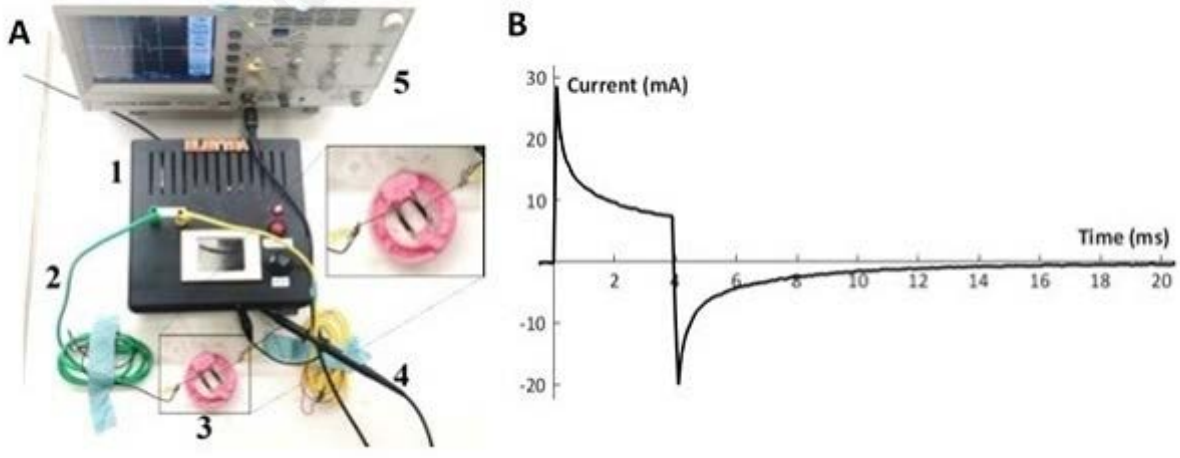

Figure 1. A) Experimental testing setup: 1. ELETTRA; 2. Connectors; 3. Electrode-holder assembly in Petri dish; 4. Sensing circuit probe; 5. Oscilloscope. Inset image: Electrode-holder assembly. B) Plot of the average current vs time for monophasic stimulation (5 V, 1 Hz, 4 ms) as measured by the sensing circuit.

icone holder at a fixed distance of 1 cm, and placed in a Petri dish with saline solution (Figure 1A). Monophasic square waves (1 Hz, 4 ms) with four different amplitudes (5.0, 7.5, 10.0, 12.0 V) were imposed. A digital oscilloscope was used to measure the total voltage and the voltage drop on the sensing resistor. For each condition, 20 subsequent pulses were recorded. Data were processed to calculate the maximum flowing current and the time constant.

Results

Preliminary tests on ELETTRA confirmed satisfactory stimulation performances, ease of use and cost-effectiveness, with an overall cost below € 100. Voltage amplitude resulted stable during stimulation. As regards the average current between the electrodes (Figure 1B), it increased instantly as the electrical stimulation was provided with a following decrease due to the induced polarization of the solution. During passive phase, the accumulated charges were released in the solution and the current reversed its direction. The maximum current flowing between the electrodes varied from 29 to 79 mA, depending on the imposed voltage. For each condition, the calculated time constant was always lower than 1 ms.

Conclusions

ELETTRA delivers a controlled and stable stimulation mimicking the cardiac electrical impulses. This device could significantly contribute to the reduction and replacement of *in vivo* animal experiments for investigation of cardiac development and disease and for preclinical validation of engineered cardiac constructs. Biological tests are ongoing on cardiomyocyte monolayers to investigate the impact of electrical stimulation on cell maturation.

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Analysis of non-animal methods and models for research in cardiovascular disease

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Abstract

Cardiovascular diseases (CVD) are disorders of the heart and blood vessels and represent 31% of all global deaths. In the contest of CVD, the use of animal experiments has been a contentious subject for many years. In recent years, *in vitro* and *in silico* models and methods have been proposed according to the 3Rs statement. However, an exhaustive report regarding the state of art in terms of *in vitro* and *in silico* experiments has not been reported yet. This work is focused on providing a collection of non-animal models and methods in use for basic and applied CVD research. The standardized descriptions of such studies will ultimately feed into EURL ECVAM database on alternative methods. Two are the research main phases. Firstly, the exclusion/inclusion criteria and the list of relevant information resources of the research have been defined. The second phase regards the search, selection and detailed description of the literature papers by analysing records on Scopus and Pubmed databases.

Introduction

Prevalence of cardiovascular disease (CVD) has been increasing worldwide, and the recent report from AHA[1] indicates over 90 million US adults have at least one CVD, which is expected to increase more in a rapid pace. In the contest of CVD, the use of animal experiments has been a contentious subject for many years. In recent years, several *in vitro* and *in silico* models and methods have been proposed according to the 3R statement. However, despite a significant amount of literature in the CVD research field, to date, a complete mapping regarding the state of art in terms of efficacy and translational research efficiency is not reported.

In line with such service, the goal of this work is to provide a collection of non-animal models and methods in use for basic and applied CVD research with information on their development status, applications or predictive value in the field of human cardiovascular diseases. The standardized descriptions of such studies will ultimately feed into a EURL ECVAM inventory on innovative methods.

Materials and Methods

Our research is organized in two main phases: the first phase is dedicated to the setting up of the methodologies, including the exclusion/inclusion criteria and format for the method summaries, the list of relevant information resources and the proposed search phrases. The second phase focuses on the actual performance of the literature search, selection of the methods, analysis and their detailed description. The search was performed analyzing records on Scopus, including Pubmed database.

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Key words: Cardiovascular diseases; non-animal methods; state of art; *in silico*; *in vitro*.

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Results

Our preliminary results depict an amount of 14743 research papers on impacted journals in CVD field without the usage of animal models. Cardiovascular Surgical and interventional Procedures (with/without devices) include about 38% of the records (Figure 1). Regarding the CVD pathologies, the myocardial ischemia is the disease where most of non-animal methods and models are applied.

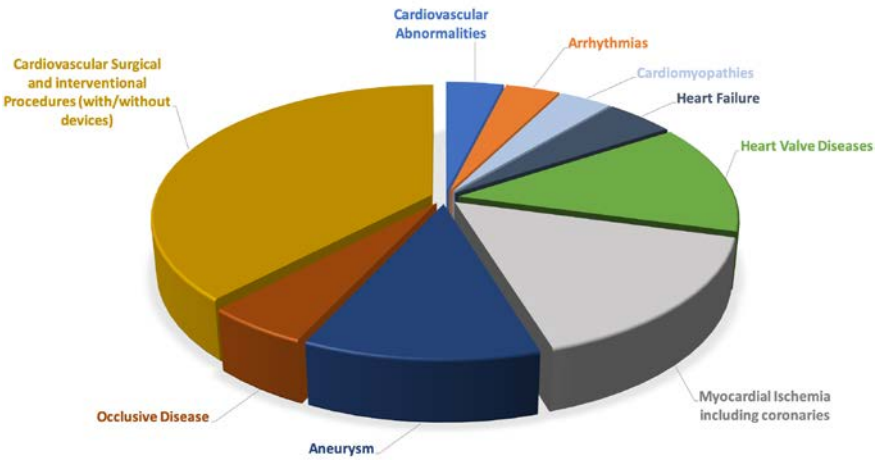

Figure 1. Records categorization.

Conclusions

These results seem to be in accordance with the effort of the EU community concerning the past projects in cardiovascular devices and point out fundamental details on further effort by the Community to cope missing research topic. The outcome of this study will be crucial to contribute to the uptake, implementation and promotion of non-animal methodologies in biomedical research, thus contributing to the reduction of the reliance on animal use.

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Clarifying mid-brain organoids: Application of the CLARITY protocol to unperfusable samples

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Abstract

The aim of this study was to apply a workflow, integrating delipidation methods and advanced 3D imaging techniques for mapping of the global neuronal organization of brain organoids. These are self-organizing constructs *in vitro* generated from human pluripotent stem cells encased in a Matrigel shell, which resemble downscaled structural and functional features of human brains. In particular, we focused on mid-brain organoids, widely considered a promising tool for studying dopaminergic neuron degeneration in Parkinson's Disease. The evaluation of the micro-anatomical alterations at a patient-level will potentially guide future research of this neuropathy, providing meaningful human specific data in line with the European Directives and the 3Rs principles.

Introduction

Brain organoids have enormous potential as downscaled *in vitro* models of the human brain. In particular, human induced pluripotent stem cells-derived organoids are crucial for exploring brain development and pathogenesis in a patient-oriented perspective.[1] Mapping organoid structural organization of the cells is important for predicting alterations in dendritic and axonal arbour associated with neuro-pathology, as well as for inferring how uniquely human features are managed at the cellular level.

In order to image the whole brain organoid micro-structure without cutting it, different optical clearing protocols have been developed in the last decades.[2] Basically, tissue clearing involves exchanging the water in the sample with organic solvents or aqueous solutions with the same refractive index as membrane lipids, such that the samples become essentially transparent, thus extending the depth range of optical microscopes. Clearing techniques such as CLARITY further allow permeability to macromolecules, also providing molecular phenotyping compatibility.[3,4] Although they were originally developed for whole animal perfusion, clearing protocols can be adapted for use on vessel-free, non-perfusable samples.

Here we describe how the integration of de-lipidation methods and advanced imaging could lead to a high-fidelity mapping of the global neuronal organization within the organoids. We applied the workflow to mid-brain organoids, generated for studying dopaminergic neuron degeneration in Parkinson's Disease.

Materials and Methods

Mid-brain organoids were generated as in Berger *et al*.[5] and clarified customizing the CLARITY protocol for unperfusable samples. Briefly, organoids were immersed in 20 mL of hydrogel monomer (4% PFA, 4% acrylamide, 0.05% bis-acrylamide and 0.25% VA-044 thermally triggered initiator) for 7 days at 4°C allowing gel passive diffusion. After hydrogel polymerization at 37°C at vacuum, each sample were immersed in 20 mL of clearing solution (200 mM of boric acid, 4% SDS, pH adjusted to 8.5 adding 1M NaOH dropwise) at 37°C, refreshing every 3 days for 3 weeks. Organoids embedded in the hydrogel and immersed in 20 mL 1X PBS solution were used as controls.

The organoids were immunolabelled with Thy rabbit anti-human (1:1000 for 48 hours at 4°C) and goat anti-rabbit Alexa Fluor 488 (1:500 for 24 h at 4°C) antibodies to identify dopamine-positive neurons. In addition, organoids were immersed in a DAPI solution (1:1000 for 30 minutes) for nuclei identification. The samples were acquired using a Nikon A1 confocal microscope and a 10x objective.

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Results

Figure 1 shows how CLARITY makes the samples permeable to both photons and exogenous macromolecules. In fact, the image stacks of the samples clarified and then acquired with the confocal microscope showed a dataset with a good contrast-to-noise (CNR) and signal-to-noise (SNR) ratio: nuclei are well defined and dopamine-positive neurons can be easily tracked. Staining procedures were not performable on unclarified mid-brain organoids, since the antibodies seem to be stuck within the Matrigel network, *i.e.* a necessary feature of current brain organoid generation protocols, surrounding the sample – image not shown – and therefore cannot penetrate within the 3D constructs.

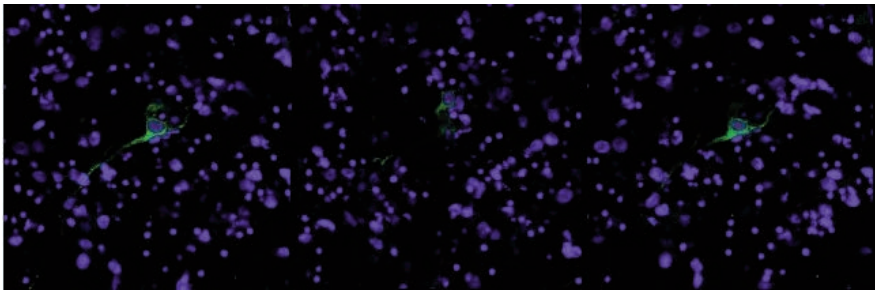

Figure 1. A clarified organoid at different optical sections.

Conclusions

We demonstrated that clarification protocols can be adapted to vessel-free 3D *in vitro* constructs. The confocal datasets obtained are characterized by an improved SNR and CNR, which can facilitate both 3D neuron segmentation and extraction of neuron morphometric features, thus obtaining an unprecedented representation of their 3D cellular structure. A rigorous workflow for establishing the best clearing practise as well as the optimization of the immunolabelling procedures for thick samples in terms of antibody concentration and staining times are on-going to avoid much of the trial and error usually affecting these methodologies.

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Application of two *in vitro* methods for the toxicity test of autogenous vaccines

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Abstract

According to the 3Rs principle (Replacement, Refinement, Reduction), this study aims to find alternative methods to evaluate the toxicity of autogenous vaccines. Currently in Italy the Istituti Zooprofilattici Sperimentali (II.ZZ.SS.) must perform the *in vivo* toxicity test for each lot of autogenous vaccine produced as laid down in the Decree of 17 March 1994. This paper describes two *in vitro* methods for assessing the toxicity of autogenous vaccines. The first is the MTT test based on the metabolic reaction of tetrazolium salt in vital cells. The second method is the test for measurement of IL-1ß production by macrophages, obtained after *in vitro* differentiation from pig monocytes in peripheral blood mononuclear cells. The two tests were performed on different vaccine antigens dilution: 1:20, 1:100 and 1:500. The results show a positive tendency between the two methods pointing out the potential of these methodologies combined for the replacement of the current *in vivo* test.

Introduction

Autogenous vaccines are immunizing products prepared against pathogenic microorganisms isolated from sick animals of one herd and only usable in the same farm. The Decree of 17 March 1994 in Italy regulates the production and control of autogenous veterinary vaccine. The legislation provides that the II.ZZ.SS. are the only agencies authorized by the Ministry of Health for the production of veterinary autogenous and autologous vaccines. Moreover, as required by the law, the II.ZZ.SS. must perform the *in vivo* toxicity test (mouse model) for each lot of vaccine produced.[1] According to the 3Rs principle, this study aims to find alternative methods to evaluate the vaccines toxicity replacing the animal model.

Materials and Methods

In this study, the MTT *in vitro* method was applied to evaluate the cytotoxicity of autogenous vaccine antigens. Moreover, an *in vitro* method was developed to measure the release of interleukin IL-1β by porcine macrophages in order to evaluate the potential *in vitro* inflammatory response triggered by the vaccine. The antigens analysed in the tests were inactivated with 0.8% formalin for 24 hours at 37°C and washed with saline solution, before the addition of the adjuvant.

The first method used is the cytotoxicity test MTT, based on the metabolic reaction of tetrazolium salt in vital cells. The MTT test is one of the most widely used, in accordance with the UNI EN ISO 10993-5 standard, concerning the biological evaluation of medical devices, which allows evaluating the safety and toxic potential of products.[2] For the test, the L-929 cell line was used, seeded at the concentration of $1.0x10^5$ cells/mL in 96-well plates in MEM culture medium added with 10% of foetal bovine serum (FBS), and incubated at + 37°C ± 1°C, 5% of CO_2. After an overnight incubation, the vaccine antigens were diluted and distributed 100 µL/well. After 24 hours of incubation, the cell monolayer was treated with MTT salt and the intensity of the colorimetric reaction was correlated to cell viability.

The second method performed is a test for the assessment of IL-1ß production by porcine macrophages in response to vaccine antigens. Interleukin-1ß (IL-1ß) is a pro-inflammatory cytokine mainly produced by activated macrophages and monocytes. The precursor (pro IL-1ß) is located at cytoplasmic level and must be cleaved by caspase-1 to generate the mature activated form.[3] Assessment of IL-1ß production by macrophages in response to vaccine antigens could help evaluate the safety and efficacy of the vaccine-induced immune response. In particular, macrophages were

obtained after *in vitro* differentiation from peripheral blood mononuclear cells (PBMC), using 10 ng/mL of Macrophage-Colony Stimulating Factor (M-CSF). Differentiated macrophages were reacted with the same antigens at different dilutions for 24 hours, followed by quantification of released IL-1ß by "Duo set ELISA for Porcine IL-1ß/IL-1F2" (R&D System).

Results

The results showed two crucial points to consider for the IL-1ß assessment *in vitro* test: i) the choice of the cell population, because macrophages with high basal reactivity tended to provide a *plateau* response; ii) the need to dilute the antigens because their high bacterial content caused an effect "endotoxin tolerance-like", that inhibits the release of IL-1ß as showed in Figure 1.[4]

Furthermore, the MTT test results showed that the samples are cytotoxic at lower dilutions, showing a cell viability ≥70% from at least 1:8. The maximum dilution, to which a cytotoxic effect was so far detected, looks to be 1:128.

Moreover, to assess the correlation between the two methods, the results of the MTT test at the dilutions 1:16 were trans-

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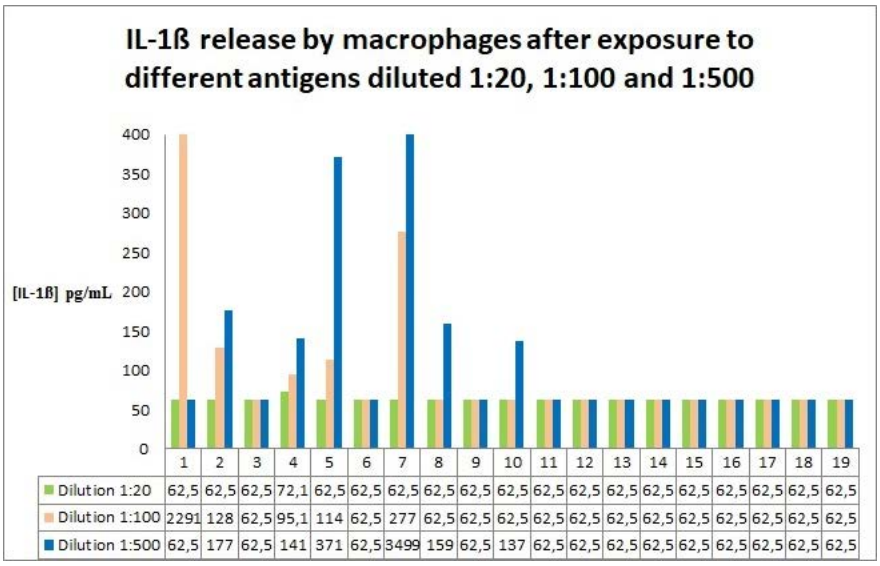

Figure 1. IL-1β release by macrophages after exposure to different antigens.

formed into a percentile value and were correlated via *GraphPad Prism software* with the concentrations of IL-1β detected at 1:500 dilutions of the antigens.

Conclusions

The statistical analysis, carried out using the Spearman coefficient, shows a positive tendency between the two methods (R=0.4955, P=0.03) pointing out the potential of these combined methodologies to examine different features of the immune/toxic response, that autogenous vaccine could trigger in the target animals.[5]

The study confirms the scientific value of the 3Rs and their applicative potentiality, especially when alternative *in vitro* methods are combined in integrated assay strategies to enhance, through multiple end-points, the effectiveness and predictive capacity of the models.

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Engineering a dynamic model of the alveolar interface for the study of aerosol deposition

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Abstract

Nano and micro particles are widely used in industrial, household and medicinal applications. To understand the interaction between particles and epithelial cells, we developed a dynamic model of the alveolar interface. This system, named DALI (Dynamic Model for the ALveolar Interface), is a modular bioreactor composed of two chambers divided by a porous membrane where epithelial lung cells are seeded. The membrane is the support of the alveolar barrier that separates the two compartments of the alveolus: the air and blood side. The system integrates the following elements: i) Air/Liquid interface, thanks to the two chambers divided by the membrane: ii) Cell culture media flow, thanks to the presence of a peristaltic pump; iii) Lung breathing motion, thanks to an airflow that allows the stretching of the membrane; iv) Aerosol deposition system, to study the effects of drug efficacy or particle toxicity on the epithelial layer; v) Quartz Crystal Microbalance, to quantify the amount of aerosolized particles.

Introduction

In order to improve the physiological relevance of the lung model and to investigate the deposition of aerosolised nanoparticles on the alveolar barrier, a bioreactor able to mimic breathing movements was designed. The system, named DALI (Dynamic model for ALveolar Interface), consists of a commercial aerosol generator, two bioreactors with a moving membrane placed between an air-liquid interface (Figure 1a), and a Quartz Crystal Microbalance (QCM) to measure the effective nanoparticle dose on the membrane (Figure 1c).

Materials and Methods

The bioreactor is composed of two polycarbonate cylindrical chambers (A and B in Figure 1b): the upper one for the air flow (height: 24 mm, diameter: 24 mm) and the bottom one for the medium flow (height: 20 mm, diameter: 24 mm). Between them there is the porous stretchable membrane fixed in a holder that consists of two annular magnets covered by PDMS (C in Figure 1b). The upper chamber is connected to an aerosol system for nanoparticles deposition (D in Figure 1b), and to a pressure regulator that ensures the cyclic stretching of the membrane. The electronics and the pressure regulators are placed in a control box. Potentiometers on the control box allow regulating the stretching level of the membrane for mimicking different stretching conditions.

The membrane was fabricated by electrospinning using a 1:1 (w/w) Bionate®:Gelatin solution. The Bionate® II 80A, a commercial poly(carbonate)urethane copolymer, was used to replicate the basement of the alveoli, as it guarantees membrane flexibility necessary to mimic the cyclic motion during the breathing. Additionally, in order to increase cell adhesion, gelatin was used in combination with Bionate® to obtain the final formulation for the membrane. Cell adhesion and biocompatibility were assessed by the Alamar Blue assay and cell staining with DAPI and rhodamine conjugated phalloidin.

A FEM model was used to simulate membrane displacement due to the pressure imbalance on the two sides of the air/liquid interface. The model was based on the Fluid Structure Interaction module of Comsol Multiphysics 4.3a software and consists of a cylindrical chamber connected to the external system with an inlet and an outlet tube (5 mm in diameter). The 80-µm thick membrane was modelled as a disk on the top of the bioreactor and undergoes a constant pressure from the top. The inlet velocity was fixed at 100 µL/min, and the fluid dynamics solved in the Laminar Flow regime. Bioreactor walls were set as walls with the no slip condition, and water chosen as a reference fluid. The FEM model was solved for different pressures (1 to 15 kPa with a step of 1 kPa), in order to establish the pressure at which the membrane displacement in z-direction is 7mm, corresponding to a linear distention of ≈ 17%, and so mimicking pathological levels of stretching.[1,2]

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Results

In the DALI, the membrane is placed between annular magnets covered by PDMS. During static experiments, the holder with the membrane can be placed both in a 6-well multiwell, or inserted between the top and bottom chambers. The bioreactor is closed tightening wing nuts and the tightness of the bioreactor is ensured by the presence of the membrane holder enclosed in PDMS, which is self-adhesive and deformable. The bioreactor can be sterilized by ethanol solution, gas plasma, or ultraviolet light.

During dynamic experiments, the basolateral chamber is connected to a commercial peristaltic pump with an inlet tube, and to the reservoir with an outlet tube (Figure 1). The apical chamber is connected to the aerosol device and to a compressed air system, with an interposed pressure regulator put inside a control box. Potentiometers on the control box allow regulating the stretching level of the membrane: <5%, 5-12%, 12-17% and >17% for mimicking different stretching conditions, both physiological and pathological.

A FEM model was used to simulate the velocity field of the fluid flowing through the bottom chamber, and to simulate the membrane displacement due to the pressure imbalance on the two sides of the air/liquid interface. When the pressure in the upper chamber increases, the membrane stretches, moving into the basolateral chamber, until it reaches an equilibrium with the hydrody-

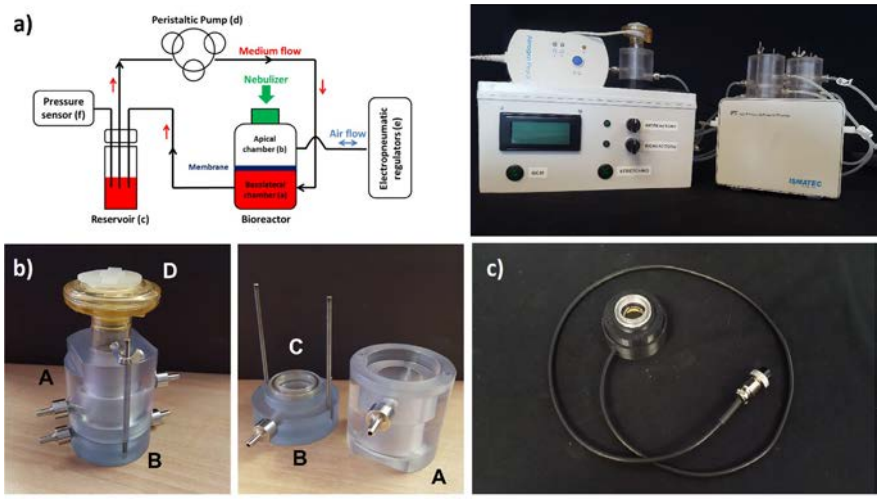

Figure 1. a) Schematic representation of the DALI system (on the left) and its picture (on the right); b) pictures of the component parts of the bioreactor; c) picture of the QCM device.

namic pressure of the flowing liquid. With the FEM model, it was possible to predict the pressure ranges that the external system must apply in order to achieve the desired stretching field on the membrane. The applied pressure corresponding to a z-displacement of almost 7 mm is 14 kPa. This displacement corresponds to a linear distention of ≈ 17%, and so mimicking pathological levels of stretching.[1,2]

Conclusions

To conclude, we present a bioreactor that is able to replicate the cyclic motion during the breathing. The flexible moving membrane causes the rhythmic stimulation of epithelial cells, leading to the study of the interaction between them and the particles, in a system that replicates *in vivo* conditions. The electrospun 1:1 (w/w) Bionate®:gelatin membrane has been selected as suitable membrane for our application, as it is biocompatible and highly flexible, allowing physiological deformation levels. This study, based on the 3R's Statement, paves the way towards the development of an actuation device for physiologically relevant studies of aerosol and drug delivery and toxicology.

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The 3Rs: Reduction and refinement through a multivariate statistical analysis approach in a behavioural study to unveil anxiolytic effects of natural extracts of *Tilia tomentosa*

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Abstract

We propose a multivariate statistical approach based on Principal Component Analysis (PCA) as an useful instrument to improve the Rules of Refinement and Reduction in *in vivo* animal experimentation. We analysed with PCA the preliminary data from a study on the effects of the oral administration of *Tilia tomentosa* bud extracts (TTBEs) on the behavioural skills of adult and aged male and female mice. PCA allows to rationalize the data set information and to dissect the results, showing connections among variables under study (behavioural parameters) and different trends in the experimental groups (control and TTBEs-administered animals). Our results show that PCA can give some important information that can be useful for the refinement of the experimental protocol, in order to reduce the number of the animals used in the experiments and/or the behavioural tests to get reliable information.

Introduction

The principles of the 3Rs (Reduction, Refinement and Replacement) are at the basis of an ethical use of animals in scientific research. Since many years, we have made efforts to set up our experimental protocols according to the ARRIVE guidelines and the 3Rs, adopting strategies such as minimizing the number of animals to get strong results and sharing tissues between research groups.

We here propose a multivariate statistical approach based on Principal Component Analysis (PCA) applied as a useful instrument to improve the rules of refinement and the reduction in *in vivo* animal experimentation.

Starting from 2018, our research group was involved in a project called FINNOVER (n° 1198), within the Interreg ALCOTRA Italy/France trans frontier call, dedicated to the study of the effects of an *in vivo* oral administration of bud derivatives in aging. The study was so far focused on investigating the impact of *Tilia tomentosa* bud extracts (TTBEs) on adult (3-6 months old) and aged (20-22 months old) male and female mice.

In 2015, Allio *et al*. investigated whether TTBEs affect hippocampal Gamma-aminobutyric acid (GABA)ergic synapses.[1] Their results demonstrated that a direct application of TTBEs on post-synaptic terminals can activate a chloride-mediated current that is blocked by bicucullin, picrotoxin and flumazenil. These data suggest that TTBEs can amplify the $GABA_A$ mediated signaling, mimicking GABA and benzodiazepines.

Based on the efficacy of TTBEs on $GABA_A$ receptors, we investigated the effects of the oral administration of TTBEs on the mice behavioural skills related to anxiety, curiosity and spontaneous motor activity in the hole-board and in the light-dark box tests, two experimental protocols useful for quantifying these behavioural parameters in animals.

In an attempt to underline changes in the behavioural responses due to the TTBEs administration, the data obtained from a preliminary set of experiments were first analyzed by using PCA. PCA, the most commonly used chemometric technique, is an unsupervised pattern recognition technique useful to rationalize the data set information. It allows to dissect the results, showing the connections among the variables under study (behavioural parameters) and the potential different trends in the experimental groups. We predicted that PCA can be useful for the refinement of the experimental protocol to rationalize the information of the data set obtained from each animal group and possibly to significantly reduce the number of the animals and/or of the behavioural tests to obtain reliable information.

Materials and Methods

Adult (3-6 months) and old (20-22 months) male and female mice were randomly assigned to three different groups: water-administered mice (control), vehicle-administered mice (EtOH /glycerin /H2O), TTBEs-administered mice (n=6 animals for each experimental group). In order to reduce stress in mice, TTBEs (1÷2000 dilution) were dissolved in the drinking water. Animals were under treatment for 14 days and monitored for the daily drinking volume intake and for the gain of weight. The behavioural tests were performed before and at the end of the treatment.

Data from the behavioural analysis were analyzed using PCA. PCA was performed by NIPALS algorithm[2] on male and female data matrix (6 rows and 5 columns) respectively, to quickly screen and rationalize the information of the experimental data. The variables under study were: n° of head dippings, % of explored area and % of entries into the centre (hole-board test); n° of transitions and time in light (light-dark box test). Autoscaling pre-treatment (column centering + column scaling) was performed in order to normalize the data, adapting the different measure units of variables. An R-based chemometric software developed by the Group of Chemometrics of the Italian Chemical Society (freely downloadable from gruppochemiometria.it /index.php/software, 2018) was used to perform the multivariate data analysis.

Results

The univariate analysis of the preliminary results from the first set of experiments showed that TTBEs affects curiosity, anxiety and the spontaneous motor activity in

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mice, depending on their age and gender. Differently, PCA of these preliminary results led to some important information helpful for the refinement of the experimental protocol.

In male mice, the PCA unveiled that young male mice diverged from the old ones on PC1 (which explains almost the 60% of the whole data set information) and that TTBEs treatment made old mice more similar to the young ones. Furthermore, both in old and in young male mice, the animals treated with TTBEs were separated from the others, while the water and vehicle treatments carried the same information on PC1 and PC2 (which together explain almost the 87% of the total information). This would indirectly suggest the possibility to eliminate one experimental group (the control or the vehicle-administered ones), so reducing the numbers of mice used in the experiments. Last but not least, on PC1 the time in the light and % of entries into the centre carried out the same information as the curiosity and the anxiety are concerned, compatible with the conclusion that just one behavioral test could be enough for highlighting the TTBEs effect.

In female mice, PC1 (which explains the 47% of the total information of the data set) separated young mice from the old ones. The latest group was characterized by a smaller number of entries into the centre when compared to young mice. Furthermore, in the group of young female mice, the ones treated with TTBEs were separated from the others, mainly on PC2 (which explains the 40% of the total information). Actually, TTBEs treatment increased the number of transitions in young female mice. However, differently from what highlighted in male mice, the vehicle influenced the behaviour in old female mice similarly to TTBEs, but differently from water.

Conclusions

The PCA analysis permits an overall view of the results from an experimental paradigm, highlighting the relations between objects (the animals) and variables (behavioural parameters) under study. This approach can be used to improve the *refinement* of the applied experimental protocol, by excluding variables that give the same information and so *reducing* the number of animals to be used within the tests.

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Health monitoring program for the control of laboratory animal diseases

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Abstract

Pathogens present in the environment are the biggest source of diseases and epidemics in the breeding of laboratory animals. In fact, the presence of microorganisms can critically influence the animal health status and, consequently, the validity and reproducibility of experimental data. In accordance with the 3Rs principle (Refinement, Reduction, Replacement), this study is part of the Refinement concept. The FELASA guidelines, formulated with the aim of guaranteeing the best animal health state, are a valid support for researchers. In this preliminary study, health-monitoring program was carried out within the breeding of laboratory animals in IZSLER facility. The main murine viruses were analyzed through molecular biology techniques (PCR, RT-PCR) and enzyme immunoassays (indirect ELISA). The established surveillance program steadily guarantees animal health and ensures the most controlled environmental and sanitary conditions. Further investigations will be needed to develop virus control strategies.

Introduction

Pathogens present in the environment play a critical role concerning the validity and the repoducibility of experimental data and animal welfare. The protection and care of laboratory animals is a bedrock in the field of health surveillance in the breeding, for these reasons and in accordance with the 3Rs principle,[1] this study is part of the Refinement concept, as by carrying out a health-monitoring program there is an improvement in animal health and environmental conditions, which are strictly dependent on the organizational and operational systems adopted in the facility. It is therefore important to establish a health surveillance program as part of a quality assurance system, with a key role in protecting animals. As mentioned in the Italian Legislative Decree 26/2014,[2] the implementation of a health-monitoring surveillance is essential and necessary as it supplies: daily microbiological surveillance, use of parameters and procedures for the introduction of new subjects and action plans in the event of overt health problems. Furthermore, guidelines provided by FELASA[3] suggest that the growing number of identified pathogens requires a constant adjustment of the diagnostic procedures in order to guarantee the optimal state of animal health. For these reasons, the National Reference Center for Alternative Methods, Welfare and Care of the Laboratory Animals of IZSLER in Brescia developed a health-monitoring program and diagnostic tools for the defense and the protection of laboratory mice.

Materials and Methods

In this preliminary study, a virus surveillance program was carried out within the breeding of laboratory animals in IZSLER facility. The main murine viruses analyzed were:

- *Polyoma virus of mice (POLY)*
- *Adenovirus type 1 (ADENO),*
- *Murine Hepatitis Virus (MHV),*
- *Murine Teilovirus (TMEV),*
- *Parvoviruses (Minute Virus of Mice, MVM and Mouse Parvovirus MPV),*
- *Pneumonia Virus of Mice (PVM),*
- *Ectromelia virus (ECTV),*
- *Polyoma virus of mice (PVM),*
- *Sendai virus (SENDAI),*
- *Reovirus type 3 (REO-3).*

Viruses were analyzed through molecular biology techniques and enzyme immunoassays (indirect ELISA). In particular, for molecular biology analysis, organs and faeces were used. Spleens, hearts, lungs, livers and kidneys were disrupted using a lysis buffer and an automatic homogenizer, while the faeces were processed with PBS and mixed with a stirrer. RNA and DNA were extracted by an automatic spin column system and PCR internal methods were performed with specific primer pair and probes for each virus.[4] The experimental conditions for RNA virus amplifications were as following: 1 cycle of reverse transcription at 50° C for 20 min, 1 cycle of initial denaturation at 95° C for 5 min, 40 cycles of denaturation (95° C for 15 sec) and annealing/extension (60° C for 45 sec). The same protocol, except for the reverse transcription, was used for the DNA viruses. The quantifications of the target sequences were analyzed through a *Real Time* thermal cycler.

Commercial kits were purchased in order to carry out the ELISA assay for the detection of Immunoglobulins G (IgG). The serum was incubated in an adsorbed plate with each viral antigen at 37° C for 45 min. The conjugate was added to the reaction plate and incubated at 37° C for 45 min. The substrate was distributed in the wells and placed in the dark for 30 min at room temperature. Using a spectrophotometer an absorbance (OD) at 405 nm was read and the data was processed in order to calculate the absorbance differential (ΔOD).

Results

A total of 42 samples were analyzed. In Figure 1 the main results are shown. From serological analysis it was observed that the mice have been in contact with MHV, highlighting the presence of the specific MHV IgG in 42% of samples, followed by ADENO (16%). The two Parvoviruses analyzed, MVM and MPV show 12% and 10% of prevalence, respectively, while REO-3, SENDAI, PVM and TMEV antibodies are present in less than 10% of the analyzed samples. Overlapping data were obtained from molecular biology tests, where in fact a

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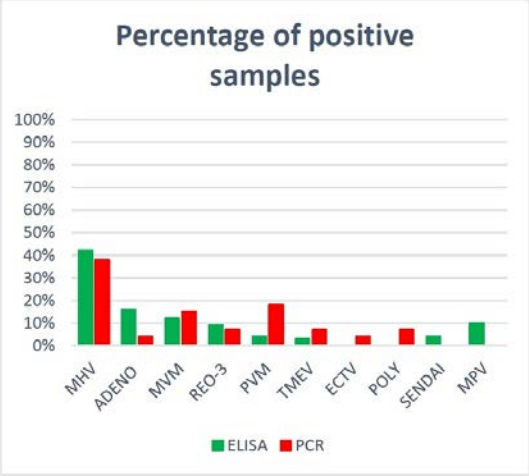

Figure 1. RT-PCR and ELISA assays results.

prevalence of MHV antigens is observed (38%). PVM is the second predominant virus in RT-PCR (18%), whereas the molecular detection of MVM and REO-3 replicates the ELISA assay test, showing 15% and 7% of predominance, respectively. The molecular analysis of ADENO and TMEV are in contrast with serological data (ADENO: 4% *vs* 16% and TMEV: 7% *vs* 3%). Finally, ECTV and POLY, whose antibodies were absent in the ELISA assay, show a prevalence of 4% and 7% of respective antigens in RT-PCR.

Conclusions

This preliminary work emphasizes the importance of welfare and care of laboratory animals in experimental research. The established surveillance program steadily guarantees animal health for the entire duration of the housing and ensures the most controlled environmental and sanitary conditions. More samples will be processed in order to perform a statistical analysis of the data obtained. However, further investigations will be needed for animal welfare monitoring and virus control strategies developing (*e.g.* quarantine).

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Zebrafish as an alternative method for toxicological studies

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Abstract

According to the Directive 2010/63/EU fish embryos do not fall into regulatory frameworks dealing with animal experimentation. Therefore, in compliance with the 3Rs principle, zebrafish embryos are considered as replacement or refinement methods. Since more and more industrial chemicals are recognized causes of skin sensitization, it is needed a thorough understanding of the toxic mechanisms of novel compounds. Thus, the FET test was performed and up to four apical observations were recorded as indicators of lethality: coagulation of fertilized eggs, lack of somite formation, no detachment of the tail bud from the yolk sac and lack of heartbeat. Then, in order to assess whether the skin sensitization due to chemical incubation was really measurable, the Fish Interleukin 8 (IL8) ELISA Kit was carried out. The preliminary results obtained so far seem encouraging. However, they need to be confirmed through further ELISA tests and compared with other *in vitro* methods.

Introduction

The assessment of chemicals for potential toxic effects on both human health and the environment generates a strong demand for robust and cost-effective assays with high predictive power. Fish models are commonly used in human and ecotoxicological research to investigate the impact of chemicals on whole organisms. In fact, many important biological functions are conserved between fish species and humans. Zebrafish embryos are currently used as vertebrate models providing an intermediate step between *in vitro* and rodent assays as regards for example ecotoxicological studies. It is needed a thorough understanding of the mechanisms to make predictions of the toxic potential of novel compounds. According to the European Directive 2010/63 and to the Decree n. 26 04/03/2014 in Italy,[1] the National Reference Center for Alternative Methods, Welfare and Care of the Laboratory Animals in Brescia has been developing an *in vitro* method using zebrafish embryos to evaluate the toxicity of some compounds recognized as skin sensitizers. The study conducted consists of a first phase to evaluate the sensitizing potential of different chemical solutions through the FET test (OECD 236: 2013);[2] which has been adapted to the needs of the aforementioned experimentation. This was followed by a second phase where Interleukin 8 (IL8) production was estimated, as there are studies showing that known skin sensitizers induce the production of proinflammatory cytokines and chemokines such as IL8.[3] Once the sublethal concentrations of the tested compounds were identified, the production of IL8 was measured.

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Key words: Zebrafish; skin sensitization; ecotoxicology.

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Materials and Methods

Zebrafish mating and eggs production

Fish selected for breeding were transferred to breeding tanks in the afternoon at a 2:1 M:F ratio. Zebrafish eggs were obtained from natural spawning of wild type animals in the early morning of the day after, following standard husbandry practices. After collecting embryos, these ones were placed in incubator at 28°C.

Dechorionation

To remove the chorion, barrier that would hinder the passage of substances, at 24 hours post-fertilization (hpf) embryos were dechorionated using a protease isolated from *Streptomyces griseus*: Pronase. Embryos were transferred to Petri dishes filled with Embryo Medium[4] and incubated with Pronase diluted at a final concentration of 0,5 mg/mL. After dechorionation, embryos were transferred to a new Petri dish with the test chemical solution for the FET test.

Chemicals

A careful and thorough review of the literature was necessary in order to identify the substances under investigation. The chemicals were selected for the availability of data on solubility (DMSO or saline) and on allergenic potency, such as 2,4-Dinitrochlorobenzene (DNCB), Lactic acid (LA) and Sodium lauryl sulphate (SLS).

Fish Embryo Toxicity Test

The experimental design involves the incubation of embryos with the aforementioned substances, the negative control represented by Embryo Medium and the solvent of the substances, of which a possible toxic effect must be excluded.

Zebrafish exposure

Dechorionated 24 hpf zebrafish were exposed to the test chemical for a period of 96 hours. Every 24 hours, several observations were recorded: lack of heartbeat as indicator of lethality, while malformations such as a pronounced yolk sac oedema, a pericardial oedema or also a spinal curvature (scoliosis) were considered developmental toxicity endpoints. At the end of the exposure period, acute toxicity was determined based on any positive outcome in one of the four apical observations, then the LC_{50} was calculated.

Tissue homogenization

For this assay, 100 zebrafish larvae were collected collected in a 1.5mL tube and homogenated: inside the Eppendorf tube was added a small bead and the instrument was setted for shaking at 30Hz for 5 minutes. After that, the sample was centrifuged again for 10 minutes at 5000g, then the supernatant was removed and centrifuged for 30 minutes at 20000g.

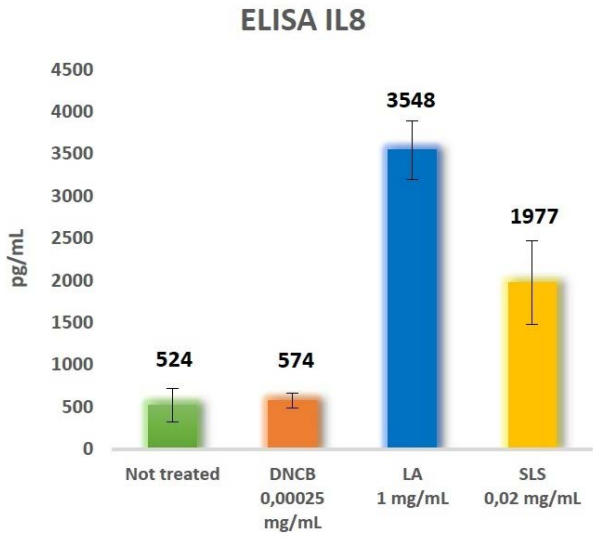

Figure 1. Quantitative evaluation of IL8 production after exposing zebrafish embryos at the highest concentration of DNCB, LA and SLS (embryos not treated were used as controls).

ELISA analysis

Through the ELISA kit (performed according to the manufacturer's directions), the IL8 was evaluated.

Results

From the LC_{50} values of each chemical agent, the substance that turned out to be the most toxic is the DNCB: 0,228 mg/L are indeed sufficient to determine the death of 50% of treated embryos. On the other hand, LA has proved to be the least toxic among those tested with an LC_{50} value of 1,172 mg/mL. As regards Sodium lauryl sulphate, its LC_{50} value observed was 0,041 mg/mL.

Once the range of concentrations in which there is 100% survival was identified, IL8 production, was measured. The results relating to the production of IL8 do not support, at the moment, a response in zebrafish larvae specifically correlated with the exposure to a sensitizing or non-sensitizing chemical. The quantitative evaluation of IL8 production was carried out by exposing the embryos for a period of 96 hours at the highest sublethal concentration of each compound (DNCB, LA and SLS) as shown in Figure 1.

Conclusions

From the preliminary results obtained, the FET test proved to be an excellent tool to evaluate the toxicity of selected sensitizing substances and to analyze phenotypes that can be correlated with them. For this purpose, the test with chemicals already in use will be implemented and the range of chemical compounds to be evaluated will also be expanded. Moreover, this experimental model is extremely advantageous in the toxicological field thanks to the high fertility of the females: it was possible, indeed, to have a very large number of experimental units and to treat and analyze many embryos simultaneously. Further investigations are needed to clarify whether the mechanism of production of IL8 in zebrafish is correlated with the allergenic potency of a substance and also to obtain a complete framework to possibly widely apply this model in larger screening.

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Assessment of an *in vitro* physiological relevant model to check therapeutic strategies for glaucoma

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Abstract

Glaucoma is a chronic, progressive and heterogeneous optic neuropathy which affects in the early stage the peripheral vision and then the central vision, leading to irreversible blindness. As known, in glaucoma the trabecular meshwork represent the main tissue which is impaired by chronic oxidative stress, aging and increase of intraocular pressure. Today, the lack of human-based models, with characteristics of high repeatability and reproducibility as well, called for an high-quality *in vitro* model with a good degree of resemblance for the tissue or organ of interest as a basis for new drug testing. Our team has been committed to this purpose by assessment of an *in vitro* 3D TM human-based model, closer to *in vivo*, using millifluidic technology, to better identify the key events underlying the pathogenesis of glaucoma and to evaluate new therapies targeted at disease treatment and prevention.

Introduction

Glaucoma is the second cause of blindness in the world affecting over 67 million people worldwide.[1] As known, the main causes of glaucoma onset are oxidative stress and vascular alteration which impaired Trabecular meshwork activities. The oxidative damage is an important step in pathogenesis of Primary Open Angle Glaucoma and might be a relevant target for both prevention and therapy.[2]

Therefore, the aim of this study was to develop an *in vitro* 3D human-based dynamic model of trabecular meshwork to define the key elements relating to the glaucoma onset.

Materials and Methods

3D cultures of Human Trabecular Meshwork Cells (HTMC, Cell APPLICATION INC). Were made by embedding HTMCs into 100% Corning Matrigel™ Matrix and were maintained in a millifluidic bioreactor system connected to the peristaltic pump (Live Box 1 and Live Flow, IV-Tech srl) with constant flow rate.[3] To simulate chronic stress conditions 3D-HTMCcultures were exposed to H_2O_2 treatment (500 µM) for 2 hours followed by 22 hours of recovery, until 15 days.

Results

Confocal imaging analysis and Alamar blue assay,[4] as index of proliferation/metabolic state of cultures, evidenced a good healthy state of HTMCs. Moreover, in our dynamic model an efficient response to stress was shown, since it was observed a NF-kB and TNF-α activation. To evaluate the feasibility of our dynamic HTMC 3D-model as a useful tool for evaluate therapeutic strategies for glaucoma disease, we analyzed the effects of a polyphenol mixture (PM), an active compound of a commercial eye drops for glaucoma. For this purpose, we studied the biological property of PM in counteracting chronic oxidative stress on 3D HTMCs. Preliminary qPCR analysis showed a modulation of gene levels of collagens and other ECM glycoproteins.

Taking into account these findings, our dynamic 3D-HTMC model can provide useful information on new prevention and therapeutic strategies for glaucoma.

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Key words: Glaucoma; *in vitro* model; oxidative stress; toxicology.

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SG-2: A promising lipolytic and pro-autophagic hit-compound to treat Alzheimer's disease

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Abstract

The identification of efficient pharmacological tools for treatment of Alzheimer's disease (AD) represents one of the main challenges of our century. Due to the complex etiopathology and the several biological processes resulting impaired in AD, the drug discovery process should focus on the development of new chemical entities able to target this multi-faceted impairment. We designed and synthetized a new analogue of 3-iodothyronamine, namely SG-2, which shares an interesting pleiotropic activity. Within this study, we explored SG-2 ability to promote beneficial effects in a C. Elegans model of AD, using a novel technique developed at Cambridge University, which exploits an automated system of high-resolution cameras to evaluate in parallel the motility of a huge number of nematodes (up to 5000 at time) in response to drug administration. Our results showed that SG-2 can promote lifespan and restores motility of worms back to the wildtype.

Introduction

Alzheimer's disease (AD) is a progressive pathological condition which affects multiple brain functions and several physiological pathways such as lipid and glucose metabolism, proteins phosphorylation and autophagic flux. This multi-faceted impairment leads to an aberrant protein aggregation and uncontrolled neuronal cell death, resulting in the well-known decline of cognitive functions. Today, there is a worldwide effort to find better ways to treat AD, delay its onset, and/or prevent it from developing. In this context, the improvement in up-to-date approaches and techniques to investigate new agents capable of interfering with AD progression still represents an urgent entail to be solved. Recently, we have designed and characterized a new class of synthetic small molecules bearing a biphenylmethane scaffold, namely SG compounds, to target the multi-faceted impairment which characterizes AD.[1,2] Among them, SG-2 was identified as a promising hit-compound able to promote a rebalancing of autophagic flux, endowed of neuroprotective effects, and able to induce a metabolic reprogramming to favor lipid consumption.[3]

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Key words: Autophagy; neuroprotection; Alzheimer's Disease; lipolytic activity; C. Elegans.

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Materials and Methods

To assess SG-2 potential in contrasting the progression of AD conditions, we tested it using a novel technique developed at Cambridge University which exploits an automated system of high-resolution cameras to evaluate in parallel the motility of a huge number of nematodes (up to 5000 at time) in response to drug administration. [4]

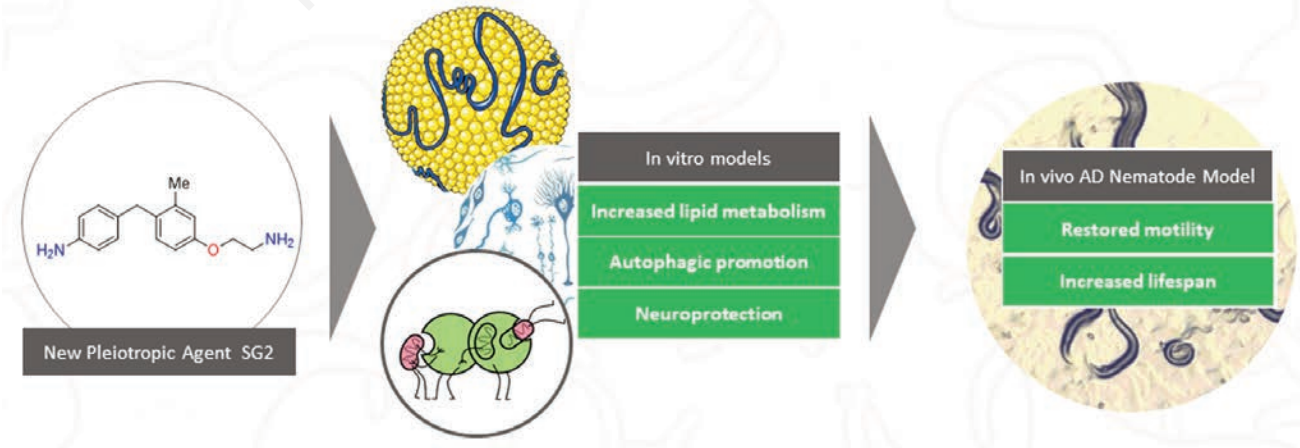

Figure 1. Effect of SG-2 in *in vitro* models and *in vivo* AD Neumatode model.

Results

Our results showed that SG-2 can alter the decline of the morbidity of AD restoring nematode's motility back to the wild type when administrated to C. Elegans at a concentration of 1μM. Moreover, we observed an enhancement of nematodes' lifespan when worms were treated with SG2 at the 4th day of life, *i.e.* when Aβ plaques are already formed. Surprisingly, no direct effect on Aβ formation has been observed *in vitro*. This result let us to speculate that the ability of SG2 to promote autophagy and induce lipid metabolism could represent a new strategy to delay or halt the progression of AD.

Conclusions

We identified a novel lipolytic and pro-autophagic hit-compound able to promote beneficial effects in several AD models. Future studies are planned to outline the specific mechanism of action of this pleiotropic agent in order to validate the potential of SG2 as novel therapeutic tool for treatment of AD.

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In vitro models of human cardiac fibrotic tissue on 'bioartificial' scaffolds

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Abstract

Cardiac infarction is a global burden worldwide that leads to fibrotic and not contractile myocardial tissue. In this work, *in vitro* models of infarcted tissue were developed as tools to test novel therapies for cardiac regeneration in the future. The models of fibrotic heart have been designed and fabricated by culturing human cardiac fibroblasts on bioartificial scaffolds, based a combination of a synthetic and a natural polymer, and having aligned or random morphology, as to mimic structural and chemical features of infarcted cardiac tissue. Early findings from *in vitro* cell tests were reported, showing an enhancement of cell attachment and proliferation in the presence of the natural polymer.

Introduction

Heart failure is a global pathological condition affecting approximately 26 million people worldwide.[1] Myocardial infarction causes the death of billions of cardiomyocytes followed by the progressive formation of a fibrotic scar mainly populated by cardiac fibroblasts. Fibrotic tissue is mechanically stiffer than healthy cardiac tissue and unable to undergo contraction.[2] *In vitro* models of infarcted tissue represent a key tool to evaluate new therapies for cardiac regeneration. In this work, a model of fibrotic heart was designed and fabricated by culturing human cardiac fibroblasts (HCFs) on bioartificial scaffolds with aligned or random morphology, mimicking structural and chemical features of infarcted cardiac tissue.

Materials and Methods

Synthetic polymer scaffolds were prepared with both aligned and random morphology by two techniques: i) Solution electrospinning (2D scaffolds); ii) Melt-extrusion additive manufacturing (3D scaffolds).

Scaffolds were surface functionalised with an adhesive protein. HCFs isolated from human ventricle were cultured onto the scaffolds. Their survival, adhesion, proliferation and morphology were studied by biochemical assays and fluorescence microscopy. HCF morphology was investigated as a function of scaffold structure and surface composition.

Results

SEM analysis demonstrated that 2D scaffolds consisted of homogeneous nanofibers without defects, while 3D scaffolds showed regular and interconnected porous structure.

Protein surface functionalisation increased wettability (measured by static contact angle measurements) and affected surface chemical composition (assessed by FTIR analysis). HCFs cultured on functionalized scaffolds showed superior attachment and proliferation compared to non-functionalized scaffolds.

Conclusions

Bioartificial scaffolds able to support the viability and proliferation of HCFs were developed and proposed as models of human cardiac fibrotic tissue. In the future, the effect of scaffold bulk and surface properties on the expression of fibroblast markers and deposited ECM will be evaluated. The study will allow the modelling of different degrees of human cardiac fibrosis by specific constructs, which will be useful for the *in vitro* testing of advanced therapies.

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Reconstituted epithelial tissues and native cornea: A comparison of the influence of surfactants on ocular permeability

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Abstract

The aim of this study was to prepare an artificial rabbit corneal epithelium (RRCE) to compare with a human corneal epithelial model and excised rabbit cornea through permeation studies to investigate differences of surfactants influence on ocular permeability of a lipophilic compound. First solubility assays with different surfactants were performed and the integrity of the RRCE was assessed by measuring transepithelial electrical resistance (TEER). The permeation parameters showed that the RRCE was more sensitive than native cornea and human cornal epithelial model to the effect of permeation enhancers.

Introduction

The purpose of this study was the evaluation of the suitability of reconstituted corneal epithelia as *in vitro* model for prediction of influence of different surfactants in ocular drug permeation. The tissues employed for the permeation studies were a homemade reconstituted Rabbit Corneal Epithelium (RRCE) and a human corneal epithelial model (COR-100 EpiCorneal™, MatTek), while the excised rabbit cornea was taken for comparison. For the permeations studies an experimental lipophilic compound (MAGL17b) with potential antiglaucoma activity was employed.

Materials and Methods

Tween® 20, Triton® X-100, Tween® 80 (T80) (Mark, Germany), Kolliphor® P188, Kolliphor® P407, Cremophor® EL (C-EL), Kolliphor® RH40 (K-RH40) (BASF, Germany), Briij 78 (Fluka, Switzerland), MAGL17b (newly synthesized compound), Rabbit Corneal Epithelial (RCE) cell line (ECACC, n95081046), human corneal epithelial model (COR-100 EpiCorneal™, MatTek).

First, solubility assay of MAGL17b in water added of different surfactant was done. The three better solutions and the suspension of drug without surfactants were employed to verify the drug permeability through different substrates: excised rabbit cornea, Reconstituted Rabbit Corneal Epithelium (RRCE) and COR-100. For the study, the tissues were accommodated in perfusion apparatus with a donor and receiving compartment. The samples of receiving phase withdrawn during the permeation studies were analysed with HPLC. The integrity of epithelial models was assessed by measuring the trans-epithelial electrical resistance (TEER) before and after the permeation experiments.

Results

The drug solubility in water was only 3 µg/mL for this reason different surfactant: Kolliphor ® P188, P407 and RH-40, Tween® 20 and 80, Triton® X-100, Briij 78 and C-EL were employed to solubilize MAGL17b.

A complete solubilisation of 0.5 mM MAGL17b (172 µg/mL) was obtained only with the surfactants: T80, K-RH40 and C-EL. In preliminary permeation studies the formulation with K-RH40 showed a poor permeability of MAGL17b, for this reason in the subsequent studies only the formulations T80 3%, C-EL 8% and C-EL 1% were employed. The permeation studies performed through the excised rabbit cornea showed a lower permeability of the lipophilic drug in suspension respect with the epithelial models, this can be explained to the presence of the stroma, a very hydrophilic compartment that contrast the passage of the lipophilic drug. In the permeation studies through the excised cornea all the formulations showed a similar permeability with P_{app} increased in presence of surfactants (Table 1). In that performed through the two epithelial models, different results with addiction of surfactants were obtained showing that RRCE is more sensitive to their action: the higher concentrations of surfactant were able to alter RRCE barrier properties. Therefore, the surfactants were able to influence the permeability through the epithelial layers altering their tight junctions. In every case, a decrease of the TEER values measured before and after permeation experiments was evident indicating some degree of suffering of the epithelial tissues caused by the test conditions. However, the results have also shown that the formulation based on C-EL8% caused a decrease of TEER in both epithelial tissues and an opacity in the excised cornea at the end of experiment indicating a higher toxicity of the surfactant at this concentration.

Conclusions

The results obtained showed that the homemade RRCE was suitable for testing some ocular permeation enhancers even if the toxic effects produced to the higher concentrations have to be considered. Moreover, the presence of a stroma equivalent might produce a barrier closer to the native cornea.

Table 1. Permeation parameters obtained for RRCE, COR-100 and rabbit excised cornea.

	APPARENT PERMEABILITY (P_{app}) X 10^2 (cm/h ± S.E.)		
	RRCE	COR-100	CORNEA
T80 3%	8.407 ± 0.6198	0.3301 ± 0.0038	0.420 ± 0.0655
C-EL 8%	11.660 ± 0.8956	0.347 ± 0.0234	0.279 ± 0.0932
C-EL 1%	1.285 ± 0.1694	0.650 ± 0.0174	0.377 ± 0.0081
SUSPENSION	0.351 ± 0.0185	0.190 ± 0.0092	0.083 ± 0.0745

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