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Parameter study of shipping conditions for the ready-to-use application of a 3D human hemicornea construct in drug absorption studies

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ABSTRACT

In this study, a shipping protocol for our 3D human hemicornea (HC) construct should be developed to provide quality-maintaining shipping conditions and to allow its ready-to-use application in drug absorption studies. First, the effects of single and multiple parameters, such as the type of shipping container, storage temperature and CO_2 supply, were investigated under controlled laboratory conditions by assessing cell viability via MTT dye reaction and epithelial barrier properties via transepithelial electrical resistance (TEER) measurements. These investigations showed that TEER is more susceptible to shipping parameters than cell viability. Furthermore, the results were used to determine the optimal shipping conditions and critical values for subsequent overnight, real-time shipping experiments. Epithelial barrier properties were then investigated via TEER and the permeation of sodium fluorescein for shipped and not shipped HC. The results underscore that acceleration forces and changes in position may have a great impact on the epithelial barrier of 3D models. Low acceleration values and short changes in position caused only minor impairments. However, combined or intensive separate effects resulted in considerably low yields after shipping. Consequently, barrier-maintaining shipping of 3D in vitro models seems to be challenging, as mechanical forces have to be reduced to a minimum.

1. Introduction

The process of drug development is both time and cost intensive. Therefore, reliable preclinical test systems are needed to reduce the time, costs and, moreover, the risk of late stage drug failures. Animal experiments and ex vivo test systems are still frequently used in the early phases of the development process, but they fail to emulate human physiology and may cause misleading results (Beißner et al., 2016; Hornof et al., 2005; Reichl, 2008). Consequently, on one hand, reliable in vitro alternatives using human cells and simulating three-dimensional (3D) in vivo-like cell environments are needed. On the other hand, scientists in research and development (R&D) should more easily benefit from existing, well-characterized 3D in vitro models.

As human cornea is an important barrier for safety and efficacy evaluations of ophthalmic drugs (Becker et al., 2008), we have already developed a corneal 3D in vitro system. Our serum-free human hemicornea (HC) construct (Hahne and Reichl, 2011), which has shown barrier characteristics similar to animal corneas, but with improved reproducibility and lower variability, was subsequently prevalidated as an in vitro model for testing transcorneal drug absorption (Hahne et al., 2012). In this prevalidation study, the cultivation protocol was successfully transferred to two other cell culture laboratories to extend the application of our model. However, the project demonstrated that the interlaboratory transfer of methods is costly and time-consuming. Furthermore, the need for extensive knowledge and elaborate equipment for cell cultivation restricts the widespread application of in vitro models, especially for 3D constructs, which are increasingly used (Colom et al., 2014). Therefore, controlled shipping methods would highly improve their applicability as well as their availability.

Unfortunately, the shipping of cells is a scarcely investigated or published topic (Kaddis et al., 2013; Rainov et al., 2000). The most investigations can be found for the shipment of human pancreatic islets for transplantation purposes (Kaddis et al., 2013; Kim et al., 2013; Rozak et al., 2008) and the shipping of blood cells (Celik and Bayazit, 2011; George et al., 1996; Olson et al., 2011; Wagner et al., 2008). Resulting from their intended application, these investigations concentrate on cell characteristics, such as cell number and cell viability, but do not consider cellular barrier integrity. However, for drug absorption models, this aspect is highly important. Even though several commercially shipped 3D in vitro models are available (MatTek®,

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Skinethic®), to our knowledge, neither a general investigation of cellular barrier characteristics during transport nor an investigation of the effects of separated or combined shipping parameters on 3D in vitro models has been performed to date. These investigations might not be required for their common application as toxicity test models, but they are highly important for drug absorption studies.

For this reason, we studied the effect of separated and combined influencing parameters during shipping on cell viability and barrier integrity of our HC model. First, a highly controllable in-house test series was performed to separately evaluate the effects of different parameters, such as shipping container, temperature and CO_2 supply. These results were used to set critical shipping parameters and optimal packaging conditions for a standardized, quality-maintaining shipping protocol. The applicability and value of the protocol were tested by subsequent highly controllable in-house multiple parameter studies as well as real-time shipping experiments in which the models were sent to our cooperation partner from the prevalidation study (Hahne et al., 2012). To determine whether the critical parameters were fulfilled during shipping, the inner temperature and the acceleration on all three axes were logged automatically and then correlated with the obtained in vitro data.

2. Materials and methods

2.1. Materials

Transwell® inserts (art. no. 3402) and cell culture flasks were obtained from Corning Costar (Kennebunk, Maine, US) and Sarstedt (Nümbrecht, Germany), respectively. Keratinocyte Growth Medium (KGM) was generated by the addition of SingleQuots® to Keratinocyte Basal Medium (KBM), which both were provided by Lonza (Basel, Switzerland). KCl and $MgSO_4 \times 7\ H_2O$ were purchased from Acros Organics (Geel, Belgium). Phosphate Buffered Saline (PBS) tablets and EDTA disodium salt solution were acquired from MP Biomedicals (Santa Ana, California, US). Trypsin-EDTA, trypsin inhibitor and NUNC™ sealing tape (art. no. 236366) were bought from Thermo Fisher Scientific (Waltham, Massachusetts, US). Acetic acid, ascorbic acid, agar, $CaCl_2 \times 2\ H_2O$, D-glucose monohydrate, HEPES, NaCl, $NaHCO_3$ and sodium dodecyl sulfate (SDS) were purchased from Carl Roth (Karlsruhe, Germany). L-Glutamine and $NaH_2PO_4 \times H_2O$ were provided by Merck (Darmstadt, Germany). Sodium fluorescein, hydrochloric acid, 10fold MEM and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (Munich, Germany). Hard paraffin (paraffinum solidum) was purchased from Caelo (Hilden, Germany). PCR mycoplasma test kit for routinely mycoplasma screening was bought from Promocell (Heidelberg, Germany). The collagen for the three-dimensional gel was extracted from rat tail following a standard protocol. Krebs-Ringer buffer (KRB) contained 6.8 g NaCl, 0.4 g KCl, 0.14 g $NaH_2PO_4 \times H_2O$, 2.1 g $NaHCO_3$, 3.575 g HEPES, 1.1 g D-glucose monohydrate, 0.2 g $MgSO_4 \times 7\ H_2O$ and 0.26 g $CaCl_2 \times 2\ H_2O$ in 1000 mL double-distilled water.

2.2. Cultivation of human corneal epithelial (HCE-T) cells

The HCE-T cell line was obtained from a 49-year-old woman with subsequent immortalization by SV40-adenovirus vector transfection. This transfection and the following characterization were performed by Araki-Sasaki et al. (Araki-Sasaki et al., 1995). The HCE-T cells for our study were provided from the RIKEN cell bank (Tsukuba, Japan) and cultured with KGM at 37 °C and 5% CO_2 in a humidified atmosphere.

2.3. Cultivation of human corneal keratocytes (HCK) cells

The HCK cell line originated from human corneal keratocytes and was also immortalized by SV40-adenovirus transfection (Zorn-Kruppa et al., 2004, 2005). For the cultivation of our HC, the HCK cells were cultured with KGM under the same conditions as the HCE-T cells.

2.4. Cultivation of human hemicornea (HC) construct

Our HC was constructed on permeable polycarbonate Transwell® filters and cultured under serum-free conditions using KGM as described previously (Hahne et al., 2012; Hahne and Reichl, 2011). First, HCK cells were incorporated in a collagen gel and aliquoted to the cell culture inserts. After a gelling time of 60 min, a suspension of HCE-T cells was seeded on top of the gel. Subsequently, the HC was cultured under submersed conditions for seven days, and it was then lifted to an air-liquid interface (ALI) to obtain a multilayered epithelium. An in vivo-like morphology and optimal barrier properties developed after three days under ALI conditions (Hahne and Reichl, 2011). For this reason, day 10 of cultivation was the established day of application for our HC. Consequently, shipping from day 9 to day 10 was envisaged for its ready-to-use application. For the evaluation of different parameters that could affect cell viability and cellular barrier integrity during shipping, the model was exposed to separate and combined influencing factors during single parameter studies and multiple parameter studies, respectively. Finally, the HC was investigated under real-time effects by overnight shipping to our cooperation partner.

2.5. Single parameter studies

Prior to complex real-time shipping, single parameter studies were performed to separately investigate different influencing factors, such as the shipping container, temperature and CO_2 supply, under highly controlled laboratory conditions.

2.5.1. Shipping container

Two different shipping containers were tested as primary packaging materials. Classic 12-well plates from Corning Costar were investigated as well as self-designed and self-produced polyamide containers for single inserts (see Fig. 1). The latter should allow tighter sealing and should thus ease submersed shipping without leakage. To eliminate the effect of other environmental conditions, well plates and polyamide containers were compared under three conditions (37 °C + 5% CO_2, 26 °C + 5% CO_2 and 26 °C without CO_2). The results were analyzed for every condition as well as averaged over all groups.

2.5.2. Temperature and CO_2 supply

As the models are normally kept under optimal conditions regarding temperature and CO_2, changes may influence cell viability and TEER. Since temperatures in Europe are usually below 37 °C, we investigated the standard value for cultivation (37 °C) and lower temperatures. The samples were stored in a cell culture incubator (HERAcell® 150i, Thermo Fisher Scientific, Waltham, Massachusetts, US) at 37 °C and 26 °C with 5% CO_2 supply. The temperature of 26 °C was selected as the

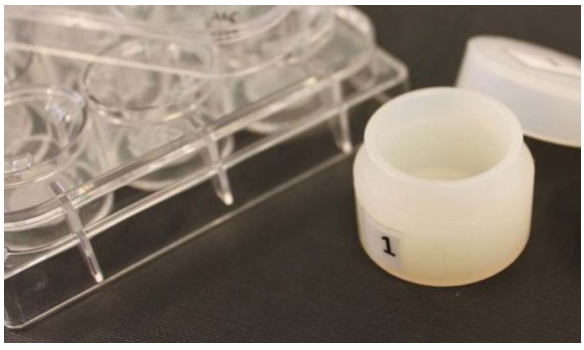

Fig. 1. Different shipping containers; left: 12-well plate from Corning Costar; right: self-designed polyamide container.