DEVELOPMENT OF IN VITRO SCREENING SYSTEM FOR FOOD HABIT RELATED RISK ANALYSIS

Mandy Bruch* and Elmar Mohr

University of Rostock, Faculty of Agricultural and Environmental Sciences, Chair of Animal Health and Animal Welfare, Justus-von-Liebig-Weg 6b, 18059 Rostock

Received – April 24, 2015; Revision – May 13, 2015; Accepted – August 31, 2015
Available Online – September 02, 2015

DOI: http://dx.doi.org/10.18006/2015.3(4).384.393

KEYWORDS
Using chamber
IPEC-J2 cells
Porcine intestine
Papain
Risk assessment

ABSTRACT

In order to assess the health risk that associated with the consumption of unknown feed or food ingredients, there is a strong need of developing an in vitro screening system. The test system should be fast, reliable, inexpensive and without the necessity of performing animal tests. Furthermore, it should also provide important clues to the potential danger of unknown substances. The present study examines the extent to which cell and tissue cultures can be used for such studies. It should be ascertained whether the cell cultures can replace the native intestinal epithelium in terms of their sensitivity and provide accurate results as a quick "screening system". As a model for intestinal operations ex vivo tissue cultures from the native intestinal epithelium of the pig and the permanent cell line IPEC-J2 were used. The cell culture was characterized in terms of their morphological and functional properties (TEER, tight-junction proteins). Various studies (short-circuit measurements, translocation of [H]-mannitol) were performed to IPEC-J2 cells and the native intestinal epithelium in order to compare the functional properties of both systems. Finally, the response of the addition of "unknown" test substances (papain and wheat extract) were investigated to determine whether the functional parameters of both systems are affected by these test substances or not. The IPEC-J2 cells show a more significant influence in their functionality by "unknown" substances than the control variant. Results of study revealed that the in vitro system reacts rapidly in response of unknown test substances and it is more sensitive. Therefore, it is possible to operate a "risk assessment" for "unknown" substances with the help of this developed screening system.

* Corresponding author
E-mail: mandy.bruch@uni-rostock.de (Mandy Bruch)

Peer review under responsibility of Journal of Experimental Biology and Agricultural Sciences.

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1 Introduction

Unknown ingredients of food and feed stuffs may pose a health risk for human and animal when consuming (Krul et al., 2000; Schmidt et al., 2011). Studies which should elucidate constructive the possible effect of these substances on an organism, often are based on animal experiments (Glaeser & Fromm, 2008; Cehak et al., 2013; Yan et al., 2013). Feeding trials with genetically modified plants (Reuter & Aulrich, 2003) or food additives (Lodemann et al., 2013) are expensive and potentially under ethically controversial agenda, because the animals are killed after food intake in order to examine the tissue and the blood. Problem related to the comparability of the results in potentially inhomogeneous test material like animals are also associated with these types of studies. For these reasons, it is feasible to support or partially replace these studies by in vitro systems? Cell and tissue cultures have long been used in medical and pharmaceutical research. Effect of drugs, macromolecules and other substances on cell absorption and transportation properties are already being studied in cell cultures (Artursson & Karlsson, 1991; Uil et al., 1997; Neumann et al., 2004; Weng et al., 2005; Grunwald et al., 2006; Cardinali et al., 2013; Jarmolowska et al., 2013).

The intestinal epithelium has been used especially for the absorption of ions (Frömter & Diamond, 1972; Frings et al., 1999), macromolecules (Warshaw et al., 1971; Bruch et al., 2008) and probiotics (Johnson et al., 2010; Lodemann et al., 2006). The ussing technique is an accepted procedure for the use of tissues and cell cultures for transport studies (Bajka et al., 2003; Rozenhal et al., 2012; Song et al., 2013). The native porcine intestinal epithelium is an established model for studies of the transport of ions and high molecular weight substances (Herrmann et al., 2012; Miyake et al., 2013). The permanent cell line IPEC-J2 is characterized and considered a suitable model for the jejunum of pig (Schierack et al., 2006; Mariani et al., 2009; Geens & Niewold, 2011; Brosnaham & Brown 2012). The combination of these already known methods of investigation can be used to estimate the possible occurring risk of an unknown substance. A rapid and easily performing screening system could be based on a cell or tissue culture giving first indications of a potential hazard. With the development of such an in vitro test system the normally required animal testing’s could be reduced and complemented by a low-cost alternative method. The present study examined the extent to which appropriate cell and tissue cultures can be used for the detection of potentially hazardous substances.

Furthermore, suitable parameters in order to detect the effect of harmful substances in an in vitro system, reactivity of cell culture and ex vivo tissue cultures (native intestinal epithelium), differences in sensitivity between cell and tissue-culture, suitability of cell and tissue-cultures, substance dependent differences between these two and the accuracy of cell culture screening system were also tested in the present study.

2 Materials and Methods

The studies used as a model for intestinal operations ex vivo tissue cultures of native intestinal epithelium of the pig (slaughterhouse material) and IPEC-J2 cell cultures. For these two systems morphological and functional parameters were determined before study. The morphological characteristics were studied by histological and intravital-microscopic analysis. Additionally the molecular biological detection of tight-junction proteins was occurred. The passive functional properties were characterized by the diffusion of tritium-labeled Mannitol (HP). As a measure of active transport properties of cell culture monolayers and the native epithelium, the response to the addition of sodium and the transport of essential amino acids was used (Rhoaads et al. 1994; Zhang et al. 2014).

In order to investigate to extend at which these system have the ability to transporting gradients with high molecular mass, GFP (green fluorescent protein) was used (Bruch et al. 2008). Papain and wheat extract, which consisted of the variety Greina, in the wild-type variant (isogenic) and in the genetically modified variant (transgenic) were used to determine the effect of an “unknown” substance on the above parameters. The statistical analysis was performed using Excel 2010 and Sigma Plot 11th. Statistical analysis of data was carried out using standard analysis of variance. The significance was determined using the F-test, least significant difference (LSD) was computed at the 5% probability level. Significant differences have been represented by different letters.

2.1 Native epithelium

The native epithelium (tissue culture) was recovered from the ileum of pigs of the breed German Landrace (male, castrated, age on average 180 days). After commercial slaughter, the tissue was removed, placed in ice-cold buffer containing indomethacin (2.8 nM) and gassed with carbogen (a mixture of 5% carbon gas and 95% oxygen gas). This part of the gut was chosen because of the occurrence of jejunal transporters in the ileum as well as M-cells mediated transport mechanisms in Peyser’s patches.

2.2 Cell line and Culture Conditions

To compare the results obtained by native epithelium to cell line, commercially available IPI-21 cells from the ileum of the pig would be the best choice. Unfortunately, they do not grow on transwell-plates so transport studies in Ussing chamber experiments are not possible. To work around this problem IPEC-J2 cells were used. The IPEC-J2 cell line is a non-transformed intestinal cell line originally derived from jejunal epithelia isolated from a neonatal, unsuckled piglet and maintained as a continuous culture (Berschneider, 1989). Cells were purchased from FLI (Federal Research Institute for Animal Health, Germany). Unless otherwise indicated, cells
were cultured on Dulbecco’s modified eagle medium (DMEM)/HAM’s F-12 (1:1) supplemented with 10% fetal calf serum (Biochrom, Germany) and maintained in an atmosphere of 5% CO₂ at 37°C. Cells reached confluence after 3–4 days and were cultivated on polyester membranes with 0.4 µm pore size (Corning Costar). The investigations were carried out in the period of 6–8 days after sowing (Schierack et al., 2006), in a passage of 20–40.

2.3 Morphological Characterization

The IPEC-J2 cells were stained immunohistochemically after 3 days growth on “cover slips”. The cell nucleus, F-actin and tubulin structures of the cells were stained with antibodies (Dapi, Bodipy and anti-tubulin, Sigma Aldrich, Germany) and fluorescent secondary antibodies (Alexa 594, Sigma Aldrich, Germany). Immunofluorescence microscopy was performed with a Zeiss LSM 510 META confocal laser scanning microscope (CLSM, Zeiss, Germany)

2.4 Functional parameters

2.4.1 Transepithelial electrical resistance (TEER)

The cells were cultured as described above and seeded with a density of 6*10³ cells/cm². Transwell filters or snapwell filters made by polycarbonate with 0.4µm pore size (Corning, The Netherlands) was used for this culturing. The TEER was measured daily with a volt-ohm-meter (WPI, Germany). The transepithelial resistance of the native epithelium was determined during the Ussing chamber experiments.

2.4.2 Ussing chamber experiments

The retrieved samples of intestine were prepared by removing the muscularis and serosa and fixed in Ussing chambers (Scientific Instruments, Germany) with an area of 1 cm². On both sides, 5 ml buffer (115 mM NaCl; 25 mM NaHCO₃; 0.4 mM NaH₂PO₄; 2.4 mM Na₂HPO₄; 5 mM KCl; 5 mM glucose; 1.2 mM CaCl₂; 1.2 mM MgCl₂; add 1.4 µM Indomethacin, pH7.4) was added. The chambers were gassed with carbogen at a temperature of 37°C. The initial resistance of the epithelium was in the range of 90-150 Ω. Confluent cell culture monolayers on snapwell filters have been investigated in Ussing chambers under the same conditions described above. During the experiments, test substances were added to the mucosal or serosal side of the Ussing chamber.

2.4.3 Short circuit (Isc) measurements

As a functional parameter the current after mucosal addition of NaCl (115 mmol) was used. After an equilibration period of 15 min (constant value of Isc) NaCl was added and the reaction of tissue or cell culture monolayer in the absence or presence of the test substance was registered. After completion of the experiment, Theophylline (10⁻³mol*l⁻¹) was added to prove the viability. Only in case of a reaction to Theophylline data were used for further investigation.

2.4.4 Diffusion of [³H]-mannitol

Tightness of tissue or monolayer was investigated with tritium-labeled mannitol (11.7 Ci/mM, conc. 3.85*10⁻⁶ mM, Amersham, Great Britian). It added to the mucosal side of native epithelium and cell culture in the Ussing chamber. Hourly a sample from the serosal compartment was taken. The samples were analyzed by liquid scintillations chromatography (Liquid Scintillation Analyser, Tri-Carb 2900TR, Perkin Elmer, USA) and the diffusion in relation to the initial amount of [³H]-mannitol was calculated in %/h*cm² or as standard permeability coefficient (Papp).

2.4.5 Transport of essential amino acids

The tritium-labeled amino acids methionine, leucine, lysine and tyrosine were added to the mucosal side of the Ussing chamber (79.7 Ci/mM, 0.57*10⁻⁶ mM). Hourly a sample was taken from the serosal side of the Ussing chamber. The samples were analyzed by liquid scintillations chromatography (Liquid Scintillation Analyser, Tri-Carb 2900TR, Perkin Elmer, USA) and the transportin relation to the initial amount of [³H]-amino acids was calculated in %/h*cm².

2.4.6 Transport of high molecular weight substances

To investigate a potentially available transport of high molecular substances, in buffer dissolved GFP (green fluorescent protein, about 4 mg/ml contain in genetically modified tobacco plants) was added to the mucosal side of the native epithelium or the IPEC-J2 cells. Hourly a sample was taken from the serosal side of the Ussing chamber. The samples were analysed by ELISA (sandwich-ELISA, Bioserv GmbH, Germany) and the transportin relation to the initial amount of GFP was calculated in %/h*cm².

2.4.7 Effect of complex test substances

As a model for the effect of „unknown” complex test substances on cell function, papain and wheat extract were selected. Papain (Sigma Aldrich, Germany) is a proteolytic enzyme from papaya. It serves the plant to repel insects and affects the fibrin structures (Wittmack & Tomaschek 1978, Konno & Barber 2014). In addition, it is considered to be allergenic and therefore it was added in the concentration of 1 mg/ml to the mucosal side of the native epithelium or to mucosal and serosal side of the cell monolayer.

Wheat extract containing gluten, known to be an agent responsible for potential incompatibilities (Smecuol et al., 1999; Menard et al. 2012) was chosen as an additional test substance. A solution prepared from wheat grains of the variety Greina (isogene (wild type variant) and transgenic (genetically modified) variant), conc. 1mg/ml buffer, was added to the mucosal side of the native epithelium and the IPEC-J2 cells.
3 Results

3.1 Morphological characterization

Results demonstrated by Figure 1 and proofed by the occurrence of Occludin (Figure 2), IPEC-J2 cells formed a dense monolayer with formation of tight-junctions. The TEER ranged between 4000-4500 Ω/cm² on day 7 of cultivation. The standard permeability coefficient (Papp) of [3H]-mannitol was 1.3386E-06 on IPEC-J2 monolayer and 3.572E-07 on native epithelium at a resistance of 110 Ω (Figure 3).

3.2 Functional characterization

To characterize electrophysiological properties the response to addition of sodium was examined. Both the IPEC-J2 cells, as well as the native epithelium showed an increase in short-circuit current Isc: about 16 µA/cm² in cell culture monolayer and about 7 µA/cm² in native epithelium (Figure 4).

3.3 Measurement of amino acid transport

Both in native epithelium and IPEC-J2 monolayer a transport of the amino acids leucine, lysine, methionine and tyrosine was measured. The transport rates for the native epithelium have been 0.007 (Lys), 0.013 (Met), 0.017 (Leu), 0.024 (Tyr) pmol/s*cm² and for the IPEC-J2 are 37.80 (Met), 58.56 (Leu), 41.55 (Lys), 104.13 (Tyr) pmol/s*cm² (Figure 5).

3.4 Transport of high molecular substances

To investigate the occurrence of unspecific transport mechanisms for high molecular substances (e.g. ABC-transporter), GFP was used. A transport of a high molecular substance such as GFP could be detected. In native intestinal epithelium a transport of GFP of 0.133 %/h*cm² of the initial concentration was measurable, in the IPEC-J2 monolayer the transport of GFP was only 0.0306 %/h*cm² (Figure 6).

3.5 Influencing the morphological and electrophysiological parameters by complex test substances

3.5.1 Papain

An influence on the functional and morphological parameters of the test systems, by papain is detectable especially on IPEC-J2 monolayer. Native epithelium did not response to papain given on the mucosal side. In contrast, IPEC-J2 monolayers are affected in most cases, only by papain donation to the serosal side. Figure 7 shows the influence of serosal addition of papain to the IPEC-J2 monolayers. It is visible already after 2 hours and further strengthened over the test period. In the same way lysine transport is affected (Figure 8).

Figure 1 IPEC-J2 cells, 7 days cultured on polycarbonate membrane

Figure 2 IPEC-J2 cells, nucleus (blue, Dapi) and Occludin (green, Alexa 488)
Figure 3. Standard permeability coefficient (Papp) of $[^3H]$-mannitol on native intestinal epithelium and on IPEC-J2 monolayer (n=8). Values followed by the same letter are not significantly different at 5% DMRT.

Figure 4. Short circuit current ($I_{sc}$) after the addition of sodium on the apical side of IPEC-J2 cells and native epithelium (n=8).

Figure 5. Transport of amino acids (methionine, leucine, lysine, tyrosine) by IPEC-J2 cells and native tissue (ileum, n=10). Values followed by the same letter are not significantly different at 5% DMRT.

Figure 6. Transport of intact GFP (green fluorescent protein) by IPEC-J2 cells and native tissue (n=8). Values followed by the same letter are not significantly different at 5% DMRT.

Figure 7. Diffusion of $[^3H]$-mannitol by IPEC-J2 monolayer and native epithelium after mucosal addition of papain (n=8). Values followed by the same letter are not significantly different at 5% DMRT.

Figure 8. Influence of mucosal or serosal addition of papain on the translocation of lysine on the IPEC-J2 monolayer (n=8) during 6 hours. Values followed by the same letter are not significantly different at 5% DMRT.

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Furthermore no reaction was reported on the mucosal addition and a significant increase in the presence of papain on the serosal side. An effect of papain is also detectable in the measurement of short circuit current caused by sodium. The sodium-induced $I_{sc}$ in IPEC-J2 monolayer is $4\mu A/cm^2$ and was significantly reduced by the addition of papain (Figure 9). Interestingly, there is no significant difference in the reaction between additions of papain to the mucosal or serosal side. The $I_{sc}$ was always reduced by about $5\mu A/cm^2$ compared to the control.

3.5.2 Wheat extract

Presence of wheat gluten with the resultant potential incompatibilities (Smeczol et al., 1999; Menard et al., 2012) could have an influence on cellular transport mechanisms. In addition, because wheat was available as isogenic and transgenic version, the possible influence of non-identical genetic material was of some interest and studied in this experiment. To evaluate the potential influence on lysine transport of IPEC-J2 cells wheat extract was admit to the mucosal side. The lysine transport showed a significant increase compared to the control variant without wheat extract (Figure 10).

There were no significant differences between the different wheat varieties. The diffusion of $[^3]$H-mannitol am IPEC-J2-monolayer increased significantly after mucosal addition (Figure 11). In contrast, a similar effect in native epithelium could not be observed. In the next step, the influence of wheat extract on the sodium-induced short circuit current ($I_{sc}$) was investigated. The results indicate a possible influence of $I_{sc}$ by wheat extract: the addition of isogenic and transgenic wheat extract was made to the mucosal side. The $I_{sc}$ increased immediately and was significantly higher than in the control variant over the entire time course (Figure 12).

4 Discussion and conclusion

4.1 Morphological and functional characterization

The IPEC-J2 cells are capable due their origin to serves as a model for the porcine intestine. Due the high compliance of height, weight, anatomy and physiology of the gastrointestinal tract of pigs and humans (Wernersson et al., 2005), this model seems also to be suitable for human (Schierack et al., 2006, Brosnahan et al., 2012). This hypothesis is strengthened by the observation of tight junctions in cells like it is detectable in human beings. The measured TEER values are consistent with the data from literature (Schierack et al., 2006). Furthermore, the diffusion of $[^3]$H-mannitol under control conditions in IPEC-J2 monolayer compared to the native intestinal epithelium is not significantly different. This result indicates that a dense intact epithelium was formed. From these facts it was derive that this in vitro system is morphologically comparable with other described in vitro systems (Geens & Niewold, 2011).

In the reaction of cells and native epithelium to the addition of sodium a significantly higher change in the short circuit current ($I_{sc}$) in the cell culture was reported. This effect can be explained by the higher resistance of the complete intestine. There are several cell layers are present, causing an overall higher resistance. This shows that the cell culture system should be preferred when investigating the influence on sodium dependent transport processes. In the study of the transport of essential amino acids in the native epithelium and the IPEC-J2 monolayer a significant difference in the rate of transport was observed by various researchers (Rhoads et al., 1994; Zhang et al. 2014). The measured rate of transportation of amino acids methionine, tyrosine, lysine and leucine is in IPEC-J2 cells was reported significantly higher than in native porcine intestine.
4.2 Use of in vitro systems for risk assessment

The suitability of the in vitro system for a risk assessment was checked using the enzyme papain and an unknown mixture of wheat extract. Papain is a protein-splitting enzyme and it serves insects repellent and affects the fibrin structures (Wittmack & Tomaszek 1978; Huby et al., 2000; Konno & Barber, 2014). The applied wheat extract was a sample from genetically modified wheat, the potential ability to influence physiological processes in the gastrointestinal tract should be investigated. Due to the higher sensitivity of cell cultures, the reaction of these is faster and more sensitive as the ex-vivo systems to a possible influence of paracellular transport routes. As the results show in figure 5, the influence of the diffusion of $[^3H]$-mannitol under test conditions, such the addition of papain (Figure, 8) and „wheat“(Figure, 10) is significantly more pronounced in cell cultures.

4.3 Response to the addition of papain

Both the diffusion of $[^3H]$-mannitol, the sodium –induced $I_{sc}$ and the transfer of the amino acid lysine influenced by the addition of papain. However, this effect could be detected only in serosal addition to the native intestinal epithelium and on the cell monolayer. The increased diffusion of mannitol on IPEC-J2 monolayer in serosal addition of papain indicates a possible change in terms of diffusion properties and in the integrity of the monolayer. May be the damage of the epithelium on the basolateral side by the addition of papain is the trigger for the significantly higher mannitolflux. Papain also showed an effect on the transport of lysine. Again, mucosal addition did not significantly influence the transport of the amino acid. But if added serosal, the transport of lysine was significantly higher. Probably proteolysis action of the enzyme causes possibly a damaging effect on the basolateral side of the monolayer. Because no blocker experiments were performed, it could not be distinguished between an increase in transport and/or diffusion. By the addition of papain the Na-$I_{sc}$ is significantly reduced. The cell monolayer has been no longer able to respond appropriately to the addition of sodium. Interestingly, this effect occurs in serosal and in mucosal addition of papain. Possibly, the epithelium was damaged or the electrophysiological properties were affected by the addition of papain. This will be investigated in further studies.
In addition, wheat extract was used as a test substance. The diffusion of mannitol was significantly increased by mucosal addition to the IPEC-J2 monolayer. Also the transport of lysine exhibits an increase under the influence of wheat extract. The Na-L, was also affected by the addition of wheat extract. Serosal addition of wheat extract was omitted because the test system was mainly developed to simulate the processes in the intact intestine. One possible explanation for the effect of wheat extract on mucosal addition to the cell culture might be bases on the effect of the wheat ingredients, because they contained gluten, which has an effect on the tight junction proteins (Smecuel et al., 1999).

Based on the response of the two test systems on the various test substances a stronger influence always shown on the morphological and functional properties of the cells culture monolayer compared to the control variant (complete ex vivo portion of the porcine intestine). The constant resistance of IPEC-J2 cells and the native epithelium during the experiment proof the stability of both test systems. The significantly higher response in the cell monolayer is therefore no artifact but inherent to the system.

The in vitro system reacts sensitively to any test substances. The observed changes in diffusion of mannitol, as well as the electrophysiological (Na-L) and transport properties (transport of lysine) are due to the influence of unknown components. In a risk assessment setup, the presented test systems should be implemented because the observed different responses make it possible to characterize the various possible influences of morphological and functional properties by the addition of various test substances.

Test systems that are based on in vitro experiments, are an effective alternative to animal experiments. The existing technical possibilities should be used to develop further in vitro test systems and apply this. The higher the accuracy and comparability with in vivo data, the number of applications would be developed. Research should invest in in-vitro systems to replace animal tests largely.

Acknowledgment
The authors thank Angelika Hauth for her excellent technical assistance.

Conflict of interest
Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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