Evaluation of the Effects of Ethanol on Monolayer and 3D Models of Human Oral Mucosa

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Abstract

The aim of this study was to examine and compare the effects of different ethanol-containing solutions on a 3D oral mucosal model (OMM) with those of fresh oral mucosal biopsies and monolayer keratinocyte cultures. 3D OMMs were engineered by the air/liquid interface culture technique using human oral fibroblasts and keratinocytes. Fresh tissue biopsies were also obtained from patients and divided into small pieces. OMMs, fresh biopsies, and monolayer cultures were exposed to serial dilutions of ethanol in PBS (5% to 50%) for 30 secondss and 60 secondss. Biological response was evaluated using basic histology and the Alamar Blue tissue viability assay. Histological examination showed that the effects of ethanol on the OMMs resembled the fresh tissue biopsies. The 3D model was also able to detect the concentration-dependent and time-dependent effects of ethanol. The TC50 values for the monolayer cultures were significantly lower than those for the 3D models. It can be concluded that ethanol in high concentrations significantly affects the oral mucosal model's viability and histological appearance. The response of the OMM to ethanol exposure was similar to the response of the fresh clinical biopsies. Monolayer keratinocytes are more sensitive to ethanol than 3D models or biopsies.

Introduction

Conventional cytotoxicity assays use monolayer cultures of cells, either monocultures or co-cultures. However it is increasingly recognised that these assays are not particularly physiologically relevant. There has been an ongoing debate as to whether or not the data obtained from monolayer epithelial cell culture systems would apply to normal human oral mucosa and can be extrapolated to the patients. One criticism is that epithelial cells in monolayer cultures lack the differentiated function and barrier properties of cells found in normal human oral mucosa. It has also been shown that when experiments are moved into three-dimensions there is often a cytoprotective effect observed with TC50 values higher for 3D models than the traditional 2D models [1]. To this end, tissue engineered 3D models of human oral mucosa have been developed [2]. These 3D oral mucosal models have been used to assess the biological effects different types of dental materials [3-6]. In our previous studies, a full-thickness 3D human oral mucosal model was developed [7] and examined for biological evaluation of restorative dental materials [8] and alcohol-containing antiseptic mouthwashes [7]. Compared to the commercially available split-thickness epithelial models, the full-thickness oral mucosal model contains both epithelium and the connective tissue and closely resembles the native human oral mucosa [9].

Alcohol is a common constituent of many oral health care products. However, recent studies have led to some concerns over potential biological toxicity of ethanol on human oral mucosa [10-13]. Alcohol has also been shown to increase the permeability of the oral mucosa [14] and can lead to a reduced barrier function in both oral mucosa and skin epidermis [15]. Thus, examining the effects of ethanol on human oral mucosa is vitally important. The data presented here is a first step towards understanding the effects of ethanol on human oral mucosa.

From a toxicology point of view, it is necessary to examine the ability of the 3D oral mucosal model to detect the concentration-dependent and time-dependent effects of potential irritant substances such as ethanol, on the oral epithelium.

Previously published studies have used monolayer cultures of human fibroblasts [13,16] or animal models [17,18]. Furthermore, the clinical relevance of the 3D oral mucosal model has not been fully established as there is no study published in the literature comparing the response of a 3D oral mucosal model to ethanol with that of the native human oral mucosa.

Therefore, the aim of this study was to examine the biological effects of different ethanol-containing solutions with different exposure times on the 3D oral mucosal model and to compare this with those of freshly excised human oral mucosal biopsies and monolayer cultures of human oral epithelial cells.

Materials and Methods

Monolayer cell culture testing

Human oral keratinocytes harvested from clinical biopsies from multiple human donors with the approval of Sheffield Research Ethics Committee were cultured in Green's medium in a humidified atmosphere of 5% CO_2 / 95% air at 37°C. Green's medium consisted of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-12 medium in a 3:1 ratio, supplemented with 10% fetal calf serum (FCS) (Biowest Ltd., UK), 10 ng/ml epidermal growth factor (EGF), 0.4 µg/ml hydrocortisone, 10^{-4} mol/l adenine, 5 µg/ml insulin, 5 µg/ml transferring, 2×10^{-7} mol/l triiodothyronine, 2×10-3 mol/l glutamine, 2.5 µg/ml fungizone, 50 U/ml penicillin and 50 U/ml streptomycin. Unless stated otherwise all reagents were purchased from Sigma, UK.

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5×10^5 mixed population of oral keratinocytes in 2 ml culture medium per well were cultured in 24-well tissue culture plates and grown to sub-confluent monolayers for 48 h. Then the cultures were exposed to 200 µl of Phosphate buffered saline (PBS) (negative control group) and serial dilutions (N=5) of ethanol (Fisher Scientific, UK) in PBS (5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, and 50%) for 30 secondss and 60 secondss and then washed with culture medium immediately and incubated in 2 ml of culture medium for further 24 hours. Then the cells were incubated in 1 ml solution of 10% Alamar Blue (Biosource, Camarilo, CA) in culture medium in a humidified atmosphere of 5% CO_2/95% air at 37°C. After 5 hours incubation, 200 µl of each well was placed into 96-well plate in duplicates, and fluorescence intensity was measured using a fluorescent plate reader (FLUOstar Galaxy, BMG Lab technologies) at an excitation wavelength of 530 nm and emission wavelength of 590 nm.

Tissue engineered 3D oral mucosal model testing

Full-thickness oral mucosal models were engineered inside tissue culture inserts as published previously [7]. The epithelial surfaces of the engineered oral mucosal models were exposed to 200 l of PBS (N=5) and serial dilutions of ethanol in PBS (5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, and 50%) for 30 seconds and 60 seconds. The 3D model was completely covered by the test solution. The tissue was then washed with culture medium immediately and cultured at the air/liquid interface for another 24 hours. After 24 hours, tissue viability was assessed by the Alamar Blue assay and oral mucosal models were processed for histology.

Tissue viability test (Alamar blue assay): The tissue was incubated in 1 ml solution of 10% Alamar Blue (Biosource, Camarilo, CA) in culture medium for 5 hours. Duplicate 200 µl samples from each model were placed into the wells of a 96-well plate, and the fluorescence intensity of each well was measured using a fluorescent plate reader.

Histology: The mucosal models were washed with PBS, fixed in 10% formalin solution for 24 hours and subsequently the samples were processed automatically for embedding in paraffin. The epithelial surface was kept flat and at 90 degrees to the plane of sectioning. Five-micrometer-thick vertical serial sections were cut and stained with haematoxylin and eosin (H and E) for histological evaluation. The histological appearance of the serial sections was assessed by more than one investigator blinded to the nature of the treatment the tissue had received. Where there were differences in the histological assessment of the tissues between investigators, these were resolved by discussion and a consensus reached before blinding was removed. Assessment criteria included the integrity and thickness of the epithelium, cell morphology, presence or absence of pyknotic nuclei, and presence of distinct interface between the epithelium and the connective tissue layer.

Fresh tissue biopsy testing

Human gingival mucosal biopsies were obtained from patients having surgical removal of impacted wisdom teeth with their written informed consent. The oral biopsies were divided into small pieces and exposed to 200 µl of PBS and serial dilutions of ethanol in PBS (5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, and 50%) for 30 seconds and 60 seconds and then washed with culture medium immediately and incubated in 2 ml of the culture medium in a humidified atmosphere of 5% CO_2 / 95% air at 37°C for another 24 hours. After 24 hours, the tissues were processed for histology as described above.
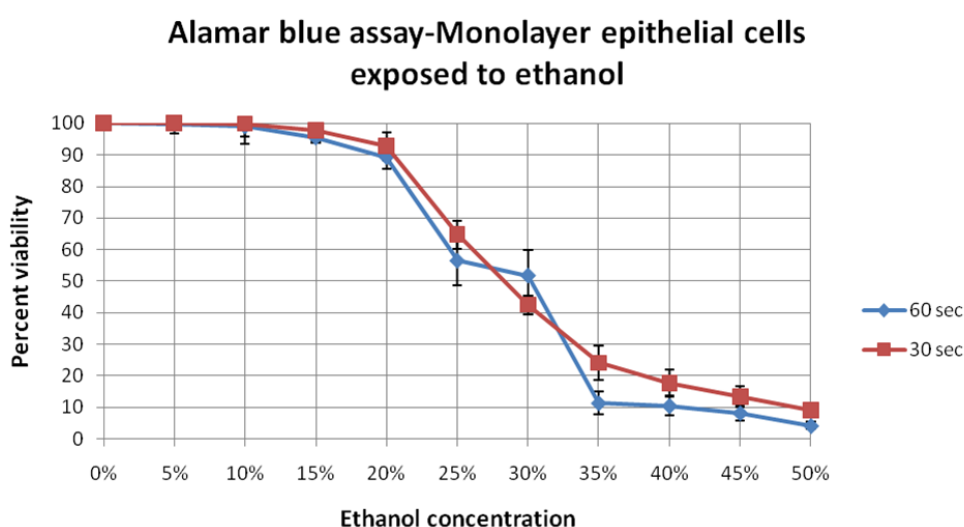
Statistical analysis

Minitab regression analysis was used to produce fitted dose-response curves for both monolayers and 3D models exposed to increasing concentrations of ethanol for 2 different exposure times. TC50 values of ethanol (concentrations which reduces the tissue viability by 50%) were calculated for both monolayers and 3D models from the line of the best fit. One-Way ANOVA was used to examine whether there was a statistically significant difference between TC50 values obtained from monolayers and 3D models.

Results

Monolayer culture testing

The results of the Alamar Blue assay for monolayer cultures of oral keratinocytes exposed to serial dilutions of ethanol are presented in Figure 1. As the concentration of ethanol increases, the cell viability

Figure 1: Quantification of cell viability in monolayer cultures of oral epithelial cells following exposure to serial dilutions of ethanol for 30 and 60 seconds as measured using the Alamar Blue assay.

drops significantly. Ethanol TC50 values calculated from the fitted dose-response regression plot were 28.99% (r^2=0.98) for 30 seconds exposure and 27.92% (r^2=0.98) for 60 seconds exposure of monolayer cultures of oral keratinocytes.
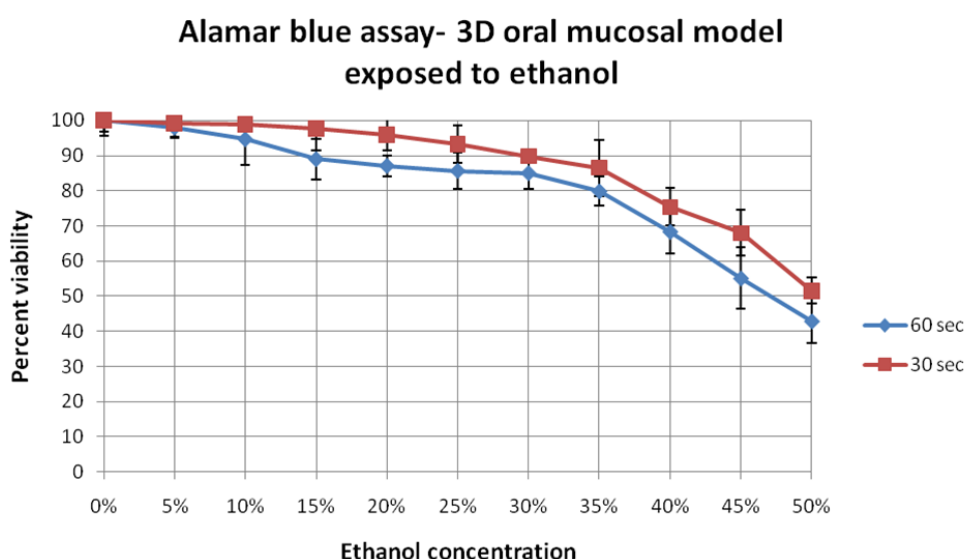
Oral mucosal model testing

The results of the Alamar Blue assay for the 3D oral mucosal models exposed to serial dilutions of ethanol are presented in Figure 2. Ethanol TC50 values calculated from the fitted dose-response curves were 50.79% (r^2=0.99) for 30 seconds exposure and 47.66% (r^2=0.99) for 60 seconds exposure of the 3D oral mucosal models.

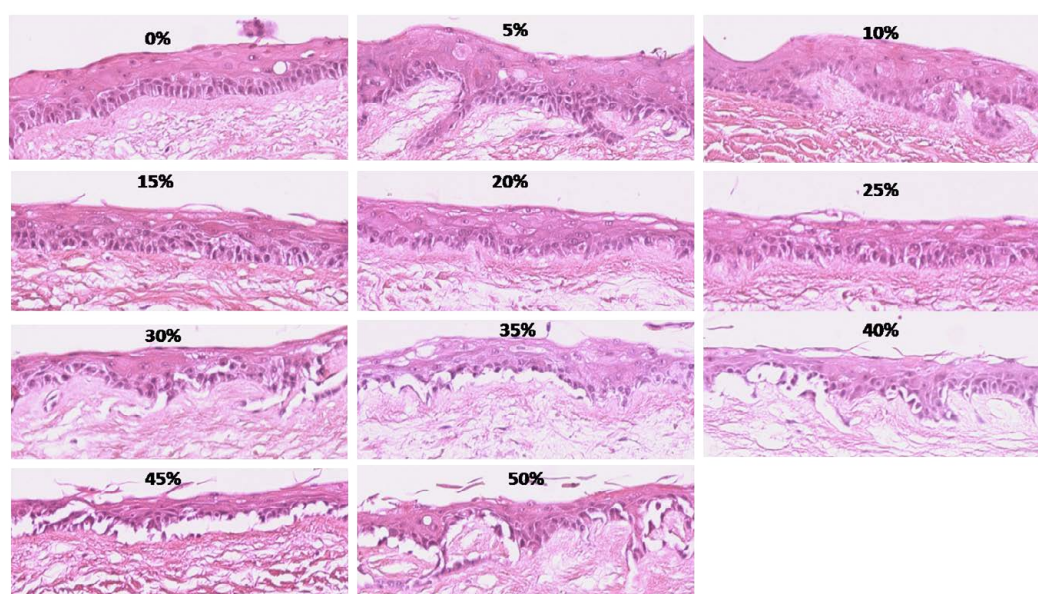Statistical analysis with One-way ANOVA followed by Tukey's test showed that there was a significant difference in TC50 values between the monolayer cultures and the 3D oral mucosal model.

Figures 3 and 4 show the microscopic views of the histological sections of engineered oral mucosal models exposed to different concentrations of ethanol for 30 seconds and 60 seconds respectively.

In oral mucosal models exposed to ethanol concentrations below 30%, the integrity of the epithelium was preserved as well as the distinct interface between the epithelium and the connective tissue layer. Slight desquamation of the superficial keratin layer could be observed in oral mucosal models exposed to concentrations below 30%. In contrast, exposure to high concentrations of ethanol above 30% caused significant cell death and disruption of the epithelial layer with
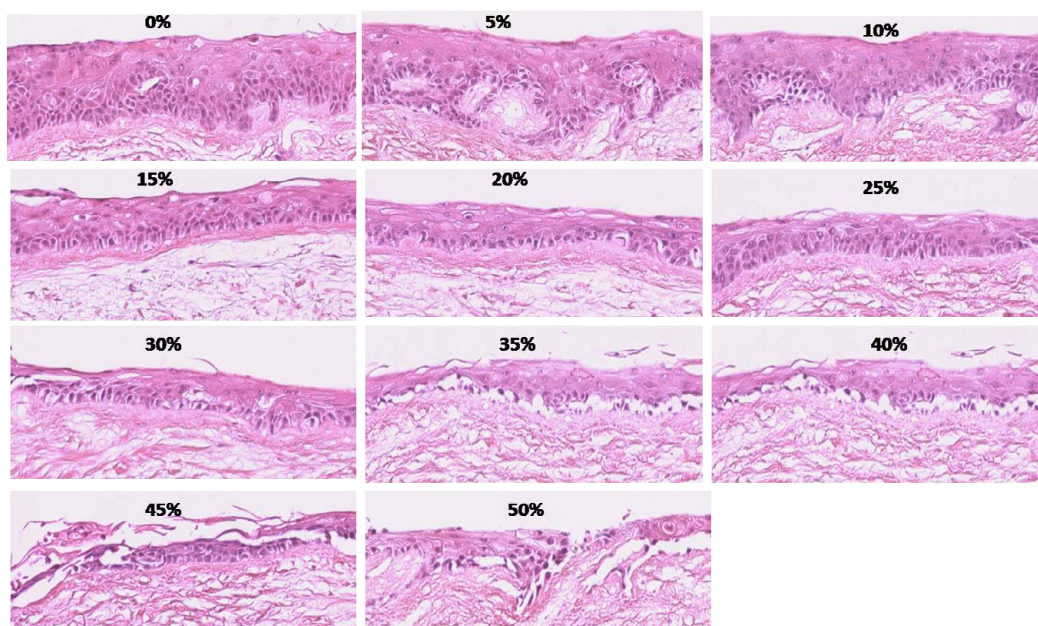

Figure 2: Quantification of tissue viability of the 3D oral mucosal models following exposure to serial dilutions of ethanol for 30 and 60 seconds as measured using the Alamar Blue assay.
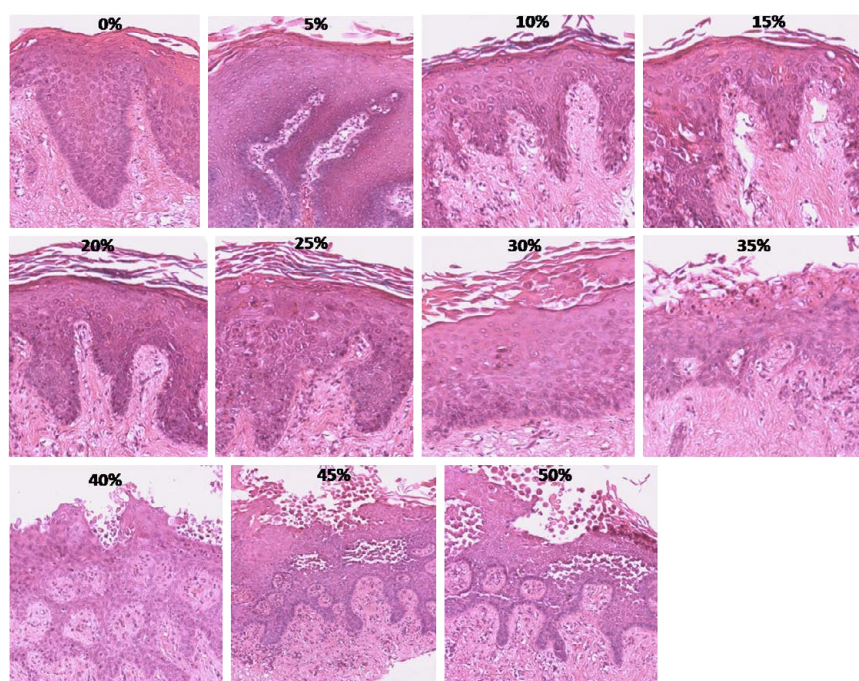

Figure 3: Histological sections of the 3D oral mucosal models exposed to serial dilutions of ethanol for 30 seconds. Haematoxylin and eosin, original magnification 10X.

Figure 4: Histological sections of the 3D oral mucosal models exposed to serial dilutions of ethanol for 60 seconds. Haematoxylin and eosin, original magnification 10X.
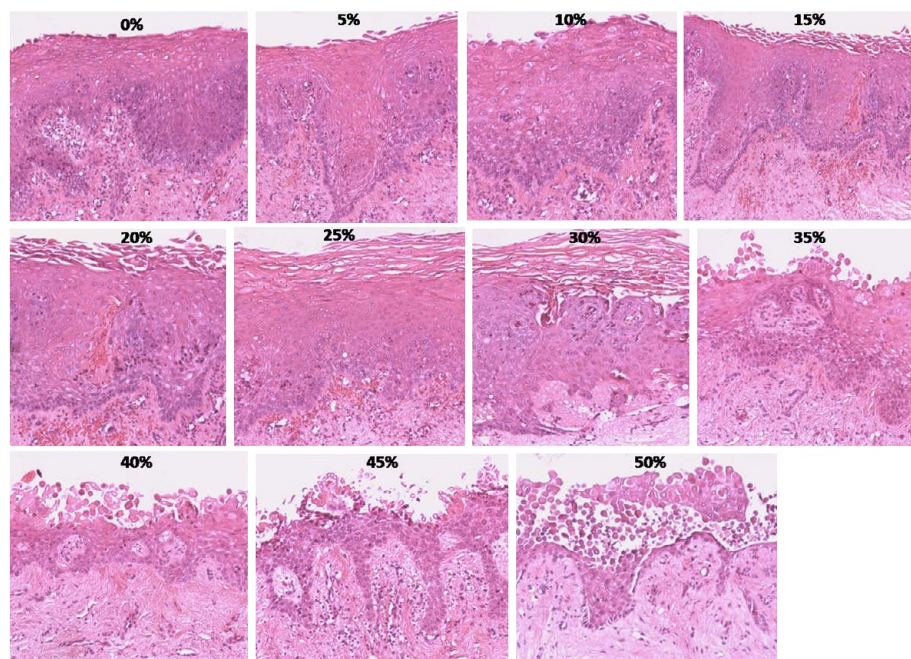

Figure 5: Histological sections of fresh tissue biopsies of oral mucosa exposed to serial dilutions of ethanol for 30 seconds. Haematoxylin and eosin, original magnification 10X.

presence of pyknotic nuclei and also separation of the epithelium from the connective tissue layer. The epithelial damage was more obvious in 3D models exposed to high concentrations of ethanol for 60 seconds compared to 30 seconds exposure.

Fresh tissue biopsy testing

Figures 5 and 6 illustrate the histological sections of fresh tissue biopsies of oral mucosa exposed to different concentrations of ethanol for 30 seconds and 60 seconds respectively. Oral mucosa appeared normal up to exposure to a 30% ethanol solution for 30 seconds exposure. The only significant change that could be observed up to 30% ethanol exposure was the expansion and slight desquamation of the superficial keratin layer. However, the underlying epithelial layers appeared normal. The higher concentrations of ethanol (above 30%)

Figure 6: Histological sections of fresh tissue biopsies of oral mucosa exposed to serial dilutions of ethanol for 60 seconds. Haematoxylin and eosin, original magnification 10X.

seemed to cause significant damage to the oral mucosa by killing and removing the suprabasal cell layers. 60 seconds exposure to high concentrations of ethanol caused more significant epithelial damage compared to 30 seconds exposure.

Discussion

Whilst tissue engineered oral mucosa is histologically similar to native tissue [7] and is used clinically for surgical transplantation [19,20] it is unclear how sensitive this model is to chemical insult. Previous work has assessed the effects of some ethanol-containing mouthwashes on the viability and tissue structure of the 3D model [9]. However, most antiseptic mouthwashes contain ethanol at various concentrations. Thus different ethanol dilutions were tested in this study. High concentrations of ethanol, beyond mouthwash levels, were also included to represent positive control groups.

Endpoints assessed in this study included histological examination of the tissue and the Alamar Blue tissue viability assay. Histology allows us to directly visualize the damage to the oral mucosa and is generally regarded as the current gold standard for imaging 3D tissue engineered constructs [21]. The Alamar Blue assay permits quantitative analysis of the viability of the oral mucosal tissue. Biological response was recorded after 24 hours incubation as our previous studies suggest that the epithelial changes due to inflammation continue for 24 hours following the exposure of the oral mucosal model to irritants [8,9].

It was not possible to use the Alamar Blue assay to assess the viability of the freshly excised clinical biopsies since it is not feasible to obtain a sufficient sample size (N=5) of equally sized pieces from the biopsy. However, histological evaluation of the fresh tissue biopsies provided very useful information in terms of changes in the morphology of the oral epithelium as a response to ethanol exposure. Conversely, when using the monolayer cultures of oral epithelial cells it was only possible to carry out the Alamar Blue assay since the monolayer cultures are not suitable for histological sectioning and evaluation.

The 3D oral mucosal models enabled the application of both histology and the Alamar Blue assay to assess the response of the oral mucosa to ethanol solutions. Thus both qualitative and quantitative data can be obtained from these models. Large numbers of the 3D oral mucosal models can be produced of the same size and the same number of cells seeded on the scaffolds. The 3D oral mucosal model system permits the use of a large pool of oral epithelial cells obtained from numerous donors. This approach produces a mixed cell population and reduces the risk of donor specific variations when the models are grown from individual donors.

The results of this study showed that the TC50 values for the monolayer cultures were significantly lower than those for the 3D oral mucosal models. Whilst this finding has not previously been reported in the literature, it suggests that monolayer cultures of oral keratinocytes are more sensitive to ethanol-containing solutions than the 3D human oral mucosal model. This is in agreement with previously reported data [1]. Some studies have reported high toxicity induced by ethanol-containing mouthwashes on monolayer cell cultures [16] and also inhibitory effects of ethanol on wound healing by assessing the proliferation of monolayer cultures of fibroblasts [13]. In monolayer cultures, all of the cells are exposed to the test materials at the same time and are therefore instantly affected by the substances. However, since the 3D oral mucosal models are sealed within the inserts and fed from the bottom connective tissue layer, only the superficial layer of the epithelium is directly exposed to the test materials. The cells in deeper layers are therefore partially protected by the barrier function of the epithelium. This arrangement is similar to what happens in the clinical situation when an irritant substance comes into contact with human oral mucosa. This arrangement is reflected in the near doubling of the TC50 values from 28.99% (30 seconds exposure) and 27.92% (60 seconds exposure) for monolayer cultures to 50.79% (30 seconds exposure) and 47.66% (60 seconds exposure) for the 3D tissue engineered oral mucosa model.

The effects of ethanol on the histology of both the tissue engineered oral mucosa and the fresh tissue biopsies are clear. When the tissue engineered oral mucosa is treated with ethanol at concentrations of 35% or greater desquamation is clearly visible. The oral epithelium is peeling away from the basement membrane. The mechanism of desquamation appears to be different in the fresh tissue biopsy but changes are still visible at ethanol concentrations of 35% or greater. In the fresh tissue biopsy the upper keratinized layers of the epithelium reduce with increasing concentrations of ethanol, until at 50% ethanol the suprabasal cells are being removed. Previously published work has shown that treatment of oral mucosa with ethanol increases the permeability of the oral mucosa. There is some debate as to the role of lipid extraction by ethanol from the superficial epithelium in this [14,15]. Whilst this study does not help identify the role of lipid extraction in the increasing permeability of the oral mucosa it does potentially provide a concentration sensitive model that could be used to investigate this further. It is important to note that the response of the excised human oral mucosa to ethanol solutions *in vitro* maybe more exaggerated than when the native oral mucosa is exposed to ethanol in real mouth environment. There are several reasons for this: Firstly, since the size of the biopsies are small, it is not possible to seal them within the inserts and they are exposed to ethanol from all sides including the connective tissue side, however, real oral mucosa in the mouth is only exposed to ethanol from the epithelial side as the connective tissue is protected by the continuous epithelium. Secondly, in the clinical situation, alcohol containing-mouthwashes are diluted with saliva in the mouth and the oral mucosa is constantly bathed with saliva. Therefore, the fresh biopsies maybe more sensitive to ethanol in vitro than native oral mucosa in the mouth environment. Thus, there is a need for clinical studies to assess the effects of real formulations of ethanol-containing antiseptic mouthwashes on human oral mucosa *in vivo* in comparison with data obtained from the *in vitro* 3D oral mucosal model.

The results of the Alamar Blue assay were consistent with the histological findings for the 3D oral mucosal models which showed that the tissue viability started to drop significantly at around 30% ethanol concentration. Also 30 seconds exposure seemed to be less toxic than 60 seconds exposure. These findings were consistent with some previous animal studies who reported that the direct toxic action of the alcohol in short-term experiments leads to a local damage of the mucous membrane which was proportional to the degree of alcohol concentration [17].

Conclusions

From the results of this study it can be concluded that all the models, monolayer, tissue engineered oral mucosa and the freshly excised oral mucosa biopsy were able to detect concentration-dependent and time-dependent effects of ethanol. The Alamar blue assay showed that the monolayer cultures of oral keratinocytes were significantly more sensitive to alcohol-containing solutions than the 3D human oral mucosal models. The histological response of the 3D oral mucosal model to ethanol exposure was similar to the response of the fresh clinical biopsies of oral mucosa. Ethanol TC50 values of approximately 28% for the monolayer cultures and 50% for the 3D tissue engineered cultures were observed. Histological analysis showed major structural changes to both native tissue and tissue engineered mucosa at concentrations of greater than 30%.

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