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COOLING OF THE ORAL MUCOSA TO PREVENT ADVERSE EFFECTS OF CHEMOTHERAPEUTIC AGENTS - AN IN VITRO STUDY

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Abstract

Background: The cytotoxic effect of chemotherapeutic agents to the oral mucosa, as a side-effect of cancer treatment, is a major problem. Cooling the oral mucosa using ice chips in conjunction with chemotherapy is known to reduce the severity of oral mucositis. However,

although the use of ice chips is of clinical value, this method of cooling has inherent problems including discomfort for the patient, non-uniformity and fluctuations in cooling temperature throughout the oral cavity. Furthermore, despite being used clinically, it is not known what reduction in temperature is required to prevent oral mucositis. The aim of the present study was therefore to determine in vitro if the cytotoxic effect of 5-Fluorouracil (5-FU) on the oral mucosa could be reduced by lowering the temperature during chemotherapeutic treatment.

Methods: Tissue engineered oral mucosal (TEOM) models were incubated at 20, 25, 30, or 35 °C for 30 minutes followed by exposure to a clinically relevant concentration of 5-FU (162 μg/ml) for two hours and compared to untreated models (35°C). Cell viability and inflammatory cytokine production (IL-6 and TNF-α) were measured using PrestoBlue® and ELISA, respectively.

Results: TEOM models incubated at 20°C showed an increased cell viability and had a reduced IL-6 and TNF-α production compared to models treated with 5-FU incubated at 35°C.

Conclusion: This study demonstrates a reduced cytotoxic effect to the TEOM by reducing the temperature of the tissue during chemotherapy treatment and suggests that decreasing the temperature to 20°C could have clinical advantages.

Introduction

Chemotherapy, along with radiotherapy and surgery are the most commonly used strategies to treat cancer (1). The main action of chemotherapy is to target rapidly dividing cells, which is a characteristic feature of cancer cells (2). However, oral epithelial cells are also highly proliferative (3) and are therefore susceptible to the cytotoxic effect of chemotherapeutic agents (4). 5-Fluorouracil (5-FU) is a well-established chemotherapeutic drug and has for a long time been used for the management of a range of different cancers including colorectal, breast and skin cancer, but is also an alternative treatment in patients with metastatic or recurrent head and neck cancer. 5-FU is an analogue of uracil and enters the cells through the same facilitated transport mechanism as the nucleotide. Intracellularly, 5-FU is converted to several active metabolites that are further incorporated into macromolecules such as DNA and RNA, inhibiting their normal functions (5-7).

The mechanism by which chemo- and radiation therapy induce cell damage is believed to arise from of a cascade of biological events taking place in the underlying subepithelium. Subsequent activation of transcription factors and release of several pro-inflammatory cytokines, including IL-6 and TNF-α, in turn leads to a compromised mucosal lining and ulcerations known as mucositis (8).

Mucositis is common debilitating side effect following chemotherapy and is frequently seen in clinical settings prior to hematopoietic stem cell transplantation (9). It affects the lining epithelium of the entire oral and gastrointestinal tract (10) and in its mildest form manifests clinically as an erythematous lesion. However, in more severe forms, painful ulceration caused by epithelial detachment from the underlying submucosa is observed and routinely necessitates narcotic analgesia (11). Mucositis may also interfere with food and liquid intake and have a negative impact on several aspects of quality of life (emotional, social and functional) (12, 13).

Chewing on ice chips before and during chemotherapy infusion is currently the standard approach for prevention of oral mucositis (14). However, ice chips are extremely cold upon first exposure in the oral cavity causing discomfort including headache and teeth sensations (15). In addition, the intra-oral temperature during ice chip treatment is not constantly maintained and declines rapidly, therefore providing only transient benefit to the patient. The aim of this study was to determine if cooling, using a constant low temperature, caused a reduction in the pro-inflammatory cytokines release and cytotoxic effect of 5-FU treatment on tissue engineered oral mucosa (TEOM) and therefore prevent tissue damage.

Material and methods

Cell culture

Normal oral keratinocytes (NOK) and normal oral fibroblasts (NOF) were isolated and cultured from oral mucosal biopsies obtained from patients with written, informed consent with ethical approval (Sheffield Research Ethics Committee 09/H1308/66) as previously described (16) and in accordance with the World Medical Association Declaration of Helsinki. NOK were cultured in flavin- and adenine-enriched medium (Green's medium) consisting of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 medium in a 3:1 (v/v) ratio supplemented with 10% (v/v) foetal calf serum (FCS), 0.1 μM cholera toxin, 10 ng/ml epidermal growth factor, 0.4 μg/ml hydrocortisone, 0.18 mM adenine, 5 μg/ml insulin, 5 μg ml transferrin, 2 mM glutamine, 0.2 μM triiodothyronine, 0.625 μg/ml amphotericin B, 100 IU/ml penicillin and 100 μg/ml streptomycin. NOF were cultured in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin.

Production of TEOM models

TEOM models were produced as previously described (16). Briefly, de-epithelized dermis (1 cm^2) was placed in each well of a 6-well plates, an 8 mm diameter steel ring laid on the upper surface and $5x10^5$ NOF and $1x10^6$ NOK added in 500 μl of Green's medium. Medium was changed twice daily for 48 h at which point the rings were removed and the models incubated at an air-to-liquid interface on steel grids for a further 10 days at 37 °C, 5% CO_2.

Drug treatments

To determine the optimum drug concentration, TEOM were treated with 2 ml of 5-FU, dissolved in Green's media, at three different clinically relevant concentrations (54, 162 and 324 μg/ml) along with a control (no drug applied) and SDS-treated (1%) control for 2 h. Following the incubation, TEOM models were rinsed in pre-warmed PBS to eliminate superficial cytostatics and replaced with pre-warmed (37 °C) medium. Models were incubated for a further 30 min at 37 °C, washed again with pre-warmed PBS to eliminate profound cytostatics, replaced with fresh medium and incubated for 48 h at 37 °C before analysis.

Temperature effects on TEOM

Based on initial experiments it was determined that a 5-FU concentration of 162 μg/ml would enable the effects of temperature to be assessed in the TEOM models. TEOM models were pre-incubated at 20, 25, 30, or 35 °C for 30 minutes before treatment with 5-FU (162 μg/ml) for 2 h or at 35 °C with no drug used as a control. Following the incubation, TEOM models were rinsed in 37 °C pre-warmed PBS to eliminate superficial cytostatics and replaced with pre-warmed medium. Models were incubated for a further 30 minutes at 37 °C, washed again with pre-warmed PBS to eliminate profound cytostatics, replaced with fresh medium and incubated for 48 h at 37 °C before analysis.

Cell viability assay

To measure cell viability, PrestoBlue® (ThermoFisher, Massachusetts, United States) was used according to the manufacturer's instructions. Briefly, TEOM models were immersed in 1.1 ml of PrestoBlue® reagent diluted 1:40 in DMEM + 10% FCS (v,v) and incubated in the dark for 2 h at 37 °C in 5% CO2. 200 μl of media was transferred to a 96-well plate in duplicate and measured using a spectrophotometer (Tecan, Männedorf, Switzerland) at excitation 560 nm and emission 590 nm. Optical density values were normalised relative to the control (0 μg/ml 5-FU), which was set at 1.

Enzyme-linked immunosorbent assay

Commercially available ELISA (OptEIA™, BD Bioscience) kits were used according to the manufacturer's instructions to measure levels of IL-6 and TNF-α in TEOM conditioned medium as described previously (17).

Histological analysis

For histological analysis, TEOM were removed from the culture medium, washed with PBS and fixed in 10% buffered formalin overnight. The model was bisected and subjected to routine histological processing and then paraffin wax embedded. Five µm sections were cut using a Leica RM2235 microtome (Leica microsystems) and stained with haematoxylin and eosin (H&E).

Statistics

Data are presented as mean ± standard deviation (SD) of three independent experiments (n=3) with each test performed in triplicate unless otherwise stated. ANOVA (One-Way ANOVA and Dunnett's multiple hypothesis correction) multiple statistical comparisons were performed using GraphPad Prism v6.00 (GraphPad Software, La Jolla, CA, USA) and differences between test and control groups considered significant when $p < 0.05$.

Results

An initial drug dose-response was performed to determine the optimum concentration of 5-FU needed to reduce cell viability in full-thickness TEOM to subsequently enable the investigation of a cooling effect during drug treatment on cell viability and cytokine release. Cell viability was measured after a 48 h incubation following a 2 h treatment with 54, 162 or 324 µg/ml 5-FU. A dose-response effect was observed with 5-FU at a concentration of 54 µg/ml having no effect on TEOM model viability

but the higher concentrations (162 and 324 µg/ml) causing a statistically significant decrease in viability with an overall reduction of 36% ($p<0.001$) and 28% ($p<0.05$), respectively (Figure 1A). Following the same trend, an increase in TNF-α production was observed for the higher concentrations of 5-FU (162 or 324 µg/ml) tested following a 2 h incubation, although these did not reach significance from the untreated control (Figure 1B). Based on these results a drug concentration of 162 µg/ml was used for all further experiments.

To investigate the effect of cooling on cell viability, TEOM models were treated with 5-FU (162 µg/ml) for 2 h at 20, 25, 30 or 35°C and compared to untreated (media alone) controls at 35°C. A statistically significant decrease in viability for all 5-FU treated TEOM models at all of the temperature conditions tested compared to untreated controls was observed in a temperature-dependent manner, suggesting that lower temperatures increase cell viability and reduce the effects of 5-FU treatment (Figure 2). Although there was a trend of increased viability at lower temperatures with 5-FU treatment, these did not reach statistical significance when TEOM incubated at different temperatures were compared to each other.

Interestingly, 5-FU-treated TEOM displayed increased secretion of the pro-inflammatory cytokines IL-6 and TNF-α at all temperatures tested compared to untreated controls. IL-6 secretion increased markedly when cells were incubated with 162 µg/ml 5-FU at 35°C and 30°C compared to cells incubated with medium alone at 35°C, although this was not statistically significant (Figure 3). Levels of TNF-α were significantly greater ($p<0.05$) in models treated with 162 µg/ml 5-FU and incubated at 35°C compared with medium alone untreated mucosal models incubated at 35°C. (Figure 4). Moreover, levels of TNF-α were significantly lower ($p<0.05$) in mucosal models treated with 162 µg/ml 5-FU at 20°C compared with TEOM treated with the same concentration of 5-FU but incubated at the higher temperature of 35°C (Figure 4). These data suggest that incubating 5-FU-treated TEOM models at 20°C reduces the pro-inflammatory cytokine burden whilst increasing tissue viability compared to those incubated at 35°C.

Histological examination of the TEOM for each condition tested demonstrates that the structural integrity of the TEOM models are maintained 48 h post-incubation with 5-FU (Figure 5).

Discussion

Oral mucositis affects approximately 80% of all cancer patients treated with high doses of chemotherapy prior to stem cell transplantation (18). Cooling the oral mucosa using ice chips has been demonstrated to reduce the risk and severity of oral mucositis (19, 20). The presumed protective mechanism of reducing the temperature of the oral mucosa is believed to be a local vasoconstriction resulting in a reduced drug delivery to the epithelial cells as well as lowered metabolic activity in the basal layer, making the epithelium less susceptible to cytotoxic agents (19).

The TEOM is a composite model of oral epithelial cells and oral fibroblasts cultured on a decellualarised dermal scaffold and enables in vitro investigations in a relevant physiological environment (21). Although being the best in vitro model for the purpose of this study, it does not completely replicate the complex structure of the oral mucosa seen in vivo (8). In particular the model lacks immune cells and blood vessels, which prevents the possibility of studying the importance of vasoconstriction. Based on these limitations our findings are most likely attributed to lowered metabolic activity to explain the prevention of irreversible cell damage after exposure to chemotherapeutic agents. Furthermore, considering the absence of essential immune components the models used in this particular experiment may not reflect the immune response in its entirety that is observed in vivo.

Pre-incubation temperatures for the TEOM models were selected based on two separate studies on healthy volunteers where an approximate mean temperature of 25-30 °C was obtained in the oral mucosa (22, 23). These temperatures were further selected with an interval of 5 °C since they represented clinically relevant temperatures.

The decision to use 5-FU was based on the fact that it is one of the most commonly used chemotherapeutic drugs in clinical oncologic practice (7). 5-FU has several known adverse effects such as gastro-intestinal and oral mucositis, which is a major complication (24). Furthermore, it has been demonstrated that 5-FU induces an inflammatory response, including the production of the pro-inflammatory cytokines IL-6 and TNF-α in animal models (25), cytokines that were investigated in this in vitro study.

Mucositis is described as a complex biological process that occurs as a result of a series of interactions, including the activation of the transcription factor nuclear factor-kappa B (NF-κB) and release of pro-inflammatory cytokines e.g. IL-6 and TNF-α (8). Previous studies have shown an increase in expression of such factors in experimental models of anti-cancer treatment (21, 26, 27). In accordance with the findings of these previous studies, we found that TEOM models exposed to 5-FU expressed pro-inflammatory cytokines to a greater extent than untreated controls. In our previous study we did not detect expression of TNF-α in an in vitro model of radiation-induced mucositis (21), whereas levels of TNF-α were increased significantly by 5-FU treatment in this study. This disparity suggests subtle differences in the cytokines released by chemo- as opposed to radiotherapy induced oral mucositis. Monocytes and macrophages are thought to be the main sources of cytokine (e.g. IL-6 and TNF-α) production in vivo. Although, these cytokines are also produced by activated lymphocytes, endothelial/epithelial cells, and fibroblasts (28), the latter being the source of immune response in our models. Since our mucosal model does not contain an immune component it is likely that pro-inflammatory cytokine release is increased further in vivo upon chemotherapy treatment, where immune cells are present.

This study demonstrated that TEOM models incubated with 5-FU at lower temperatures had an improved ability to preserve their cell viability compared to models incubated at higher temperatures. The reverse temperature dependent pattern was seen for IL-6 production and the pattern was even more obvious for TNF-α expression, as treated models incubated at lower temperatures had

significantly lower production of pro-inflammatory cytokines. This was particularly true in models incubated at 20 °C compared to those incubated at 35 °C. These results are consistent with previous clinical reports showing that cooling has a protective effect on mucosa exposed to chemotherapeutic agents (19, 20).

In contrast to the extensive epithelial damage caused in radiated TEOM-models, the histological examination of TEOM-models exposed to 5-FU showed an intact structural integrity at 48 h post incubation. The preservation of the epithelium is considered positive as clinical ulcerations are characterized by a loss of epithelium leaving an exposure of the underlying connective tissue (29). It is difficult to state if a correlation exists between preservation of the integrity of epithelial and incubation at lower temperatures in TEOM-models exposed to 5-FU since models were followed only for 48 h. However, based on our histological examination no such correlation exists. Further studies with longer follow-up periods are needed to investigate if a true correlation exists. Furthermore, it would be of interest to study the model's biological mechanism and its cellular response to 5-FU as well as other chemotherapeutic agents when blood vessels and immune components are incorporated in the model. Partly because these are key components in the pathobiology of the immune system but in particular to further mimic the physiology of the oral mucosa seen in vivo.

An example of other chemotherapeutic agents that could have been used and evaluated in this study is Melphalan, a bifunctional alkylating agent that does not require metabolic activation. Similar to 5-FU, it incorporates into macromolecules but instead Melphalan exerts its effects through DNA inter-strand cross-linking, which leads to replication arrest and cell death (30).

In summary, this is the first study to evaluate the cytotoxic effect of a chemotherapeutic agent on oral mucosa in vitro and also the first in vitro study to investigate if the cytotoxic effect could be reduced by lowering the temperature. Our findings show that models incubated at lower temperatures have a higher cell viability and reduced cytokine production and further suggest that decreasing oral mucosal temperature to 20 °C in conjunction with chemotherapy could have clinical advantages.

Conflict of interest

This study has been funded by BrainCool AB, Foundation Olle Engkvist Builder (2015/555), Swedish Dental Association (1109), The Royal and Hvitfeldtska Foundation (2016-0216) and Wilhelm & Martina Lundgrens Research fund (2015-0294). Dr. Walladbegi is currently in receipt of a PhD scholarship funded by BrainCool AB. Prof. Jontell is a scientific adviser for BrainCool AB. All other authors report no personal conflict of interest.

Figure legends:

Figure 1: Dose-response effects of 5-FU on tissue-engineered oral mucosa models. Tissue-engineered oral mucosa model viability (A) and TNF-α production (B) following incubation with increasing 5-FU concentrations (54, 162 and 324 µg/ml) after a 2 hours incubation period compared to untreated controls. Data are presented as mean ± standard deviation (n=3), ***<0.001 and *p<0.05.

Figure 2: The effects of cooling on tissue-engineered oral mucosa model viability. Tissue-engineered oral mucosa model viability was measured 48 hours post incubation following treatment with 5-FU (162 µg/ml) for two hours at different cooling temperatures (20, 25, 30 and 35°C) compared to untreated controls (35°C). Data are presented as mean ± standard deviation (n=3), * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$.

Figure 3: The effects of cooling on tissue-engineered oral mucosa model IL-6 secretion. Tissue-engineered oral mucosa model IL-6 secretion was measured 48 hours post incubation following treatment with 5-FU (162 µg/ml) for two hours at different cooling temperatures (20, 25, 30 and 35°C) compared to untreated controls (35°C). Data are presented as mean ± standard deviation (n=3).

Figure 4: The effects of cooling on tissue-engineered oral mucosa model TNF-α production. Tissue-engineered oral mucosa model TNF-α production was measured 48 hours post incubation following treatment with 5-FU (162 μg/ml) for two hours at different cooling temperatures (20, 25, 30 and 35°C) compared to untreated controls (35°C). Data are presented as mean ± standard deviation (n=3), * $p \leq 0.05$, ** $p \leq 0.01$.

Figure 5. Histological examination of tissue-engineered oral mucosa models post-treatment with cooling. Structural integrity of tissue-engineered oral mucosa models following 5-FU treatment (162 μg/ml) at specified temperatures (20, 25, 30 and 35°C) compared to untreated controls (35°C). Scale bar=100 μm.

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Figure 1

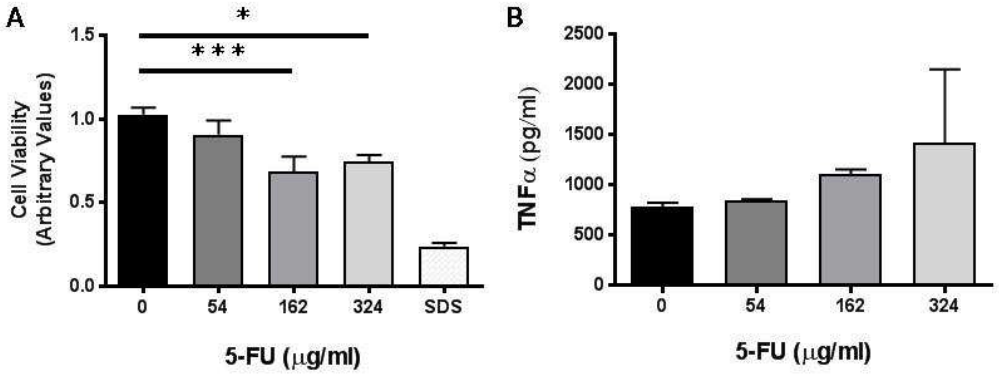

Figure 2

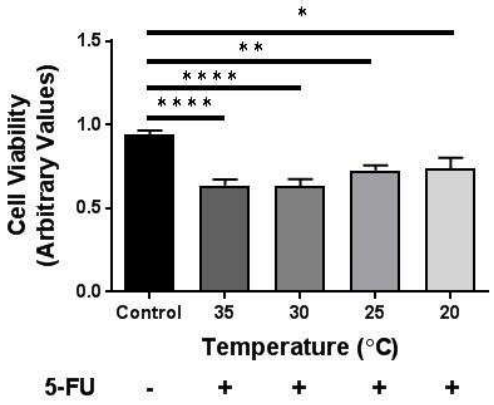

Figure 3

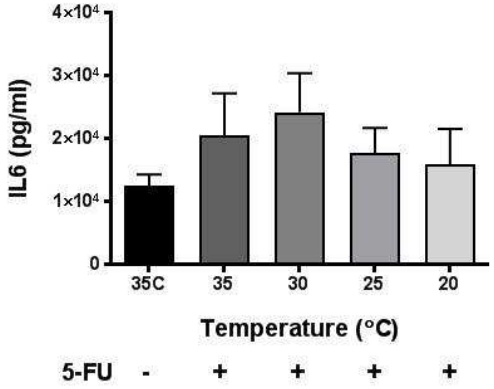

Figure 4

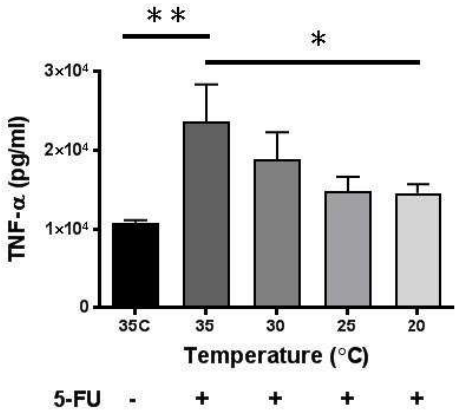

Figure 5