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Caco-2 cell line as a model to evaluate mucoprotective proprieties

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ABSTRACT

Physical protection of mucosa surface and reduction of inflammatory processes are currently considered the main strategies in the treatment and prevention of mucosal diseases. However, the majority of models used to verify the activity of new mucoprotective agents are based on limiting instrumental assessment or the sacrifice of experimental animals. In this study, for the first time, some *in vitro* experimental methods using Caco-2 cell line are proposed as predicting *in vivo* behaviour and action of mucoprotective agents. To this purpose, hyaluronic acid and natural polysaccharides for their bioadhesive activity, hydrocortisone and natural polyphenols as anti-inflammatory agents have been chosen. The obtained results demonstrated that the techniques (Con A/o-pd assay and Franz cell system) of mucoadhesive evaluation on Caco-2 cells are useful to compare the activity of each experimental sample and to assess the adhesion time to the mucosal cell surface. Moreover, the reduction of intercellular adhesion molecule-1 (ICAM-1) expression in Caco-2 cells can be considered directly correlated to the mucosal anti-inflammatory effect induced by the hydrocortisone and natural polyphenols. In conclusion, the study supported the use of Caco-2 cell as a model to compare and investigate the effect of different active substances on the mucosa and its diseases.

1. Introduction

Mucosal diseases have become more prevalent especially in Western industrialized countries and their incidence is expected to increase dramatically in the coming years. Lifestyle change, dietary constituents and environmental components were identified as the main cause of increasing predisposition of individuals to mucosal dysfunctions (Groschwitz and Hogan, 2009; Liakakos et al., 2009; Liu et al., 2009). Examples of mucosal diseases include inflammatory bowel diseases (IBD), ulcerative colitis, Crohn's disease, gastroesophageal reflux in the gastrointestinal tract; sinusitis and rhinitis in the nasal mucous membrane; interstitial cystitis in the bladder. In addition, there are also painful ulcerative disorders of mucosal surfaces which result in adverse side effects due to specific therapies, such as chemotherapy and radiation therapy (mucositis and esophagitis) (Allende and Yerian, 2009; Holgate, 2007; Lazard et al., 2009).

It is well known that mucosal disorders are usually characterized by two main processes: alterations of mucosal integrity and tissue inflammation. Alterations of the mucosal surface barrier can increase mucosal permeability leading to the absorption of toxic and aggressive factors into the body. Moreover, damages of the mucous membrane induce the activation of specific repair mechanisms that involve inflammatory processes both affecting deeper layers and non-epithelial cell population (Romier et al., 2009; Sturm and Dignass, 2008). As a consequence, mucosa protective agents can improve healing acting on the mucosal surface by the application of bioadhesive protective barrier and the reduction of inflammation processes.

Current active investigations search for efficient protective agents, especially natural active substances that are preferred for many advantages such as biocompatibility, safety, nontoxic and non-irritant properties. However, the majority of models used to evaluate and investigate the activity and effects of new mucoprotective agents are based on limiting instrumental assessment (such as tensile tester) or the sacrifice of experimental animals (Belgamwar and Surana, 2010; Davidovich-Pinhas and Bianco-Peled, 2010). In our knowledge, little is known about the use of *in vitro* cell lines to evaluate the property of mucosa protective agents.

In the present work, *in vitro* experimental models using Caco-2 cell line are proposed as methods predicting *in vivo* behaviour, and investigating the action of mucoprotective agents. Until now, the Caco-2 cell line is mainly considered as model of the intestinal barrier in the determination of drug absorption and permeation (Levy et al., 1995; Meunier et al., 1995; Shah et al., 2006). The

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in vitro models used in this work are able to evaluate the bioadhesive properties of active agents, the time adhesive in condition that simulate the continuous contact with physiological fluid and the capacity of them to counteract the overproduction of the intercellular adhesion molecule-1 (ICAM-1), specifically involved in mucosal inflammatory processes (Malizia et al., 1991).

Physical protection of mucosa often represented a first important strategy in the treatment and prevention of mucosal diseases. Mucoadhesive materials can be used as therapeutic agents in their own right, to coat and protect damaged tissues (gastric ulcers or lesions of the oral mucosa) or as lubricating agents (in the oral cavity, eye and vagina) (Smart, 2005).

Moreover, mucosal diseases include conditions often characterized by complex pathophysiologies. It was demonstrated that inflammation events occur in damaged or altered mucous membranes. Consequently, in mucosal diseases an unbalanced production of pro-inflammatory factors such as growth factors, cytokines, adhesion molecules and neuropeptides occurs (Rafiee et al., 2003). For example, mucosal membranes in the gut are constantly exposed to luminal antigens including bacterial lipopolysaccharide (LPS), a component of outer membrane of Gram-negative bacteria (Pang et al., 1994; Panja et al., 1998). The signalling events induced by LPS lead to the NFkB activation and to pro-inflammatory gene expression. In turn, NFkB regulates the expression of ICAM-1, a cell surface glycoprotein that plays a pivotal role in the recruitment of leucocytes at the sites of intestinal inflammation. Consequently, this adhesion molecule is up-regulated in the inflamed mucosa (Kim et al., 2005; Malizia et al., 1991; Strugess et al., 1990). In the *in vitro* model proposed in this study, the reduction of ICAM-1 expression in Caco-2 cells can be considered directly correlated to the mucosal anti-inflammatory effect.

In our work, various agents affecting mucosa had been chosen for their different nature and mechanisms of action. The well-known mucoadhesive hyaluronic acid and the anti-inflammatory hydrocortisone are used as positive control in *in vitro* experiments, whereas natural mucoprotective polysaccharides from *Opuntia ficus indica* (L.) and polyphenols from *Olea europaea* (L.) had been investigated. *Opuntia* polysaccharides are used as mucoprotective agent because they are able to form a protective layer on mucosal surface and to accelerate the re-epithelization of dermal wound (Galati et al., 2001, 2002; Wittschier et al., 2009); but in our knowledge not much is known about the adhesive capacity and the adhesive time of polysaccharides on mucosal cells. Recently, compelling scientific evidence supports the action of olive biophenols as protective agents against oxidative damage and inflammation associated to mucosal disorders (Min et al., 2005, 2006; Romier et al., 2009). In particular, Dekanski et al. (2009) pointed out the gastroprotective activity of olive leaf extract against cold restraint stress-induced gastric lesions in rat. It was generally accepted that the activity of olive phenols could be related to their antioxidant and radical scavenger properties (Mylonaki et al., 2008; Obied et al., 2007; Pieroni et al., 1996). However, further studies may be useful to investigate the ability of this active substances to affect mucosal inflammation processes.

2. Materials and methods

2.1. Materials

Polysaccharides extract from *O. ficus indica* (L.) cladode and polyphenols extract (contained 18% (w/w) of polyphenols determined by Folin-Ciocalteu method) from *O. europaea* (L.) leaf were kindly supplied by Bionap (Bionap, Italy). Streptavidin peroxidase, biotinylated concanavalin A from Canavalia ensiformis (Con-A), o-phenylenediamine dihydrochloride (o-pd), trypan blue, hydrogen peroxide, and other reagents of analytical or high-purity grade were purchased from Sigma–Aldrich (Italy); all solvents from Carlo Erba (Italy).

2.2. Cell cultures

Caco-2 cells were maintained in minimum essential medium (MEM) (Sigma–Aldrich, Italy) supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin, and incubated at 37 °C in a humidified, 95% air/5% CO_2 atmosphere. The medium was changed every 2–3 days.

2.3. Con A/o-pd assay

Con A/o-pd assay was based on that described by Cardile et al. (2008) and Patel et al. (1999). Caco-2 cells were trypsinized, washed in saline solution and divided into seven groups treated as follows: (1) control group (saline solution alone); (2) hyaluronic acid 0.1%; (3) hyaluronic acid 0.25%; (4) hyaluronic acid 0.5%; (5) natural polysaccharides 0.1%; (6) natural polysaccharides 0.25%; (7) natural polysaccharides 0.5%.

Each experimental solution (5 mL) was added to the cell suspension (2 mL) and incubated for 15 min at 30 °C under gentle shaking. The cells were then washed twice by adding 5 mL isotonic 0.05 M Tris buffered saline (TBS) followed by centrifugation at 2000 rpm for 5 min. After the second wash the cells were transferred to a clean tube and given a final wash. This step was necessary so that any material bound to the walls of the tube would not be carried over and interfere with the assay. The cells were sedimented by centrifugation at 2000 rpm for 5 min, after which all but 2 mL of the supernatant was removed. The residue was then vigorously stirred with a vortex mixer and washed by adding 12 mL isotonic 0.05 M TBS followed by centrifugation at 2000 rpm for 5 min. The washing step was repeated twice, after which the cells were transferred to a clean tube and given a final wash prior to the addition of the next reagent. 5 mL of 0.05 M TBS containing 1 mM calcium chloride and 10 mg/L biotinylated Con-A were added to the cells and the mixture was incubated at 30 °C for 30 min under gentle shaking. It was then centrifuged at 2000 rpm for 5 min and the supernatant was removed leaving 2 mL buffer. The cells were washed twice with TBS, and their suspension transferred to a clean tube. 5 mL of 0.125 M TBS containing 5 mg/L streptavidin peroxidase was added and each tube was incubated at 30 °C for 60 min under gentle shaking. The cells were then washed twice, transferred to a clean tube and washed again. 1 mL of o-phenylenediamine dihydrochloride (o-pd) solution (containing 0.4 mg o-pd and 0.4 μL 30% H_2O_2 in 1 mL 0.05 M citrate phosphate buffer) was added to each pellet, and the suspension was constantly stirred. The oxidation of o-pd was stopped after 2 min with 1 mL of 1 M H_2SO_4 after producing a yellow color and the optical density measured at 492 nm (spectrophotometer Genesis, Sigma–Aldrich, Italy).

2.4. Evaluation of adhesive time

The adhesion time of hyaluronic acid and natural polysaccharides was evaluated by using sets of eight Franz cells (Fig. 1). The Franz cell is constituted from a donor and a receptor chamber and membrane is placed between these two compartments. The receptor is thermostated at 36 °C by means of water circulation placed in an external shirt to mimic the real application. In our experiment, we have placed in donor the Caco-2 cells suspension treated with hyaluronic acid 0.5% and natural polysaccharides 0.5%. The donor has been fed with a continuous flow (0.5 mL/min) of saline solution, thermostated at 36 °C and constituted from an isotonic solution containing phosphate buffer, pH 7. For the circulation of saline solution, a peristaltic pump with eight channels was employed to serve