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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

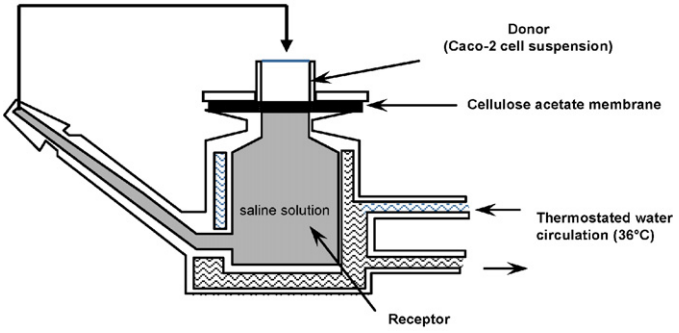

Fig. 1. Scheme of Franz cells used in the evaluation of mucoadhesive time.

as the entire battery of Franz cells. A cellulose acetate membrane was placed to the base of the donor, in the zone of separation with the receptor, to allow the outflow of the saline solution from the donor to the receptor, keeping the cells in the donor chamber. The saline solution flowed through the cells treated with hyaluronic acid or natural polysaccharides for 0, 30, and 60 min. At the end of each point time, the cells were transferred from the donor to appropriate test-tubes, treated with biotinylated lectin (Con-A) and then with streptavidin in presence of o-phenylenediamine dihydrochloride, in order to determine the activity of residual mucoadhesion after treatment with saline solution at different times. Results were expressed as a percentage reduction relative to the control. Each experiment, relative to a determined exposure time range to the saline solution flow, was run three times.

2.5. Western blot analysis

Caco-2 cells were stimulated or not (untreated controls) with LPS 1 μg/ml for 24 h in the absence or presence of hydrocortisone (10^{-5} M), polysaccharides (100 μg/mL), polyphenols (100 μg/mL), or both polysaccharides and polyphenols. Each sample was tested for the expression of intercellular adhesion molecule-1 (ICAM-1), and evaluated by Western blot analysis. Briefly, the untreated and treated Caco-2 cells were washed twice with ice-cold PBS and collected with lysing buffer (10 mm Tris–HCl plus 10 mm KCl, 2 mm $MgCl_2$, 0.6 mm PMSF and 1% SDS, pH 7.4). After cooling for 30 min at 0 °C, the cells were sonicated. Sixty micrograms of total protein present in the supernatant was loaded on each lane and separated by 4–12% Novex Bis-Tris gel electrophoresis (NuPAGE, Invitrogen, Italy). Proteins were then transferred to nitrocellulose membranes (Invitrogen, Italy) in a wet system. The transfer of proteins was verified by staining the nitrocellulose membranes with Ponceau S. The membranes were blocked in Tris buffered saline containing 0.01% Tween-20 (TBST) and 5% non-fat dry milk for 1 h at room temperature. Mouse monoclonal anti-ICAM-1 (1H4: sc-51632, Santa Cruz Biotechnology, Santa Cruz, CA) antibody (1:200) and mouse monoclonal α-tubulin antibody (Sigma, Milan, Italy) (1:5000) were diluted in TBST and the membranes incubated at 4 °C overnight. Antibodies were detected with horseradish peroxidase conjugated secondary antibody using the enhanced chemiluminescence detection Supersignal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL). Bands were measured densitometrically and their relative density calculated based on the density of the α-tubulin bands in each sample. The values were expressed as arbitrary densitometric units corresponding to signal intensity.

2.6. Statistical analysis

Statistical analysis was performed with statistical software package SYSTAT, version 9 (Systat Inc., Evanston, IL, USA). Each result was calculated as a mean value ± standard error (SEM).

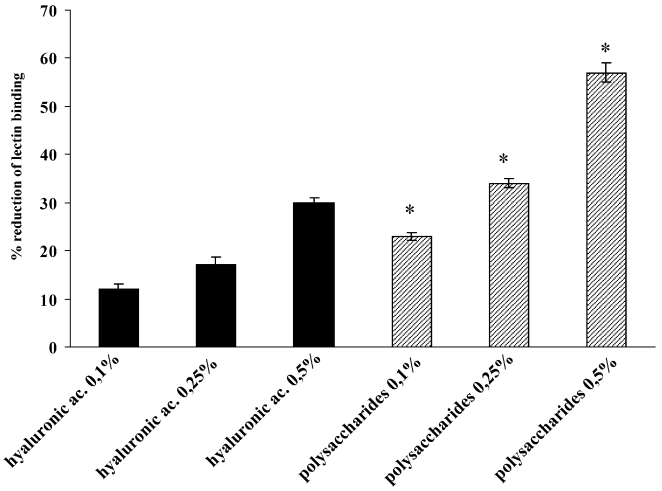

Fig. 2. Mucoadhesion of natural polysaccharides determined as % reduction of lectin binding on Caco-2 cells at different concentrations; *$p < 0.05$ vs hyaluronic acid.

Evaluation of the statistical significances was performed by Student's *t*-test. Values of $p < 0.05$ were considered to represent statistical significance.

3. Results

In this study, a technique predicting *in vivo* behaviour was used to evaluate the bioadhesive ability of active substances. The basis of the assay is as follows: if an experimental sample binds to mucosal cells, it will mask cell-surface glycoconjugates, therefore proportionally suppressing Con-A lectin binding. Lectin is a protein with a high affinity to glucosidic and mannosidic groups of membrane glycoproteins and it is artificially bound to the biotin; streptavidin peroxidise added to the cellular suspension binds to the biotin and form the complex protein–glucose–lectin–biotin–streptavidin-peroxidase. If streptavidin peroxidase is present (thus the complex protein–glucose–lectin–biotin–streptavidin-peroxidase) it catalyses the polymerization reaction to obtain the yellow coloration of the cellular sample, due to the production of 2,3-diaminofenazine (yellow) from o-phenylenediamine dihydrochloride. Moreover, if the cells are subjected to a continuous flow of physiological fluid by the use of a Franz cell system, the evaluation of yellow coloration in different time intervals is correlated to adhesive force between the cell surface and the bioadhesive substances. Our results showed a dose-depending mucoadhesivity of hyaluronic acid and natural polysaccharides from *Opuntia* cladode (Fig. 2). Reduction of lectin binding ranged from 12% to 30% for the hyaluronic acid and from 23% to 57% for herbal polysaccharides. Moreover, at the same concentration (0.5%, w/w), polysaccharides showed a higher lectin binding reduction in comparison to hyaluronic acid in different time intervals (Table 1). Hyaluronic acid, in fact, induced no lectin binding reduction after 60 min when subjected to a continuous flow

Table 1
Evaluation of adhesive time of hyaluronic acid and natural polysaccharides (0.5%, w/w) on Caco-2 cell line by Franz cell system and Concanavalina (Con) A/o-phenylenediamine dihydrochloride (o-pd) assay.

Time of treatment	Reduction in lectin binding (mean% ± SEM)		
	0	30 min	60 min
Hyaluronic acid	30 ± 1	10 ± 0.4	None
Natural polysaccharides	47 ± 2[a]	34 ± 2[a]	18 ± 1

Data are expressed as mean percentage reduction in lectin binding on Caco-2 cells relative to the control at different times (0, 30, 60 min).
[a] $p < 0.05$ compared to hyaluronic acid.

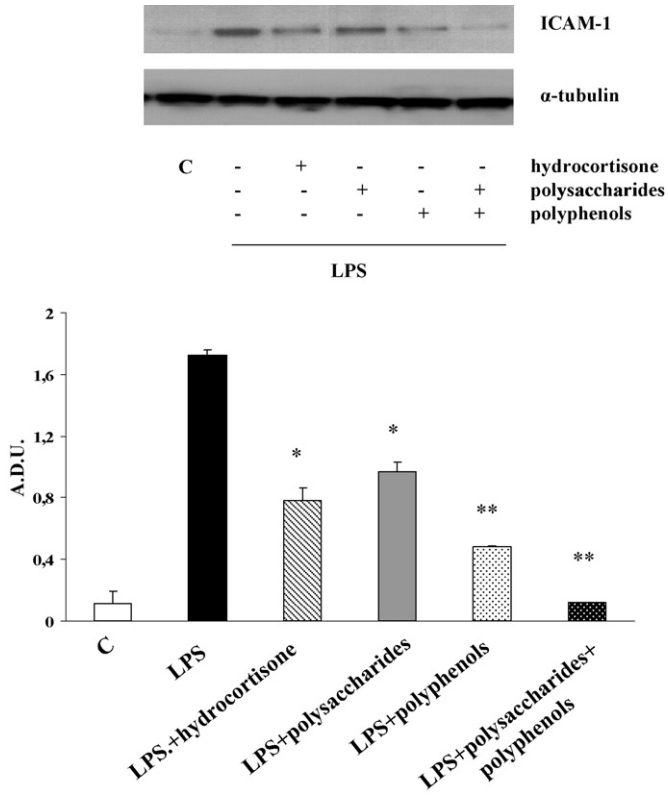

Fig. 3. Effects of natural polysaccharides and polyphenols on ICAM-1 expression induced by LPS on Caco-2 cells determined by Western blot analysis. Caco-2 cells were treated with LPS 1 μg/mL for 24 h in the presence of hydrocortisone (10^{-5} M), polysaccharides (100 μg/mL), polyphenols (100 μg/mL), or both extracts. Data show the relative expression (mean ± SEM) of ICAM-1 calculated as arbitrary densitometric units (A.D.U.) collected from three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$ compared with LPS-induced ICAM-1.

of physiological fluid. Finally, as expected, no mucoadhesive properties were attributed to olive polyphenols at any concentration (data do not showed).

About the anti-inflammatory cell model, untreated Caco-2 cells showed a very low expression of ICAM-1, whereas incubation of cell cultures with LPS 1 μg/mL for 24 h induced a strong expression of ICAM-1. Addition of hydrocortisone (positive control), natural polysaccharides and polyphenols induced a significant reduction of ICAM-1 expression in comparison with LPS treated cells (Fig. 3). Finally, to evaluate possible interaction between active substances, the inhibition of ICAM-1 expression was observed (Fig. 3). The exposure of Caco-2 cells to polysaccharides with polyphenols lead to strong inhibition of ICAM-1 expression, with values similar to control. The inhibition of the LPS-stimulated expression of ICAM-1 in Caco-2 cells by polyphenols and polysaccharides was not due to their cytotoxicity, as assessed by MTT assay (data not shown).

4. Discussion

Changes in structure or damage of the mucosal surfaces may result in severe diseases that significantly interfere with day activities and quality of life. Nowadays, the treatment of mucosal alterations involves the protection of the mucosa, reduction of the healing time and down-regulation of tissue inflammation (Rajendran and Kumar, 2010; Romier et al., 2009; Sturm and Dignass, 2008). Several agents are commonly proposed in the protection of mucosa but it is very difficult to investigate their mechanisms of action and to predict their efficacy *in vivo*. Common protocols proposed to evaluate 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 this research, we assessed for the first time some *in vitro* methods on Caco-2 cells with the aim to evaluate the physical protection and the anti-inflammatory action on the mucosa, two important strategies used in the treatment and prevention of mucosal diseases. Mucoadhesive models proposed in our study can quantify the adhesive capacity of samples and compare each others. A more similar condition to *in vivo* behaviour than traditional models was realized by the use of Franz cell system and the continuous flow of physiological fluid on the cell surface. Finally, anti-inflammatory effect was evaluated monitoring the expression of a marker specifically associated with mucosal inflammation events. Different active agents such as hyaluronic acid and polysaccharides from *Opuntia* as bioadhesive agents, hydrocortisone and olive polyphenols as anti-inflammatory agents have been investigated.

The models adopted herein showed a good capacity to compare and differentiate the activity of samples in correlation to their nature: hyaluronic acid and natural polysaccharides evidenced a good and prolonged adhesion to the cell surface even if they were subjected to continuous flow of physiological fluid. As expected, no bioadhesive property can be attributed to the polyphenolic substances.

About the anti-inflammatory activity assayed on Caco-2 cells, besides hydrocortisone, both natural substances were able to counteract the overproduction of inflammatory mediators correlated to mucosal diseases, even if a less activity was associated to *Opuntia* polysaccharides than olive polyphenols. Finally, an interesting activity was kept when polysaccharides and polyphenols were used at the same time on Caco-2 cells stimulated with LPS. The simultaneous presence of both extracts lead to a very significant reduction of ICAM-1 protein. Our results showed that the anti-inflammatory cell model was able to evaluate the effect of mixed active substances and their interaction on ICAM-1 expression.

Consequently, in agreement with current scientific knowledge, *Opuntia* polysaccharides mucoprotective activity could be mainly related to their bioadhesive proprieties on epithelial mucosa (Galati et al., 2001, 2002). As reported by Galati et al. (2001, 2002), treatment with *O. ficus indica* cladodes prevented the development of ethanol-induced ulcers (preventive treatment) or promoted a faster recovery time (curative treatment). It is known that natural polysaccharides can interact with proteins such as mucin and the polar head of membrane phospholipids. They could have a protective effect once they replace hydrogen bonds of water molecules, generating and increasing local viscosity (Vázquez-Ramírez et al., 2006). Results obtained in this work validated this hypothesis and the capacity of *Opuntia* polysaccharides to adhere on the cell surface and to form a physical protection showing a good resistance to the continuous contact with physiological fluid flow.

Another interesting result of our study is that, compared to hydrocortisone, polyphenols more efficiently blocked some pro-inflammatory actions of LPS on intestinal epithelial cells, reducing the expression of mediators strictly correlated to mucosal diseases (Kim et al., 2005; Malizia et al., 1991; Strugess et al., 1990).

As proposed by other authors (El and Karakaya, 2009; Mylonaki et al., 2008; Obied et al., 2007; Pieroni et al., 1996), polyphenols such as simple phenolic compounds, flavonoids, secoiridoids, cinnamic acid or lignans possess a strong anti-inflammatory and lenitive activity. From the results obtained, we can support that olive polyphenols are able to protect mucosa not only as simple antioxidant and radical scavengers but they can block LPS-induced NFkB activity of cells and, consequently, they can affect the inflammatory processes associated with mucosal diseases.

5. Conclusion

In conclusion, the *in vitro* techniques proposed in this study using Caco-2 cell line as a model can be considered simple and rapid preliminary methods useful to predict the *in vivo* behaviour and able to evaluate the protective effect of new substances on the mucosa.

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