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Inflammatory Bowel Disease (IBD): a novel biological role of saffron petal extracts as a modulator of phlogistic pathway *via* FBW7/ NF-kB in Caco-2 cell line LPS-stimulated

Federica De Cecco*, Sara Franceschelli, Lorenza Speranza

Department of Medicine and Aging Sciences, University "G. d'Annunzio" Chieti- Pescara, Via dei Vestini 31, 66100 Chieti, Italy
*Corresponding author. E-mail: federica.dececco@unich.it

Abstract. Inflammatory bowel disease (IBD) is a chronic pathology characterized by extensive inflammation, which causes a functional alteration of the intestinal barrier. Today most of the drugs applied for IBD have adverse consequences. In this scenario, the development of new therapeutic agents for the treatment of IBD is necessary. A new approach to develop effective therapeutic strategies is the study of natural compounds with anti-inflammatory properties. Saffron petals contain flavonolic glycosides (kaempferol), carotenoids (crocin and crocetin) and anthocyanin pigments, with antioxidant and anti-inflammatory activity. Recently, kaempferol and crocin identified in Saffron Petal Extract (SPE), has been able to reduce oxidative stress in intestinal epithelial cells. Our aim was to evaluate the therapeutic potential of SPE on inflamed human intestinal Caco2 cells that mimic the intestinal microenvironment. We have demonstrated that SPE, down-regulating the expression of the ubiquitine FBW7, inhibits the degradation of the IKB-α subunit and keeps NF-κB in the inactive state. This leads downstream to a reduced activation of inducible molecules (iNOS, COX-2 and HO-1) involved on intestinal inflammatory process. In conclusion, since FBW7 increases in colon tissue of IBD patients, SPE may represent an attractive and promising supplementary treatment for the therapeutic management of IBD with conventional therapies.

Keywords: IBD, inflammation, intestinal epithelial cells, saffron petals extract.

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic, relapsing inflammatory disorder of the gastrointestinal tract resulting from a loss of homeostasis between the gut immune system and the gut microbiome (Chang JT). The pathophysiology of IBD involves genetic, environmental, epithelial, microbial and immune factors (Hyun CK).

Effective treatment of IBD is one of the most challenging health problems for humans worldwide. New treatment options for IBD are continually

being explored. Chemically synthesized drugs are always accompanied by adverse reactions (Cai Z, et al.). In the search for new pharmacologically active substances, natural ingredients, as saffron and their extracts, are receiving great attention. The *Crocus sativus* plant, saffron, is known for several medicinal uses (Butnariu, et al.). The petals are discarded, but possess flavonolic glycosides (kaempferol), carotenoids (crocin and crocetin), anthocyanin pigments, and other bioactive compounds with anti-inflammatory and other therapeutic effects (Cerdá-Bernad D, et al.). It has been shown that the F-box and WD repeat domain-containing protein 7 (FBW7) was augmented in colon tissues from IBD patients. Whether and how FBW7 participates with IBD remain unknown (Meng Q, et al). Therefore, this study investigated the possible effect of Saffron Petal Extract (SPE) on FBW7 pro-inflammatory pathway using a Caco-2 intestinal epithelial cells that mimic the intestinal microenvironment.

MATERIALS & METHODS

Cell culture

The human colon adenocarcinoma Caco-2 cell line (ATCC® TIB-202™ Rockville, MD, USA) was cultured as previously reported by (Wu XX, et al.).

Cytotoxicity assay

The Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay (Sigma-Aldrich, St. Louis, MO, USA) was performed, as previously described (Franceschelli S, et al.), to assess the cell viability of Caco-2 cells treated with different concentrations of SPE (from 50ng/ml to 1mg/ml) provided by Hortus Novus (L'Aquila, Abruzzo, Italy) after 24h and 48h.

ROS Detection

An NBT (nitroblue tetrazolium) assay was performed as previously described to detect intracellular ROS levels (Franceschelli, S. et al.).

RNA extraction, reverse transcription, and Real-Time PCR

Cells were collected in 1mL QIAzol lysis reagent (Qiagen, Hilden, Germany), total RNA extraction and Real-Time PCR was performed as previously described (Pesce M, et al.).

Western Blot Analysis

Western blot analysis was performed as described previously (Patruno A et al.) using the following antibodies against ICAM (OTI1E5; 1:700), IKB alpha (NFKBIA) (OTI1D4; 1:400), FBXW7 (OTI6B1; 1:1000), COX-2 (ab52237; 1:500), and β-actin (Santa Cruz Biotechnology).

Statistical analysis

Quantitative variables are summarized as the mean value and standard deviations (SD) in the Tables and Figures. To assess the accuracy of fold change data, the 95% confidence interval (95% CI) and standard error (SE) were determined. A Student's t-test for unpaired data was applied to evaluate the significance of differences. All tests were two-tailed. The threshold of statistical significance was set at $p=0.05$. Data analysis was performed on GraphPad Prism 6 Software, version 6.01, 2012.

RESULTS

SPE does not affect the viability of intestinal cells

To determine the non-toxic concentrations of SPE, we examined the cell viability (at 24h and 48h) on Caco-2 cells treated with different concentrations (see Fig. 1A,B). SPE did not affect the viability cells (Fig. 1A,B). Furthermore, the superoxide anion (O_2^-) radical-scavenging activity was also measured in a non-enzymatic method at 24h. The generation of O_2^- was markedly inhibited (~50%) from the concentration of 50 µg/ml in respect to cells LPS-stimulated (Fig. 1C). Thus, SPE at 50 µg/ml was more often used in the following experiments to test its anti-inflammatory activity.

Effect of SPE on inducible proteins

To strengthen our hypothesis about the effect of SPE on intestinal epithelial cells, the expression of inducible proteins, known to be controlled by NF-kB and up-regulated in the inflammatory state of IBD, were checked. SPE-treatment induces a down-regulation of both mRNA and proteins expression of iNOS (Fig. 2A), COX-2 (Fig. 2B) and HO-1 (Fig. 2C) compared to activated cells.

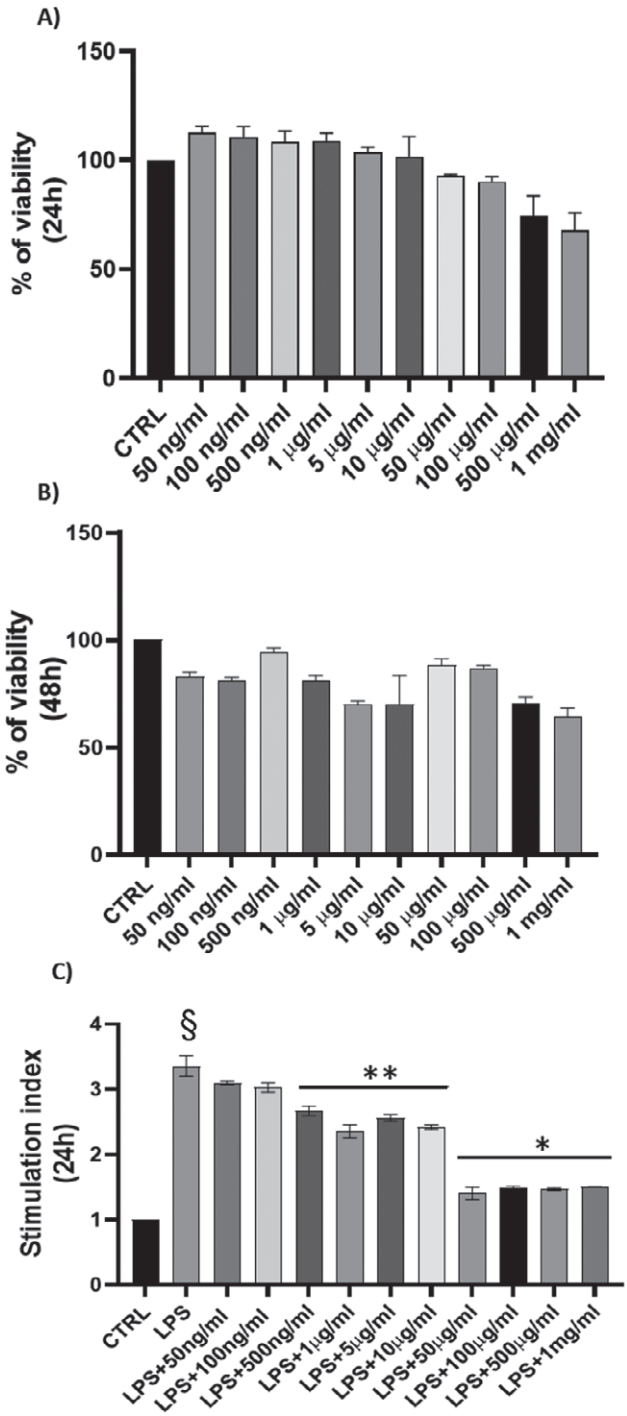

Figure 1. Cytotoxyc effect of SPE on Caco-2 cells. Cells were treated with SPE for 24h (A) or 48h (B). Cells viability was measured by MTT assay as reported in Materials and Methods. Date are reported as % of viability in respect to control cells. Each bar represent means ±SEM (n=3); (C) Antioxidant activity of SPE against oxidative stress LPS-induced measured by NBT test. Results were registered as stimulation index (SI). SI value of 1 was assigned to control cells. Each bar represent means ±SEM (n=3). §p<0.005 vs CTRL; **p<0.05 and*p<0.01 vs LPS.

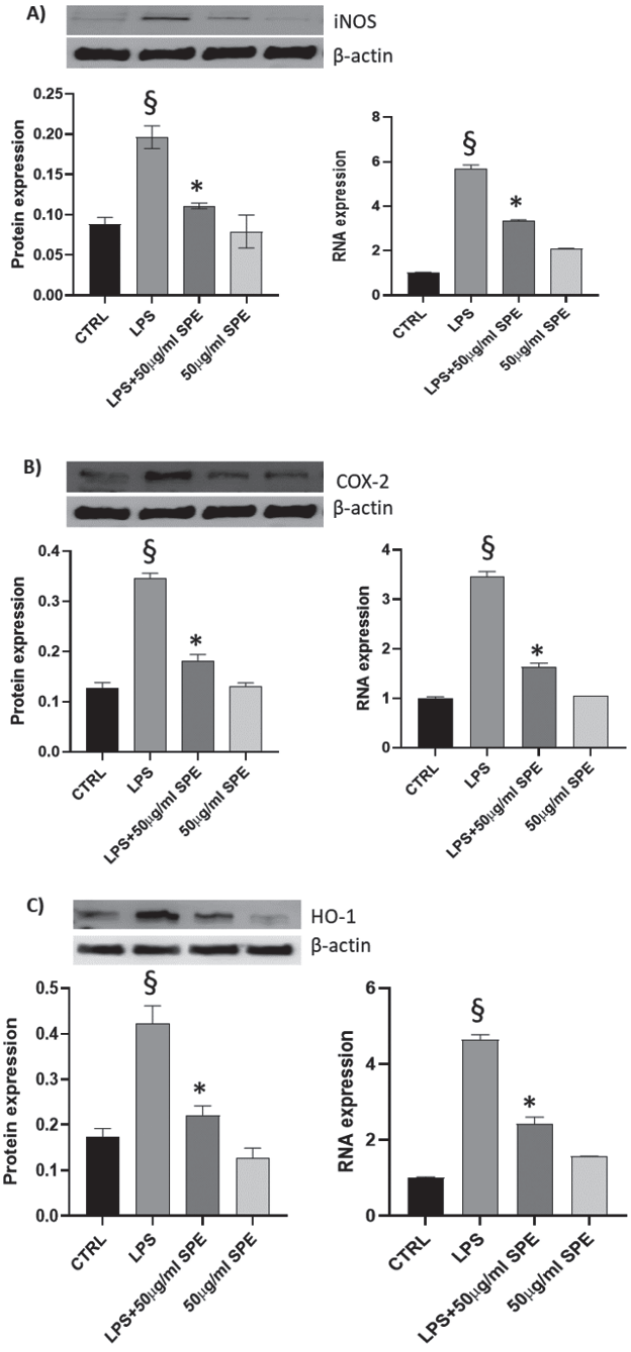

Figure 2. Effect of SPE on inducible molecules in Caco-2 cells. Representative image of Western blot analysis with relative densitometry and real-Time PCR analisys for iNOS (A), COX-2 (B), and HO-1 (C). To the left, in the densitometric analysis (n = 3), each bar is reported as the intensity of optical density (IOD) ± SD. The following primer pair sequence was used: iNOS (F:5'- CATTGCTGTGCTCCATAGTTTC-3'-, R:5'- CAGGACGTAGTTCAGCATCTC-3'); COX-2 (F:5'-CGATGCTGTGGAGCTGTAT-3'; R:5'-CATTGCTGTGCTCCATAGTTTCG-3'); HO-1 (F:5'- CCAGCAACAAAGTGCAAGAT -3'; R: 5'-TCCACCGGACAAAGTTCAT-3'). §P < 0.05, vs control cells and *P < 0.05 vs LPS-stimulated cells.

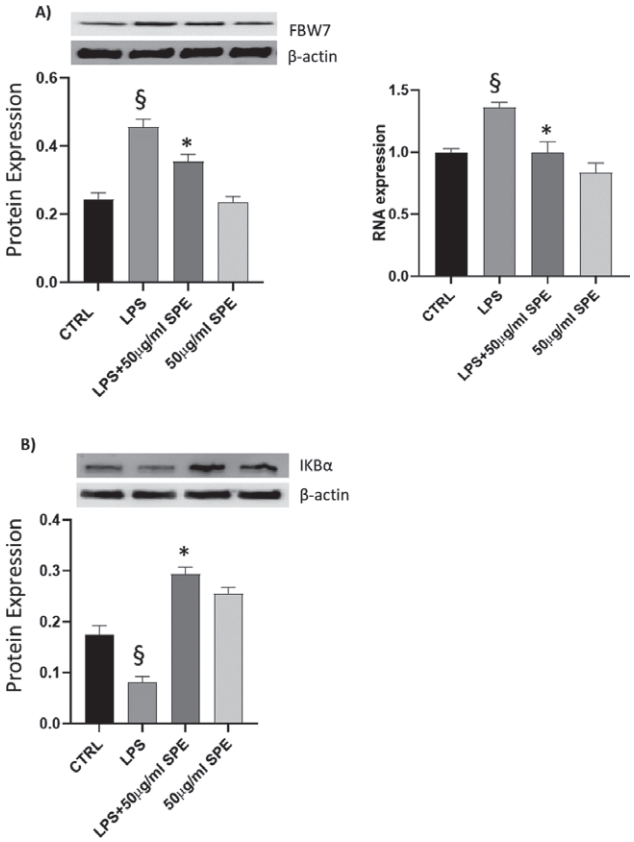

Figure 3. Effects of SPE on FBW7/ IKB-α Signaling in Caco-2 cells. Representative image of Western blot analysis for FBW7 (A) and IKB-α (B). To the left, in the densitometric analysis (n = 3), each bar is reported as the intensity of optical density (IOD) ± SD. §$P < 0.05$, significance vs control cells. For qRT-PCR analysis, the following primer pair sequence was used: FBW7 (F:5'-CAGTCCGCTGTGTTCAATATG-3', R:5'-GCCCTGTTAACGTGTGAATG-3'); 18S (F:5'- CTTTGCCATCACTGCCATTAAG -3', R:5'-TCCATCCTTTACATCCTTCTGTC-3'). *$P < 0.05$ significance vs LPS-stimulated cells.

Effect of SPE FBW7/NF-κB signaling

We study the effect of SPE on FBW7, a novel E3 ubiquitin ligase for IKBα related to NF-kB activation and intestinal inflammation. Protein and mRNA analysis of FBW7 were significantly up-regulated in cells LPS-stimulated confirming its role in regulating the inflammatory response. SPE-treatment induces a down-regulation of the FBW7 expression. This could make us think of its possible involvement in the negative regulation of a process of inflammation. IKBα level was significantly expressed by SPE-treatment respect to cells LPS-activated, making us hypothesize that SPE exerts its negative effect *via* NF-kB/FBW7 pathway.

DISCUSSION

IBD is a chronic, relapsing inflammatory disorder of the gastrointestinal tract resulting from a loss of homeostasis between the intestinal immune system and the gut microbiome in genetically predisposed individuals (Chang JT).

Despite the incredible progress of modern medicine, significant obstacles remain in the treatment of IBD. There is high interest in alternative natural agents in the management of IBD (Duan L, et al.). Saffron has been used for health management since ancient times for its several pharmacological effects as well as peptic ulcer and ulcerative colitis (Butnariu M, et al.). Recent studies have demonstrated the efficacy of SPE as a protective agent against several diseases (Maccarone R, et al.). For the first time, we demonstrated that the SPE significantly reduce the expression of FBW7, involved on regulation of inflammatory pathways, in activated Caco-2 cells.

SPE induce a significative reduction of the ROS production (Fig. 1C), as well as the expression of inducible iNOS, COX-2 and HO-1 proteins (Fig. 2). Nuclear factor κB (NF-κB), a transcriptional factor, is the hallmark of inflammatory response and several studies have reported that NF-κB activation contribute to colitis. Upon stimulation with LPS, the inhibitor of κB (IκB) kinase (IKK) complex activates leading to phosphorylation and ubiquitin-dependent degradation of NF-κB inhibitory protein IκBα (Franceschelli S, et al.). This allows NF-κB to translocate into the nucleus and trigger a variety of target genes transcription as iNOS, COX-2 (Pesce M, et al.). FBW7 is a ubiquitin ligase that regulate the development of colorectal cancer and has recently emerged as an important regulator of NF-κB in intestinal inflammation (Zhang Z, et al.). Our results evidenced that SPE, down-regulating the expression of the ubiquitine FBW7, inhibits the degradation of the IKBα subunit and keeps NF-κB in the inactive state (Fig. 3). Our findings demonstrate that the SPE inhibited the expression of pro-inflammatory proteins of iNOS, COX-2 and HO-1 in LPS-stimulated Caco-2 cell line by suppressing the NF-κB activation *via* FBW7. Since proteins degradation plays a critical role in the pathogenesis of human diseases (Hanna J, et al.), the inhibition of FBW7 by the SPE could alleviate a multitude of NF-kB-driven inflammatory disease as IBD.

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