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Bovine and soybean milk bioactive compounds: effects on inflammatory response of human intestinal Caco-2 cells.

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ABSTRACT

In this study, the effects of commercial bovine and soybean milks and their bioactive compounds, namely genistein, daidzein and equol, on the inflammatory responses induced by lipopolysaccharide (LPS) treatment of human intestinal Caco-2 cells were examined in terms of nitric oxide (NO) release and inducible nitric oxide synthetase (iNOS) expression.

Both milks and their bioactive compounds significantly inhibited, in a dose-dependent manner, the expression of (iNOS) mRNA as well as protein expression, resulting in a decreased NO production. In addition, the activation of NF-κB in LPS-stimulated intestinal cells was examined. In all cases it was observed that cell pre-treatment before LPS activation inhibited the phosphorylation, as well as the degradation, of the IkB complex. Accordingly, quantification of bioactive compounds by solid phase microextraction coupled with liquid chromatography has shown that they were absorbed, metabolized and released by Caco-2 cells in culture media.

In conclusion, we have demonstrated that milks and compounds tested are able to reduce LPS-induced inflammatory responses from intestinal cells, interfering with the activation of NF-kB dependent molecular mechanisms.

Keywords: Bovine and Soybean milks; isoflavones-aglycones; equol, Caco-2 cells; SPME-HPLC-UV/DAD.

1. INTRODUCTION

The mucosal surface of the intestinal tract is a complex ecosystem combining the gastrointestinal epithelium, immune cells and resident microbiota. The intestinal mucosa is not only involved in absorbing nutrients, but it is constantly exposed to an array of foreign antigens. Robust protective immune responses must be generated in response to pathogenic insult, leading to the production of mediators (cytokines, growth factors, adhesion molecules, etc.) which promotes and amplifies the inflammatory response (Kaser et al., 2010).

Whereas an adequate inflammatory response is essential to protect the host from pathogens attack, an excessive production of inflammatory mediators may alter the homeostasis of bowel epithelium. Inflammatory bowel disease (IBD), including Crohn's disease (CD), and ulcerative colitis (UC), are chronic inflammatory disorders of the gut with complex etiologies (Kaser et al., 2010). One of the worst complications of IBD is the ultimate development of colon cancer. Therefore, the employments of agents that block the inflammatory responses have been particularly successful in IBD therapy. Currently there is no cure for IBD, thus the search for new molecules able to control IBD and their delivery to the site of inflammation are the goal of many researchers.

Flavonoids, natural phenolic compounds, are normal constituents of the human diet and are known for a variety of biological activities. Some of them act as enzyme inhibitors and antioxidants, and have been reported to have anti-inflammatory properties. However, the molecular mechanisms explaining how flavonoids suppress the inflammatory response are not yet known in detail (Panaro et al., 2012; Cianciulli et al., 2012; Havsteen, 2002).

Genistein, an isoflavone very abundant in soy (*Glycine max*), has been found to play an important role in the prevention of various chronic diseases including cancer (Sarkar and Li, 2003). Various epidemiological, *in vitro* and animal studies have evaluated the anti-cancer, cardio-protective, anti-osteoporotic, antioxidant, anti-inflammatory activities of genistein. It has been also associated with the inhibition of tyrosine-specific protein kinase in malignant cells (Caldarelli et al., 2005; Zhang et

al., 2008; Park et al., 2010; Rusin et al., 2010). It was reported that genistein modulates the expression of NF-κB and MAPK (p-38 and ERK1/2), thereby attenuating D-Galactosamine induced fulminant hepatic failure in Wistar rats (Ganai et al., 2015)

Like genistein, daidzein is an isoflavone physiologically active that occurs mainly in free and glycosylated form in soy. Isoflavone glucosides of daidzein and genistein, namely daidzin and genistin, are more abundant and soluble but less active than free forms (Di Cagno et al., 2010). In the gastrointestinal tract, each sugar-conjugated is hydrolysed by isoflavone β-glucosidase from gut bacteria to release the aglycone and sugar part. Then, all free forms are rapidly absorbed, metabolized or reconjugated with sugars by intestinal epithelial cells to assure their utilization or removal by the cells (Di Cagno et al., 2010). An interesting metabolite of daidzein is equol because it has a greater structural similarity to estrogens than its parent (Antignac et al., 2004; Di Cagno et al., 2010; Andres et al., 2011). It produces major effects on vascular reactivity, thrombosis, lipid profiles and cellular proliferation (Andres et al., 2011).

As a result, it can be hypothesized that the dietary intake of isoflavones may be helpful in the treatment of IBD. In this regard, bovine milk has been reported to have beneficial effects on gastrointestinal health both in infants and in adults (Haug et al., 2007). Moreover, soy flavonoids may have some benefits for people with intestinal inflammation beyond simply nutritional support. Similarly, soy milk, the most popular alternative to cow's milk, could also possess anti-inflammatory activity, which is suitable in IBD therapy.

The aim of this study is investigating the potential anti-inflammatory effects of bovine and soybean milks, as well as of the equol, daidzein and genestein using an *in vitro* model of human intestinal epithelial cells, Caco-2 cell line, submitted to Lipopolysaccharide (LPS) treatment as pro-inflammatory stimulus. LPS, the principal component of the outer membrane of Gram-negative bacteria, plays a pivotal role in triggering an early inflammatory response through the interaction of several receptors for microbial products.

2. MATERIALS AND METHODS

2.1.Cell cultures and treatments

Caco-2 human colorectal cancer cells (ICLC HTL 97023) were obtained from Interlab Cell Line Collection (Genoa, Italy). The cells were cultured in MEM medium supplemented with 10% fetal bovine serum (FBS, UE approved origin), 100 U/mL penicillin, 100 μg/mL of streptomycin, L-glutamine (2 mM), 1% nonessential amino acids (NEEA), referred to as complete medium (all reagents were purchased from Life Technologies-Invitrogen, Milan, Italy).

Cultures were maintained at 37° C in an atmosphere with humidity containing 5% CO_2 and expanded in tissue culture flasks (75 cm^2, BD Bioscences, USA), changing the medium daily. Caco-2 cells were grown up to 80% confluency in 6-well cell culture plates.

For the experiments, cells were treated with *Salmonella enterica* serotype typhimurium LPS (Sigma). Preliminary experiments were performed in order to establish the optimal dose (1 μg/mL) of LPS and time of exposure to LPS (48 h). Before LPS stimulation, some wells were pre-treated with different concentrations (5, 10, 50 μM) of daidzein (4′,7-dihydroxyisoflavone, Sigma), genestein (4′, 5, 7-trihydroxyisoflavone, Sigma) and equol (dihydroxyisoflavone, Sigma) or with different amounts (10, 30 and 50 μL) of the commercial (bovine or soy) milks. After 1h of incubation at 37°C, cell cultures were then stimulated with endotoxin as previously indicated. Untreated cells were used as control.

2.2. Cell Viability Assay

In order to test cells viability, we used MTT (3,4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Briefly, cells (2.5×10^4) were seeded in a 96-well plate (BD Biosciences, USA). After cell treatment, culture media were carefully removed and 100 μL of 0.5 mg/mL MTT in cell culture medium was added to each well and incubated for 4 h. Finally, 100 μL of 10% SDS, 0.01 M HCl solution was added to each well to dissolve the formed formazan

crystals. The amount of formazan was determined measuring the absorbance at 560 nm using a microplate reader.

2.3. Quantitative analysis of equol, daidzein and genistein from different samples by SPME-HPLC-UV/DAD method

In order to clarify the role of the isoflavones in the anti-inflammatory responses observed *in vitro,* their concentration has been determined both in cell culture media and cell lysates, as well as in bovine and soybean milk samples, by solid-phase micro-extraction coupled to liquid chromatography (SPME-LC).

Milk was diluted 1:100 with a 0.2% formic acid - 30% (w/v) NaCl solution and stirred for 30 min before SPME. An enzymatic deconjugation was accomplished to estimate the total amount of each isoflavone-aglycone. A solution (0.75 mL), containing 10 mg/ml β-glucosidase (≥2 units/mg solid, Sigma-Aldrich, Milano, Italy) in acetate buffer (0.1 M, pH 5.0), was added to 0.75 ml of milk, and incubated overnight at 37◦C. Finally, 30 µL, pure or diluted (1:10), were subjected to SPME. Culture media, taken from wells after 48 h, were diluted 1:10 with 0.2% formic acid with 30% NaCl before SPME analysis. Analytes extraction from the cell lysate supernatant was performed by diluting an aliquot of each sample 1:4 with saline after protein total assay. Then, variable aliquots were transferred into a vial containing the usual extraction mixture, in order to have 1.5 mL of solution at proteins total concentration of 0.025 mg/mL to be submitted to SPME analysis.

The SPME step was conducted in stirred solutions for 20 min using a 65 µm PDMS-DVB fiber (Supelco, Bellefonte, PA, USA). Desorptions were performed for 15 min in mobile phase in the SPME interface before switching the injector valve in the inject position.

The LC-UV system was composed by a P2000 pump coupled with a UV6000LP photodiode array (all Thermo Finnigan, San Jose, CA), a SPME manual interface (Supelco, Bellefonte, PA, USA), a KinetexTM C18 (2.6 µm, 100 x 4.6 mm i.d., Phenomenex, USA) column.

142 Instrumental conditions were as follows: isocratic elution using 0.05 % (v/v) formic acid in
143 acetonitrile/methanol/water (8:32:60, v/v/v) as mobile phase, flow rate 0.7 ml/min; detection
144 wavelengths were 260 and 280 nm (5 nm band-width).
145 The quantification of the analytes in bovine and soybean milk samples was performed with the
146 standard addition method.
147 Fortified samples were equilibrated at 37 °C in a water bath for 30 min before being processed as
148 described. Three replicates for each concentration, including blank, were made.
149 Calibration curves for the target analytes in Caco-2 cells culture media were constructed in the
150 range 0.015 - 20 μM. Fortified samples were diluted 1:10 in 0.2% formic acid at 30% NaCl before
151 analysis.
152 *2.4. Nitric oxide (NO) production*
153 Nitrite, a stable end-product of NO metabolism, was estimated using the Griess reaction. After
154 treatment of Caco-2 cells with LPS for 48 hours, culture media were collected and mixed (1:1v/v)
155 with the Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylenediamine dihydrochloride, and
156 2.5% phosphoric acid) for 10 min at room temperature, followed by spectrophotometric
157 measurement at 550 nm. Nitrite concentrations in the culture media were determined by comparison
158 with a sodium nitrite standard curve and expressed as μmol/mL. To avoid interference by nitrites
159 possibly present in the medium, in each experiment the absorbance of the unconditioned medium
160 was assumed as the "blank".
161 *2.5. Electrophoresis and Western Blotting*
162 After treatments, cells were lysed with lysis buffer [1% Triton X-100, 20 mM Tris-HCl, 137 mM
163 NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μM leupeptin
164 hemisulfate salt, 0.2 U/ml aprotinin (all from Sigma-Aldrich)] for 30 min on ice. The lysate was
165 vortexed for 15-20 sec and then centrifuged at 12,800x g for 20 min; the protein concentration in
166 the supernatant was spectrophotometrically determined with Bradford's protein assay (Bradford,

1976). Protein samples were diluted with sample buffer (0.5 M Tris HCl pH 6.8, 10% glycerol, 10 % w/v SDS, 5% β2-mercaptoethanol, 0.05% w/v bromophenol blue) and then boiled for 3 min. Proteins (25 μg/lane) and prestained standards (BioRad Laboratories, Hercules, CA, USA) were loaded on 7% SDS precast polyacrylamide gels (BioRad Laboratories).

After electrophoresis, the resolved proteins were transferred from the gel to nitrocellulose membranes. A blotting buffer [20 mM Tris/150 mM glycine, pH 8, 20% (v/v) methanol] was used for gel and membrane saturation and blotting. A blocking solution [bovine serum albumin (BSA), 0.2%–5% (w/v), Tween-20,(0.05–0.1%), non fat dry milk (0.5–5%), Casein(1%), all from BioRad Laboratories] was used in order to prevent nonspecific binding of unoccupied membrane sites. Then, membranes were incubated in the dark with (1:200 diluted) primary antibody anti human NOS II, anti human IKB α anti human pIKB α (all from Santa Cruz Biotechnology, Heidelberg, Germany), for 60 min at room temperature. The membranes were washed with T-PBS (for 20 min, 3 times) and then incubated with the secondary antibody anti-human IgG diluted 1:2000, horseradish peroxidase (HRP)-conjugate (Santa Cruz Biotechnology) for 60 min. Bands were visualized with the chemiluminescence method (BioRad, Laboratories).

2.6. Densitometric analysis

The bands obtained after immunoblotting were submitted to densitometric analysis using ID Image Analysis Software (Kodak Digital Science). Results were expressed as arbitrary units.

2.7 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and quantitative real-time PCR analyses.

Briefly, total tissue RNA was extracted from treated and untreated cells by the Trizol isolation reagent (Invitrogen, Milan, Italy) according to the manufacturer's instructions. Reverse transcription was performed in a final volume of 20 μL containing 3 μg of total RNA, 40 U of RNase Out (Invitrogen), 40 mU of oligo dT with 0.5 mM dNTP (PCR Nucleotide Mix, Roche Diagnostics, Milan, Italy), and 40 U of Moloney Murine Leukemia Virus Reverse Transcriptase

192 (Roche Diagnostics). The reaction tubes were incubated at 37°C for 59 min, and then at 95°C for 5
193 min and at 4°C for 55 min. The cDNA obtained was then amplified by a thermal cycler (Eppendorf,
194 Milan, Italy) under the following conditions: 95°C for 1 min, 55°C for 1 min and 72°C for 1 min
195 (30 cycles of amplification). The reaction tube contained, in a final volume of 50 μL, 2 μL of
196 cDNA, 200 μM dNTP (PCR Nucleotide Mix, Roche Diagnostics), 4 U TaqDNA Polymerase
197 (Roche Diagnostics), 5μL of $MgCl_2$ buffer stock solution, and 50 pmol of the specific primers.
198 The mRNA levels of various genes were quantified using the SYBR Green QuantiTect RTPCR Kit
199 (Roche, South San Francisco, CA, USA). β-actin was used as endogenous reference. Data were
200 analyzed using the relative standard curve method according to the manufacturer's protocol. The
201 mean value of each gene after β-actin normalization at the time point showing the highest
202 expression was used as calibrator to determine the relative levels. The primers used for
203 amplification for iNOS (NCBI reference sequence NM_000625.4) were: forward primer 5'-
204 CGGCCATCACCGTGTTCCCC-3'; reverse primer, 5'-TGCAGTCGAGTGGTGGTCCA-3'; and
205 for β-actin forward primer, 5'-GGCGGCACCACCATGTACCCT-3'; reverse primer, 5'-
206 AGGGGCCGGACTCGTCATACT-3'.

207 *2.8. Data Presentation and Statistical Analysis*

208 Student's t test and analysis of variance (one-way ANOVA) on the results of at least five
209 independent biological replicates were performed. Values of $p < 0.05$ were considered statistically
210 significant.

211 **3. RESULTS**

212 *3.1. Viability test*

213 The MTT assay was used in order to test the viability of cells exposed to isoflavones and/or
214 endotoxin treatment. Low concentrations of all tested isoflavones resulted no toxic for Caco-2 cells
215 in comparison with untreated cells, whereas relatively higher concentration (50 μM) were resulted
216 cytoxic (Fig. 1, Panel A). We also found that the viability of cells exposed for 48 h to 1 μg/mL LPS

217 was significantly reduced in comparison to controls (Fig. 1, Panel B). Interestingly, the viability of
218 LPS stimulated cells was significantly improved after 1h pre-treatment with isoflavones, in a dose-
219 dependent manner (5-10 μM) (Fig. 1, Panel C). Cell viability of LPS stimulated cells resulted
220 significantly improved by treatment with tested milks, in a dose dependent manner (data not
221 shown).

222 *3.2. Quantitative analysis of equol, daidzein and genistein from different samples by SPME-HPLC-*
223 *UV/DAD method*

224 To clarify the role of equol, daidzein, genistein in the anti-inflammatory responses, firstly, their
225 concentration in bovine and soybean milks was determined by a SPME-HPLC analysis; then, *in*
226 *vitro* experiments were performed and compounds were also quantified in culture media and Caco-2
227 cells lysates, slightly modifying the previously validated method for the quantification of
228 compounds in milk.

229 Table 1 shows concentrations of analytes in milks before and after β-glucosidase deconjugation.

230 **Insert Table 1**

231 As evident, the predominant natural forms are sugar-conjugated, and lower concentrations of
232 aglycones were found in cow's milk compared to soy. Equol was never detected in the two milk
233 types.

234 Calibration curves were obtained for quantitative analysis of compounds in culture media.
235 Therefore, the method was validated in terms of linearity, limits of detection (LOD), quantification
236 (LOQ), precision, and repeatability.

237 The responses were found linear in the range of 0.05-15 μM for all analytes, with correlation
238 coefficients always >0.999 and intercepts not significantly different from zero at the 95%
239 confidence level.

240 The estimated limits of detection (LOD) and quantification (LOQ) obtained, calculated as three and
241 ten fold the standard deviation of the intercept of each calibration curve, were 0.02 μM for daidzein

242 and genistein and 0.03 μM for equol, and 0.07 μM for daidzein and genistein and 0.10 μM for
243 equol, respectively.
244 The precision of the method was investigated at the concentration levels of 0.5, 5 and 10 μM, by
245 performing daily three replicates (within day). The same solutions were analyzed for three times
246 each day, for a period of five days (between days). The within-day and between days precision of
247 the method, estimated by an ANOVA test, ranged from 3.1 to 3.7% and from 5.1 to 6.7%,
248 respectively. Average amounts of compounds in 5 or 10 μM culture media, picked up after 48 h
249 incubation by wells with Caco-2 cells plus LPS, were 23.0 ± 2.0 16.0 ±1.8 and 2 ±0.2 % of
250 estimated quantities in corresponding control wells (without Caco-2 cells and with LPS) for equol,
251 daidzein and genistein, respectively.
252 To certify absorption analytes by cells, equol, daidzein and genistein were checked in related
253 supernatants of cellular lysates. Given the complexity of the matrices, the method of standard
254 additions was used for SPME analysis.
255 Caco-2 cells pretreatments have produced the results listed in table 2, where concentrations of
256 compound are expressed in relation to total proteins estimated in each sample analyzed.
257 All amounts estimated are lower than expected, thus demonstrating their probable involvement in
258 cellular responses at stimulus.
259 In case of cellular pretreatments with soybean milk, the concentrations of isoflavones-aglycones in
260 culture media were found higher than original levels. For example, in culture media by Caco-2 cells
261 pretreated with 50 μl of soybean milk before of the stimuli with LPS, daidzein and genistein were
262 estimated at levels 2.54±0.09 and 2.67±0.10 μM, instead 0.23 ± 0.01 and 0.05 ± 0.01 μM of control,
263 respectively. Whereas, in respective cellular lysates, only genestein was found at level of 2.29
264 nmoles/mg of total proteins. Similar results were obtained also with the other amounts tested (10, 30
265 μL) of soy sample.

Insert Table 2

However, isoflavones-aglycones were not detected in samples related to bovine milk pretreatments, probably due to their lower biological contents in cow's milk.

3.3. NO production and iNOS expression.

Undetectable levels of NO production were observed in cell cultures treated with isoflavones alone (data not shown). In Caco-2 cells, LPS treatment significantly increased the level of NO as compared to untreated cells (control). A significant reduction ($p<0.01$) of LPS-induced NO production was observed in cells submitted to 1h pre-treatment with isoflavones in comparison to cells treated with LPS alone; this effect was dose-dependent, where the maximal reduction ($p<0.01$) was observed at 10 μM, as shown in Fig.2 (Panel A). In particular, from our results it emerged that both daidzein and genistein are more efficacious than equol in the reduction of LPS induced NO release.

We also investigated the effect of milks (bovine and soy) incubation on the NO release after LPS stimulation. Also in this case, 1h pre-treatment with milks, reduced LPS-induced NO production in a dose-dependent manner; the maximal reduction ($p<0.01$) was observed with 50 μL milk (Fig.2 Panels B and C). In cell cultures treated with milk alone we observed NO levels comparable to those detected in controls.

In addition, RT-PCR data indicated that treatment with LPS significantly increased the expression of iNOS at 6 hours; however, pre-treatment with isoflavones resulted in a significant dose dependent decrease of the iNOS gene expression (Fig. 3, Panel A). Similar results were obtained in the experiments performed in the presence of commercial milks tested. Also in this case we observed that milks pretreated cells before LPS stimulation exhibited a significant dose-dependent reduction of iNOS mRNA expression in comparison to cells stimulated with LPS alone (Fig. 3, Panels B and C).

Consistent with the results of RT-PCR, western blot analysis showed that treatment with LPS significantly increased iNOS protein synthesis at 48 hours; however, iNOS expression resulted significantly down-regulated in the presence of compounds (Fig. 4, Panel A1). Similar results were obtained in the experiments performed in the presence of tested commercial milks, where milk pre-treatment of cell cultures determined a significant reduction of iNOS protein expression in comparison to cells stimulated with LPS alone. Also in this case the effects were resulted dose dependent (Fig. 4, Panels B1 and C1).

Taken together, these results indicate that isoflavones as well as milks, both bovine and soy, are able to suppress the release of NO in LPS-stimulated intestinal cells modulating the expression of iNOS both at transcriptional and at post-transcriptional levels.

3.4. NF-κB activity in LPS-induced intestinal cells

Activation of NF-κB is closely related to regulate the iNOS expression, in this regard the phosphorylation and degradation of IκB-α are essential in the translocation of NF-κB p65 in the nucleus from cytosol. Therefore, we determined the effect of our compounds on LPS-induced degradation and phosphorylation of IκB-α protein. For this purpose, we evaluated the expression of p-IkB in cell lysates obtained from LPS treated Caco-2 cells. In this context, we observed that cells exposed to LPS exhibited a significant increase of IκB-α expression as compared to unstimulated cells, after 48 h of cell stimulation. Little phosphorylation of IκB-α was observed in unstimulated cells, as revealed by densitometric analysis (Fig. 4, Panels A2, B2, C2).

Pretreatment with isoflavones reduced, in a dose dependent manner, IκB-α phosphorylation, reaching a maximal reduction at 10 μM (Fig. 4) in LPS stimulated Caco-2 cells (Fig. 4, panel A2). Moreover, milk pre-treatment of LPS stimulated cells determined a dose-dependent inactivation of the NF-kB (Fig. 4, Panels B2 and C2).

Overall, these data indicate that both isoflavones and milks inhibited NF-κB activity in LPS-stimulated intestinal cells by suppressing degradation of IκB-α, and, consequently, attenuating the expression of iNOS mRNA.

4. DISCUSSION

The Caco-2 cell line, which is derived from human colon adenocarcinoma, exhibits enterocyte-like characteristics and has been used widely as an *in vitro* model of absorption by intestinal epithelial cells. Using Caco-2 cell line, we previously examined the cellular uptake and metabolism of milk isoflavones. We found that compounds were taken up into Caco-2 cells and metabolized in accord to previous works (Steenmsa et al., 2004).

Several analytical methods have been suggested for the determination of isoflavones and their metabolic derivatives in biological fluids and food matrices. (Antignac et al., 2004; de Rijke et al., 2006; Rostagno et al., 2009, Aresta et al., 2015) Solid phase microextraction is a widespread sample preparation technique that allows a solventless extraction of the target compounds. Recently, it has proposed a new simple, selective, sensible, accurate and low cost method for the determination of major isoflavones in soy drinks based on SPME coupled with liquid chromatography (Aresta et al., 2015). In this study, all SPME – HPLC-UV/DAD parameters suggested have been carefully considered and applied, obtaining results comparable to previous works. Equol was not detected in two types of milk. However, according to literature data, it is not a natural component of vegetable milks, unlike those of bovine, whose levels range from 0.236±0.004 to 4.140±0.152μM, quantities lower or near to LOD of method used (Antignac et al., 2004; Di Cagno et al., 2010).

The intestinal cells are also able to mount an adequate inflammatory response, by releasing of pro-inflammatory mediators, such as cytokines and free radicals, to protect against infections (Caricilli et al., 2014). However, when excessive, inflammatory responses may alter the homeostasis of the intestinal mucosa, destroying the intestinal epithelial monolayer. The reactive NO is a major mediator of the inflammatory responses. NO is enzymatically generated in a variety of cells from L-

arginine pathway by three isoforms of NO synthetase. Three major isoforms of nitric oxide synthase include neuronal, endothelial and inducible nitric oxide synthase (nNOS, eNOS and iNOS, respectively). The iNOS form is calcium independent and inducible by inflammatory cytokines and bacterial products, such as LPS, in various cell types, including intestinal cells. Increased expression of iNOS leads to synthesis of micromolar quantities of NO, which causes damage to cells and tissues through the formation of NO-reactive products, used as measurement for toxicity marker (sHaug et al., 2007). NO-induced oxidative stress is associated with many diseases, including IBD. Excessive production of NO, and the presence of nitric oxide synthase protein and iNOS mRNA have been observed in intestinal mucosa of affected areas in patients with ulcerative colitis and Crohn's disease (Ikeda et al., 1997). This is consistent with data obtained from animal models of inflammatory bowel disease (Kankuri et al., 1999). Nitric oxide, in conjunction with superoxide anion, generates significant amounts of peroxynitrite anion, a potent oxidizing agent, which inflicts cellular injury and necrosis probably via mechanisms including DNA fragmentation and lipid oxidation (Valko et al., 2007). For these reasons, down-regulation of NO has been used to treat such diseases (Lanas et al., 2008). The anti-inflammatory agents, such as anti-inflammatory drugs (NSAIDs), have been widely used to treat inflammation-associated diseases. However, using NSAIDs to inhibit the expression of iNOS and NO can lead to severe gastrointestinal damage (Sasso et al., 2015). As a result, NSAIDs use is actually avoided for the treatment of IBD. Searching for effective treatments is urgent needed for the development of therapies and for the prevention of IBD. Many people with IBD turn to alternative medicine including traditional plant based remedies. In the current study, we found that equol, daidzein and genistein, isoflavones, which are present as natural components of milk, down-regulate the release of NO as well as the expression of iNOS mRNA and protein in LPS-stimulated intestinal cells in a dose-dependent manner. These effects on the LPS induced NO production were also observed in Caco-2 cells pre-treated with bovine and soy milks prior LPS stimulation. Similar results were obtained by Di Cagno et al., (2010) reporting that

soy milk as well as equol, genistein and daidzein, markedly inhibited the inflammatory status of Caco-2/TC7 cells as induced by treatment with interferon-γ and LPS, although the exact molecular mechanism responsible for this action is not reported.

Interestingly, we also observed that isoflavones treatment, as well as milks treatment, of LPS stimulated cells is able to reduce, although not completely depress, NO production, which remained yet significantly higher in comparison with control cells. This result is consistent with previous studies reporting NO to play a protective role in the intestinal epithelium (Wallace & Miller, 2000; Zhang, Urbanski, & McCafferty, 2007). In this respect it was reported that in the iNOS-deficient mice iNOS induction the sites of intestinal injury result protective rather than detrimental, since an increase in the macroscopic damage was seen during acute phase of the experimentally-induced colitis (McCafferty et al., 1997). These data suggest that induction of iNOS plays a critical role in the healing and in the gut inflammatory processes. Consequently, a controlled NO production by intestinal cells may contribute to the normal homeostasis of the intestinal epithelium.

It has been reported that NF-kB plays a critical role in the IBD, and previous studies showed that NF-kB inhibitors suppress pro-inflammatory responses thus ameliorating the clinical scenario (Sun et al., 2015). In this context, it is well accepted that several natural antioxidants, such as polyphenol compounds, mediate anti-inflammatory activities by directly suppressing the expression of NF-κB dependent pro-inflammatory mediators, including iNOS (Panaro et al., 2012; Bai et al., 2005; Kumar, Takada, Boriek, & Aggarwal, 2004).

Therefore, blockade of NF-κB signal transduction pathways may be one of the major mechanisms underlying the prevention of the development of IBD.

The isoflavones, daidzein and genistein, have been reported to inhibit iNOS protein and mRNA expression and NO production in LPS induced murine J774 macrophages in a dose-dependent manner down-regulating the NF-κB activation (Hämäläinen et al., 2007). Protective effects of genistein are also demonstrated in other experimental models. For example Valles et al. (2010) have

found that genistein in astrocytes in primary culture attenuates inflammation, preventing the expression of inflammatory mediators, including iNOS. Also Comalada et al. (2006) have indicated that flavonoids inhibit TNF-α production as well as iNOS expression and NO production in LPS-activated macrophages, an effect associated with the inhibition of the NF-κB pathway. Furthermore, Lu et al. (2009), investigating the anti-inflammatory properties of genistein in primary astrocytes treated with hemolysate, demonstrated that genistein inhibited the expression of iNOS and COX-2 mRNA and the level of hemolysate-stimulated NF-κB.

Finally, other results demonstrate that equol is able to impair LPS-induced NO production and iNOS gene expression in RAW 264.7 murine macrophage and that these effects are mediated, at least in part, through NF-κB activity inhibition (Kang et al., 2007).

The present study demonstrated that isoflavones tested, as well as bovine and soy milks, attenuate NO production in LPS activated intestinal cells, interfering to NF-kB activation. In particular we observed that natural compounds used in this study, genistein, daidzein and equol, were able to reduce in a dose dependent manner NF-kB activation, thus leading to mRNA and protein reduced expression in LPS intestinal cells.

Many milk-derived components have immunomodulatory and anti-inflammatory properties, and some of these reduce intestinal inflammation when orally administered to animal models of colitis. The most commonly consumed milk in Western countries is bovine milk. Like human milk, bovine milk contains a number of potentially beneficial bioactivities, as well as carbohydrate (including lactose and oligosaccharides), vitamins, minerals and antioxidants (Haug et al., 2007).

However, milk composition varies between different species (Haug et al., 2007) and is affected by nutrition and health status (Mele et al., 2009), which depends on a variety of factors, such as genotype, milk protein allergies, or lactose intolerance (Haug et al., 2007). In this regard, the IBD patients are often likely to eliminate milk from their diet due to a widespread belief that is deleterious, using milk substitutes soy-based, that have been shown more beneficial compared to

cow's milk. This could lead to the commercialization of soy as a healthy alternative to milk. For IBD patients who can tolerate dairy products, milk may provide a valuable nutritional support. Some components of milk, such as equol, may also have beneficial actions in the control of the inflammatory status of the gut, although these components may not be present in concentrations high enough to provide substantial benefits. The impact of milk on human health and the functionality of each bioactive component need to be further investigated, so that the use of bovine or soy milk as well as of the milk components, as isoflavones, in the diet can be optimised to provide maximum benefits for intestinal inflammation, thus minimizing adverse effects.

Conclusions

In conclusion, our actual findings indicate that isoflavones present in the milk are able to control LPS-induced NO production down-regulating iNOS expression in activated intestinal cells. This action seems to be directly attributable to the selective inhibition of NF-kB signaling pathway, thus providing the first molecular description of the anti-inflammatory properties of isoflavones in an in vitro model of IBD. As a result, our data represent a valid rationale for the use of isoflavone-enriched milks as a complementary approach, with lower incidence of side effects, during the management of IBD. However, additional in vivo research is needed in vivo to confirm these results in order to suggest the use of isoflavone-enriched milks as functional beverages for medical purposes.

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Table and Figure Captions

Table 1

Concentration of isoflavones-aglycones in two commercial bovine and soybeans milks before and after β-glucosidase deconjugation.

Table 2

Concentration of proteins and analytes in Caco-2 cells lysates.

Figure 1

Cell viability assessed by MTT assay. Panel A: Caco-2 cells were incubated (48 h) in the presence of different concentrations of isoflanones (5-50 μM). Panel B: Caco-2 cells were incubated in absence (control) or in presence of LPS (0,5-1 μg/mL). Panel C: cells were stimulated with LPS alone or after pre-treatment of isoflavones. Ctr: control; LPS: LPS-treated cells. Data, expressing the percentage of cell viability, are reported as means ± SD of five independent experiments. *$p < 0.05$; **$p < 0.01$ compared with the control value (medium); #$p < 0.01$ versus LPS alone.

Figure 2

NO release in Caco-2 cells. Panel A: intestinal cells were exposed to LPS alone or after 1h pre-treatment with isoflavones. Panel B: effect of bovine milk (B) treatment (see in the text for concentration and time used) on NO release; Panel C: effect of soy milk (S) treatment (see in the text) on NO. Ctr: control; LPS: LPS-treated cells Results are expressed as means ± SD of five independent experiments. E: equol; D: daidzein; G: genistein. *$p < 0.01$ versus control value (medium); #$p < 0.01$ versus LPS alone; §$p < 0.05$ and §§$p < 0.01$ between LPS+isoflavones or LPS+milks.

Figure 3.

iNOS mRNA expression. Panel A: intestinal cells were treated with LPS alone or after 1h pre-treatment of isoflavones. Panel B: iNOS mRNA expression in Caco-2 cells treated with bovine milk (B) alone or in presence of LPS; Panel C: iNOS mRNA expression in Caco-2 cells treated with soy

568 milk (S) in absence or in presence of LPS. Values represent the mRNA fold changes relative to β-
569 actin used as resident control and expressed as means ±SD of five independent experiments. Ctr:
570 control; LPS: LPS-treated cells; E: equol; D: daidzein; G: genistein. *p < 0.01 compared with the
571 control value (medium); #p <0.01 versus LPS alone; §p <0.05 and §§p<0.01 between
572 LPS+isoflavones or LPS+milks.

573 **Figure 4**

574 Western blot analysis of iNOS in Caco-2 cells. Panel A1: intestinal cells were treated with LPS
575 alone or after 1h pre-treatment of isoflavones. Panel B1: Caco-2 cells were treated with bovine milk
576 (B) alone or in presence of LPS. Panel C1: Caco-2 cells treated with soy milk (S) in absence or in
577 presence of LPS. Protein expression levels were normalized to β-actin and results of densitometric
578 analysis are expressed as means ± SD of five independent experiments. E: equol; D: daidzein; G:
579 genistein; Ctr: control; LPS: LPS-treated cells *p < 0.01 compared with the control value (medium);
580 #p <0.01 versus LPS alone; §p <0.05 and §§p<0.01 between LPS+isoflavones or LPS+milks.
581 Signalling pathway analysis. Panel A2: Western blot analysis of p-IkBα in Caco-2 cells treated with
582 LPS alone or after 1h pre-treatment of isoflavones. E: equol; D: daidzein; G: genistein. Panel B2:
583 Immunoblot analysis of p-IkBα in Caco-2 cells treated with bovine milk (B) alone or in presence of
584 LPS. Panel C2: Immunoblotting analysis of cells treated with soy milk (S) in absence or in presence
585 of LPS. Protein expression levels were normalized to β-actin and results of densitometric analysis
586 are expressed as means ± SD of five independent experiments. *p < 0.01 compared with the control
587 value (medium); #p <0.01 versus LPS alone; §p <0.05 and §§p<0.01 between LPS+isoflavones or
588 LPS+milks.

589589

Highlights

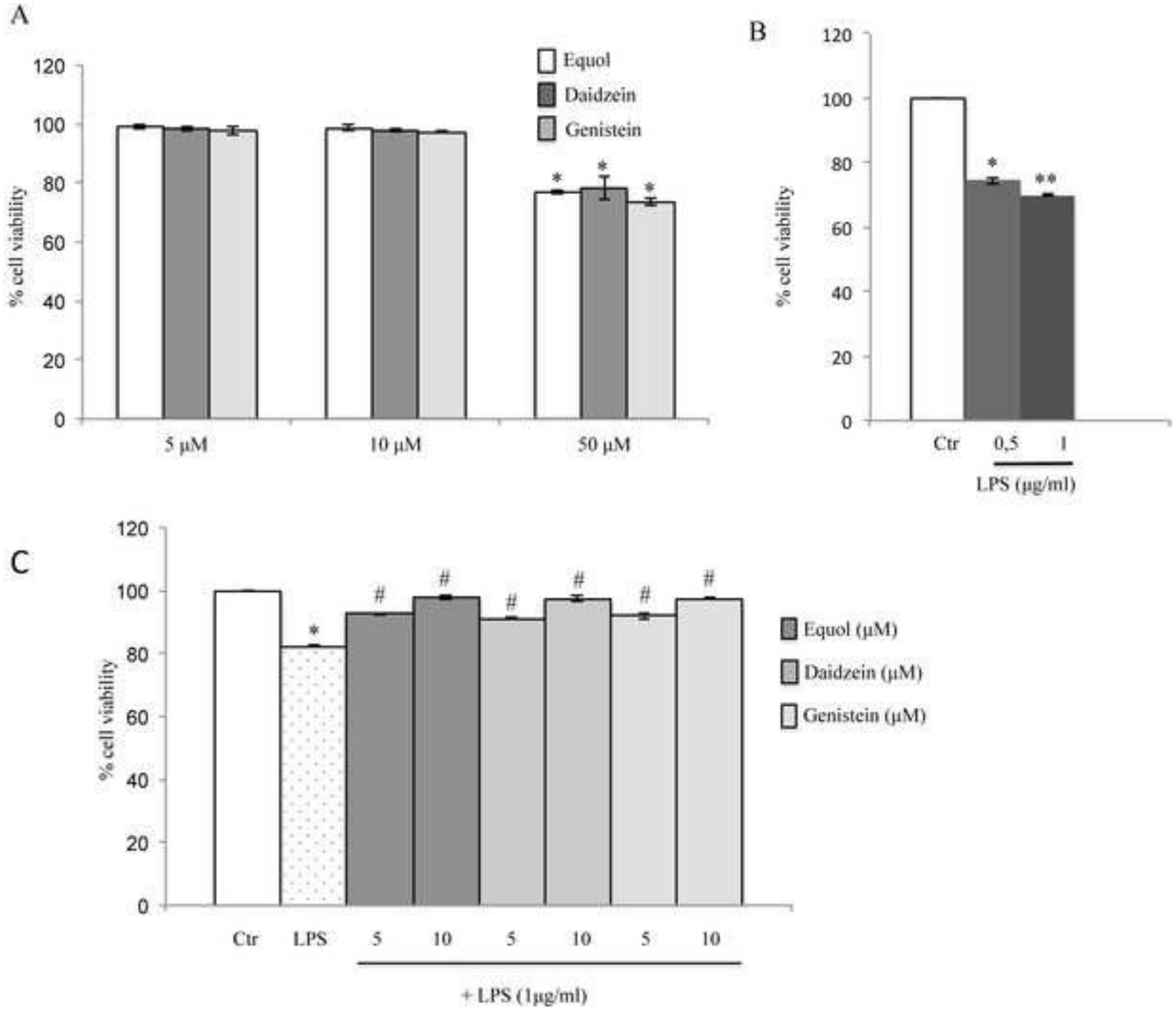
1. milks and bioactive compounds tested are able to reduce LPS-induced inflammatory responses
2. B and SB milk bioactive compounds significantly inhibited the expression of (iNOS) mRNA
3. B and SB milk bioactive compounds significantly inhibited protein expression
4. the inhibition is dose-dependent
5. bioactive compounds were absorbed, metabolized and released by Caco-2 cells in culture media

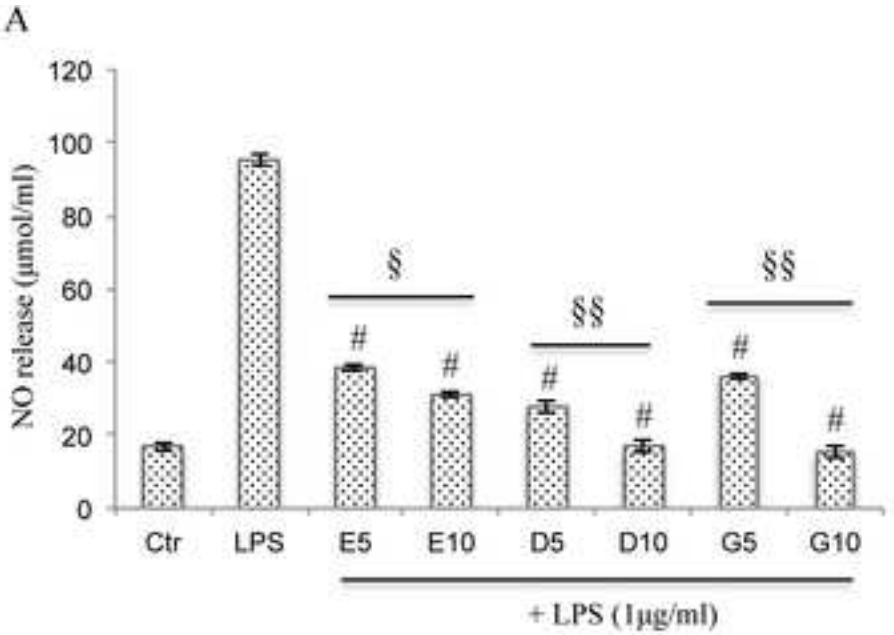
Isoflavones	Soybean milk		Bovine milk	
	before	after	before	after
Daidzein	3.6± 0.3 μM	160.7± 13.5 μM	0.06± 0.01 μM	0.12± 0.05 μM
Genistein	0.9± 0.1 μM	121.8± 12.1 μM	0.03± 0.01 μM	0.04± 0.01 μM

Table 1

Pretreatment		[proteins] (mg/mL)	[analyte]$_{found}$ (nmoles/mg proteins)
equol	5 μM	8.6±0.5	0.44±0.25
	10 μM	6.8±0.3	1.16±0.54
daidzein	5 μM	9.4±0.6	0.46±0.19
	10 μM	18.4±1.5	0.44±0.36
genistein	5 μM	17.0±1.2	0.28±0.15
	10 μM	19.6±1.6	0.49±0.28

Table 2


A
Equol
Daidzein
Genistein
% cell viability
120
100
80
60
40
20
0
5 µM
10 µM
50 µM
B
% cell viability
Ctr
0,5
1
LPS (µg/ml)
C
% cell viability
Ctr
LPS
5
10
5
10
5
10
+ LPS (1µg/ml)
Equol (µM)
Daidzein (µM)
Genistein (µM)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NO release (μmol/ml)
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100
80
60
40
20
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§
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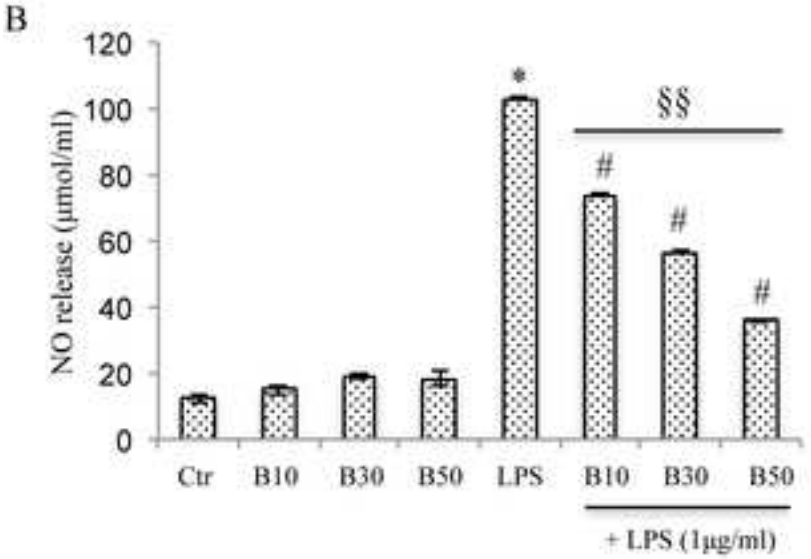
#
Ctr
LPS
E5
E10
D5
D10
G5
G10
+ LPS (1μg/ml)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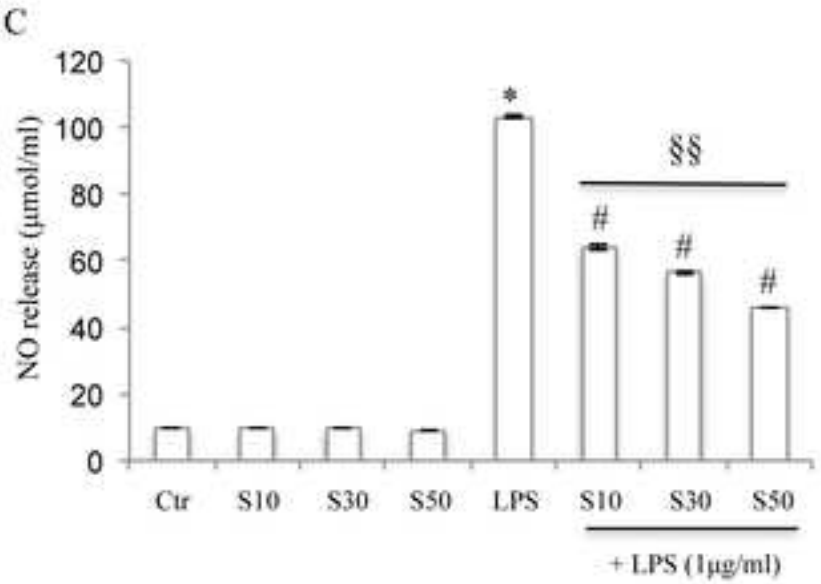
LPS
B10
B30
B50
+ LPS (1μg/ml)


C
NO release (μmol/ml)
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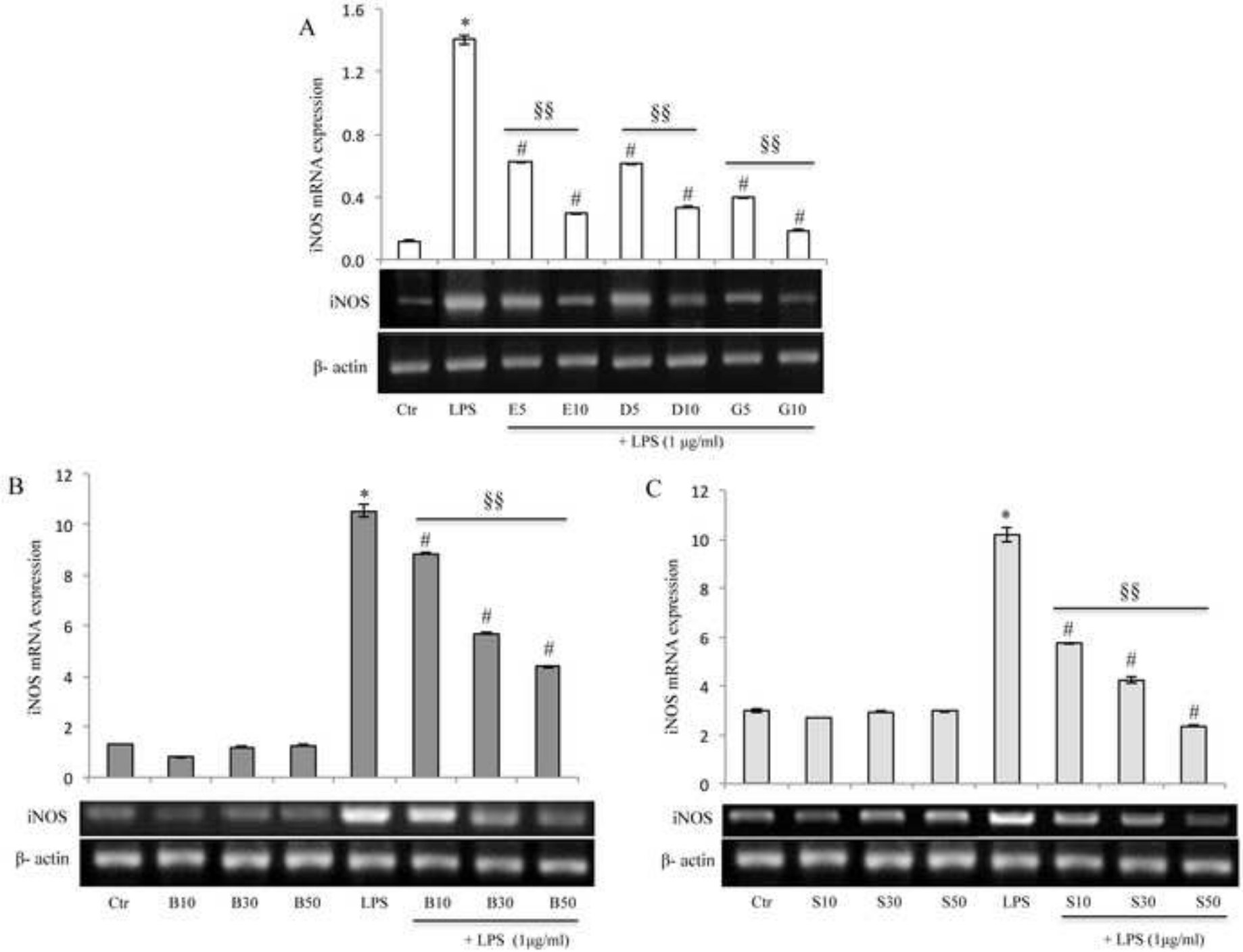
S30
S50
+ LPS (1μg/ml)


A
iNOS mRNA expression
1.6
1.2
0.8
0.4
0.0
*
§§
§§
§§
#
iNOS
β- actin
Ctr
LPS
E5
E10
D5
D10
G5
G10
+ LPS (1 μg/ml)
B
iNOS mRNA expression
12
10
8
6
4
2
0
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§§
#
iNOS
β- actin
Ctr
B10
B30
B50
LPS
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B30
B50
+ LPS (1μg/ml)
C
iNOS mRNA expression
12
10
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iNOS
β- actin
Ctr
S10
S30
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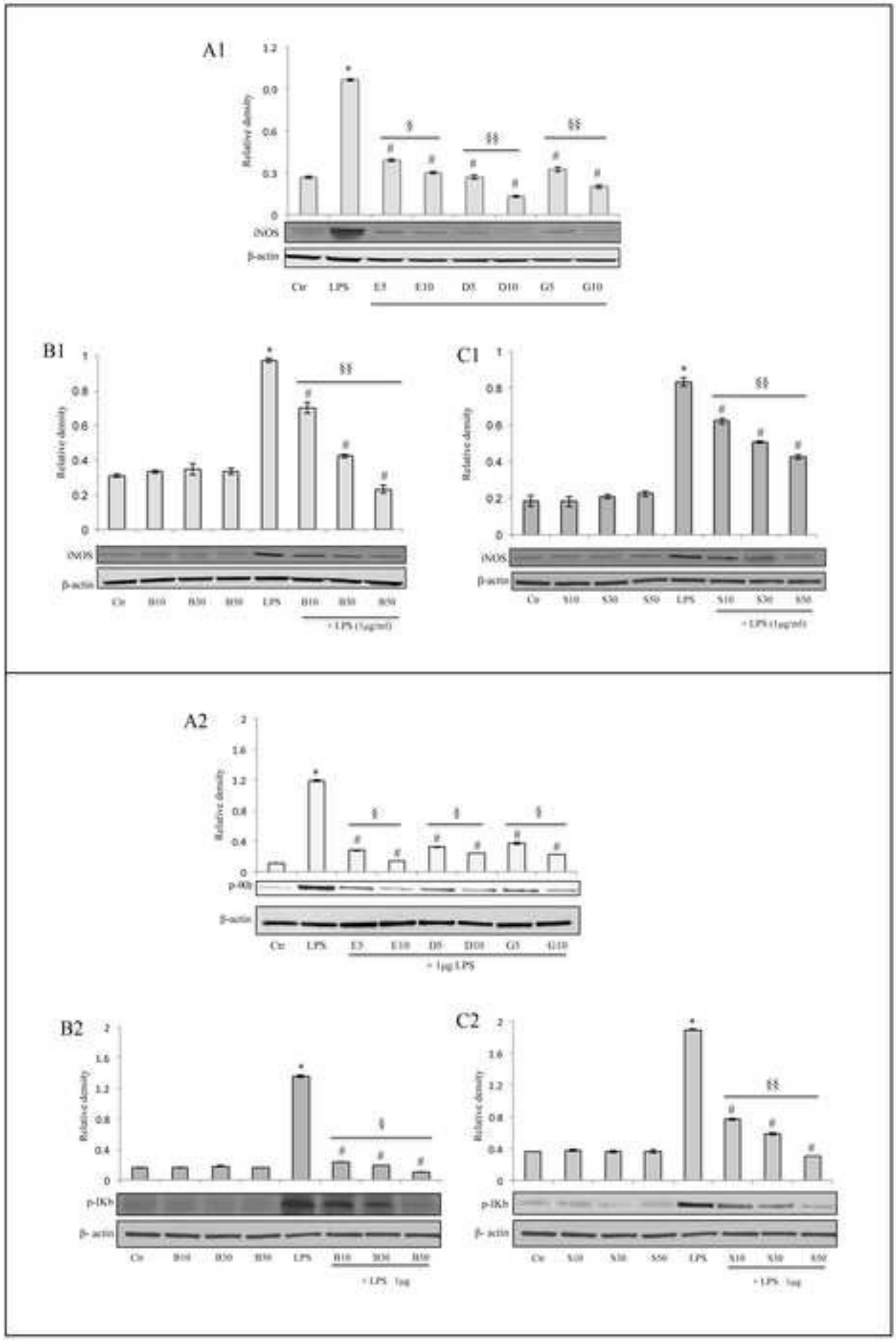
S50
+ LPS (1μg/ml)


A1
Relative density
iNOS
β-actin
Cr
LPS
E5
E10
D5
D10
G5
G10
B1
C1
B2
C2
p-IKb
β- actin
+ LPS (1μg/ml)
A2
p-IKb
+ 1μg LPS