EFFECT OF GRAPE POMACE EXTRACT ENRICHED IN POLYPHENOLS IN COUNTERACTING INTESTINAL INFLAMMATION BY USING AN *IN VITRO* INTESTINAL CELLULAR MODEL

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Abstract

The rapidly increase of the incidence of severe chronic intestinal inflammation diseases have a huge impact on animal and human life quality. The counteracting of intestinal inflammation using prebiotics was lately investigated. Grape pomace (GP) is an industrial waste from the wine processing rich in polyphenols which could be an alternative, less expensive source of anti-inflammatory bioactive compounds (polyphenols). Using an in vitro model of intestinal inflammation, we evaluated the effect of the grape pomace extract enriched in polyphenols on several key markers of inflammation. Thus, expression of cytokines, TLRs and signalling molecules were analysed in epithelial CaCO2 cell culture. After cell culture treatments, tRNA was extracted and gene expressions were evaluated by qPCR. Our results showed that grape pomace extract is able to counteract the LPS-induced intestinal inflammation by decreasing the gene expression of pro-inflammatory cytokines: TNF-α, IL-1β, IL-6 and Il-8 and by slightly increase of the IL-10, anti-inflammatory cytokine mRNA. Also, our results showed that grape pomace extract could modulate the expressions of TLRs genes, as modulators of the innate immune response.

In conclusion, our study contributes to a better understanding of the grape pomace extract enriched in bioactive compounds, in modulation the intestinal inflammation, as well as the consequence in terms of susceptibility to enteric disease.

Key words: intestinal inflammation, grape pomace, polyphenols, cellular model

INTRODUCTION

The rapidly increase of the incidence of severe chronic intestinal inflammation diseases have a huge impact on animal and human life quality. The intestinal inflammatory disease is a complex disorder, an excessive cell-mediated response to antigens of the normal bacterial microflora that leads to an impairment of the barrier function of the gut. Natural therapy approaches using natural products (phytochemicals, antioxidants, probiotics, dietary fiber, lipids) may be useful for treating intestinal inflammation [1]. The counteracting of intestinal inflammation using prebiotics with a rich content in bioactive compounds, such as polyphenols was lately investigated. Polyphenol extracts from a variety of plants have been shown to have immune-modulator and anti-inflammatory effects. For example, the dietary intake of green tea polyphenols can ameliorate intestinal inflammation in a mouse model of intestinal inflammation [2]. Other studies showed that polyphenols from apple conferred protection against colitis and dampened pro-inflammatory cytokine expression in mice [3]. Grape pomace (GP) is an industrial waste from the wine processing rich in polyphenols which could be an alternative, less expensive source of anti-inflammatory bioactive compounds. Red wine polyphenols have been indicated as chemo-preventive agents against cancer and other degenerative diseases associated with inflammatory processes [4]. Thus, red GP, easily available in winemaking areas, could be exploited as a source of the beneficial

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polyphenols of red wine. Due to its composition in polyphenols, GP could be used as an alternative dietary treatment in intestinal inflammatory pathologies. In the present study we used an *in vitro* model of intestinal inflammation to evaluate the effect of the grape pomace extract enriched in polyphenols on several key markers of inflammation.

MATERIALS AND METHODS

Preparation of GP extract: Polyphenols from GP were extracted in acetone 80% solution, for 24 hours. The resulted extract was concentrated and the total polyphenols concentration was determined using Folin-Ciocalteu method.

Cell culture: CaCO2 intestinal cells were purchased from the American Type Culture Collection (ATCC). The cells were cultured in Minimum Essential Medium (MEM), supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic and were maintained at 37 C in a 5% CO_2 atmosphere. The inflammatory condition was simulated by treating the cells with LPS 5 µg/ml, for 4 hours. After induction of intestinal inflammation, cells were cultured in presence of GP extract for 24 hours.

Cell proliferation assay: Cell proliferation was measured in a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in 96-well flat bottomed plates at a density of 5×10^4cells/100µl and treated with GP extract. The culture medium was removed after 24h. Next, 10µl of MTT reagent and culture medium was added to each well for another 4h at 37 C prior to measurement of cell viability. The absorbance was determined in an ELISA micro-plate reader (TECAN Sunrise) at a test wavelength of 450nm.

Nitric oxide production: Total nitrite concentration, as an index of NO production, was determined in cell medium using a total NO//nitrite determination kit (Cayman Chemicals).

Extraction of total RNA and cDNA synthesis. Total RNA was extracted and measured using Qiagen RNeasy mini kit (QIAGEN GmbH), according to the manufacturer's recommendations. The total RNA isolated from each sample was further used to generate cDNA using M-MuLV Reverse Trascriptase kit (Thermo Fischer Scientific) according to the manufacturer's protocol.

qPCR. Briefly, the PCR reaction was performed in Rotor-Gene-Q (QIAGEN GmbH) machine using 1µl of cDNA, 12.5 µl Maxima SYBR Green/ Fluorescein qPCR Master Mix 2X (Thermo Fischer Scientific) and 0.3µM each of gene-specific primer. The PCR cycling conditions consisted in: UDG pre-treatment at 50°C for 2 min, initial denaturation step at 95°C for 15s, followed by 40 cycles of 95°C for 15s, 60°C for 15s and 72°C for 15s with a single fluorescence measurement; a final elongation step was carried out at 72°C for 10 min. Three reference genes were used for data normalisation. Results were expressed as relative fold change (Fc) compared with control.

Statistical analysis: Result data are expressed as mean ± standard error of the mean (SEM). One way ANOVA analysis was used to determine the statistical differences between groups for all parameters analysed. Values of $P< 0.05$ were considered significant.

RESULTS AND DISCUSSIONS

The effect of GP extract on cell proliferation ability of intestinal cells:

In order to choose the most appropriate concetration of GP extract for the inflammation model a MTT assay was conducted. Our results presented in Figure 1 indicated that no significant differences in the number of viable cells were found at 24 h (P>0.05) after the tratment with different concentration of GP extract. Our results are similar with those reported by Melo et al. [5], which found no effect of different winery by-products on macrophage cells proliferation. The concentration of 5 µg was choosing for the further experimentation in our study.

The effect of LPS and GP treatments alone or in combination on total nitrite concentration in intestinal cells:

In order to evaluate the capacity of GP extract rich in polyphenols to counteract intestinal inflammation induced by bacterial stimuli like LPS, the levels of NO generated by stimulated CaCO-2 cells were monitored.

As shown in Figure 2, treatment with 5 μM LPS increased nitrite levels by 77,92% in comparison with medium alone. GP extract alone did not affect the cellular nitrite formation in comparison with control. The addition of 5 μg/ml GP extract to LPS-treated cells induced a decrease of nitrite levels with only 9% in comparison with LPS-treated cells. NO is a free radical produced from the amino acid L-arginine by nitric oxide synthase (NOS) enzyme. Although NO, in constitutive levels, has a physiological role in maintaining adequate perfusion and regulation of microvascular and epithelial permeability [6], persistent overproduction of NO via up-regulation of iNOS is associated with inflammatory response leading to gut barrier injury [7]. The result obtained herein showed that higher concetration of GP is necessary to decreased the LPS-induced NO.

The effects of GP extract treatment on LPS-modulated cytokine gene expression:

To have a comprehensive overview on the ability of GP extract to attenuate intestinal inflammation, we evaluate the mRNA levels of some important inflammatory mediators, pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-8) and anti-inflammatory cytokine IL-10 in CaCO-2 cells. To induce intestinal inflammation, the cells were treated with LPS for 4h, and after that GP extract was added for another 24h. As expected, LPS treatment induced a strong increase in pro-inflammatory cytokine gene expression (TNF-α: 5.31 ± 1.22 Fc, $P<0.05$; IL-1β: 8.16 ± 2.44 Fc, $P<0.05$; IL-6: 6.72 ± 2.46 Fc; IL-8: 4.71 ± 0.99 Fc, $P<0.05$, Figure 3) and decrease of IL-10 mRNA level (0.24 ± 0.04 Fc, $P<0.05$, Figure 3). The GP extract treatment alone was associated with a decrease of mRNA of pro-inflammatory cytokines TNF-α (0.63 ± 0.20 Fc), IL-1β (0.72 ± 0.34 Fc), IL-6 (0.83 ± 0.33 Fc) and has no effect on IL-8 (1.02 ±0.43 Fc) and IL-10 (1.29 ± 0.24 Fc) gene expression (Figure 3) in comparison with untreated cells. The addition of GP extract to LPS-treated cells reduced the levels of pro-inflammatory cytokines induced by LPS. Briefly, the TNF-α mRNA level was decreased by 61%, IL-1β by 82%, IL-6 by 88%, IL-8 by 78%, while the IL-10 gene expression was increased by 400% in comparison with LPS-treated cells. In a LPS-induced model of inflammation, using intestinal cells, Kaulmann et al [8] demonstrated the potential of phytochemical-rich fruits and vegetables (plum and cabbages) to ameliorate LPS-induced intestinal inflammation by reducing the levels of inflammatory mediators such as cytokines.

The effect of GP treatment on toll-like receptors (TLRs) in cellular model of intestinal inflammation:

Toll-like receptors are expressed mainly in the intestinal epithelial cells and in the inate immune cells. TLRs belong to the pattern-recognition receptors and represent the first line of defense against pathogens, playing a pivotal role in both innate and adaptive immunity. Dysregulation in the activity of such receptors can lead to the development of chronic and severe inflammation as well as immunological disorders [9] caused by the entrance of pathogens into the cells. Under homeostatic conditions intestinal epithelial cells have low expression of TLR2 and TLR4 (which recognise the *E. Coli*, transduce the signal into the nucleus and trigger the inflammatory response), and therefore in a healthy context, TLR activation is low; however, in an inflammatory scenario, TLR expression on these cells increases and then TLR signaling is triggered [10].

In order to evaluate the effects of GP extract on Toll-like receptors in LPS and GP-treated CaCO-2 cells, we analysed by qPCR the TLRs gene expression. As expected, our results presented in Table 1 showed that LPS treatment increase the mRNA level for all analysed TLRs. A concentration of 5 μg/ml of GP extract induced a decrease of TLR1, -4, -5, -6, -7, -8, -9 and -10 gene expression, while the mRNA level for TLR-2 was increased and of TLR-3 was unmodified by GP treatment. Addition of GP extract to LPS-treated cells was associated with a decrease of TLR-1 (12.5%), TLR-2 (37%), TLR-3

(70%), TLR-4 (45%), TLR-5 (90%), TLR-6 (48%), TLR-8 (80%), TLR-9 (98%) and TLR-10 (18%) (Table 1).

There are studies that have evidenced the influence of different classes of flavonoids on TLR gene and protein expression [9]. Our results showed that GP extract enriched in polyphenols can modulate the TLRs gene expression induced by LPS activation, the initial activation process of the receptor. Further studies are needed to evaluate the effects of GP extract on the upstream and downstream signaling molecules involved in the TLR pathway.

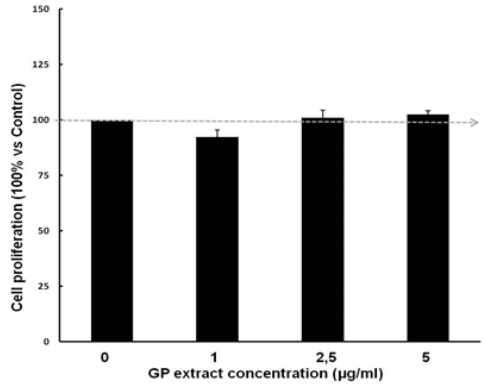

Figure 1 *Assessment of the effect of GP extract on cell proliferation in CaCO-2 cells.* Cell proliferation was determined using the MTT assay at 24h after incubation with GP extract. The results are representative for three independent experiments and are presented as means ± SEM.

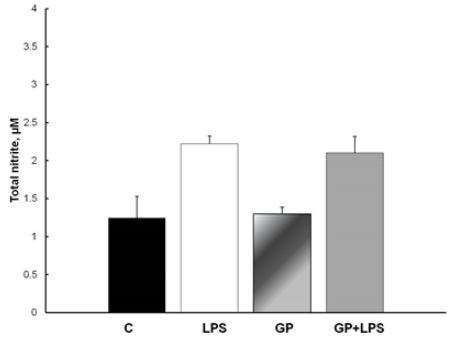

Figure 2 *Total nitrite concentration in CaCO-2 cell medium after LPS and GP treatment.* C:control group; LPS:LPS-treated group (5µg/ml); GP:GP-treated group (5µg/ml); GP+LPS:LPS and GP-treated group. The results are representative of six experiments. Values are presented as means ± SEM.

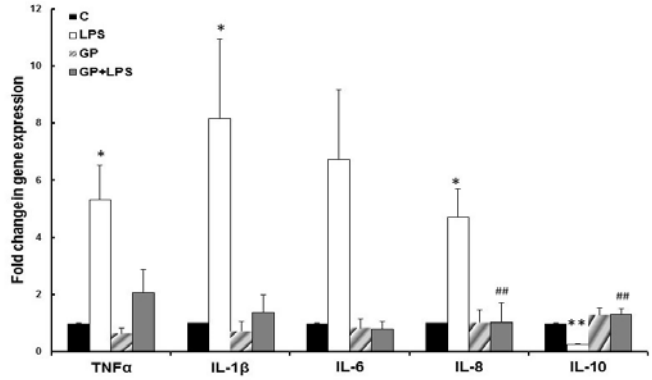

Figure 3. *Relative mRNA abundance of inflammatory markers (cytokines) in LPS and GP-treated CaCO-2 cells*. C:control group; LPS:LPS-treated group (5μg/ml); GP:GP-treated group (5μg/ml); GP+LPS:LPS and GP-treated group. The results are representative of six separate experiments. Bars represents means ± SEM. *,## indicates significant differences in comparison with control and LPS-treated group, respectively (P < 0,05).

Table 1 Relative gene expression (fold change, Fc) for Toll-like receptors in LPS and GP-treated CaCO-2 cells

Gene	Experimental treatment			
	Control	LPS	GP	GP+LPS
TLR1	1.00±0.00^{a}	3.92±0.82^{b}	0.48 ±0.15^{c}	3.43±0.93^{bd}
TLR2	1.00±0.00^{a}	3.60 ±0.79^{b}	3.63±1.01^{b}	2.26±0.52^{ab}
TLR3	1.00±0.00^{a}	4.78±0.92^{b}	1.21±0.26^{c}	1.43 ±0.15^{c}
TLR4	1.00±0.00^{a}	2.94±0.63^{b}	0.83±0.24^{a}	1.59 ±0.34^{a}
TLR5	1.00±0.00^{a}	3.59±1.04^{b}	0.65±0.23^{a}	0.34±0.12^{c}
TLR6	1.00±0.00^{a}	4.11 ±0.73^{b}	0.14 ±0.08^{c}	2.12 ±0.65$^{a, d}$
TLR7	1.00±0.00^{a}	2.12 ±0.30^{b}	0.28 ±0.11^{c}	4.35 ±0.97^{d}
TLR8	1.00±0.00^{a}	2.43±0.65^{b}	0.64±0.23^{a}	0.48 ±0.10^{c}
TLR9	1.00±0.00^{a}	4.01 ±0.53^{b}	0.55 ±0.21^{c}	0.05±0.03^{d}
TLR10	1.00±0.00^{a}	3.94±0.83^{b}	0.63 ±0.24^{a}	3.21 ±0.57^{b}

*Relative mRNA abundance of inflammatory-related markers (Toll-loke receptors, TLR) in LPS and GP-treated CaCO-2 cells. C: control group; LPS: LPS-treated group (5μg/ml); GP: GP-treated group (5μg/ml); GP+LPS: LPS and GP-treated group. The results are representative of six separate experiments. Values represents means ± SEM.^{a,b,c,d} = Mean values within a row with unlike superscript letters were significantly different (P<0·05).

CONCLUSIONS

Our results showed that grape pomace extract is able to counteract the LPS-induced intestinal inflammation by decreasing the gene expression of pro-inflammatory cytokines: TNF-α, IL-1β, IL-6 and Il-8 and by significant increase of the IL-10, anti-inflammatory cytokine mRNA. Also, our results showed that grape pomace extract could modulate the expressions of TLRs genes, as modulators of the innate immune response. In conclusion, our study contributes to a better understanding of the grape pomace extract enriched in bioactive compounds, in modulation the intestinal inflammation, as well as the consequence in terms of susceptibility to enteric disease.

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