Polyphenol-rich extract of pomegranate peel alleviates tissue inflammation and hypercholesterolaemia in high-fat diet-induced obese mice: potential implication of the gut microbiota

Audrey M. Neyrinck, Vincent F. Van Hée, Laure B. Bindels, Fabienne De Backer, Patrice D. Cani and Nathalie M. Delzenne*

Metabolism and Nutrition Research Group, Louvain Drug Research Institute, Université catholique de Louvain, Avenue Mounier 73, Box B1.73.11, B-1200 Brussels, Belgium

(Submitted 18 November 2011 – Final revision received 25 April 2012 – Accepted 27 April 2012 – First published online 7 June 2012)

Abstract

Pomegranate extracts have been used for centuries in traditional medicine to confer health benefits in a number of inflammatory diseases, microbial infections and cancer. Peel fruit are rich in polyphenols that exhibit antioxidant and anti-inflammatory capacities in vitro. Recent studies strongly suggest that the gut microbiota is an environmental factor to be taken into account when assessing the risk factors related to obesity. The aim of the present study was to test the prebiotic potency of a pomegranate peel extract (PPE) rich in polyphenols in a nutritional model of obesity associated with hypercholesterolaemia and inflammatory disorders. Balb/c mice were fed either a control diet or a high-fat (HF) diet with or without PPE (6 mg/d per mouse) over a period of 4 weeks. Interestingly, PPE supplementation increased caecal content weight and caecal pool of bifidobacteria. It did not significantly modify body weight gain, glycaemia, glucose tolerance and inflammatory markers measured in the serum. However, it reduced the serum level of cholesterol (total and LDL) induced by HF feeding. Furthermore, it counteracted the HF-induced expression of inflammatory markers both in the colon and the visceral adipose tissue. Together, these findings support that pomegranate constitutes a promising food in the control of atherogenic and inflammatory disorders associated with diet-induced obesity. Knowing the poor bioavailability of pomegranate polyphenols, its bifidogenic effect observed after PPE consumption suggests the involvement of the gut microbiota in the management of host metabolism by polyphenolic compounds present in pomegranate.

Key words: Pomegranates; Microbiota; Obesity; High-fat diet; Inflammatory disorders; Prebiotics; Polyphenols

Pomegranate (Punica granatum L.) has been used for centuries to confer health benefits in a number of inflammatory diseases (1,2). Fruits are widely consumed either fresh, or as a beverage. Dietary supplements containing pomegranate extracts are becoming popular in the Western world for the treatment and prevention of arthritis and other inflammatory diseases (1,2). The health benefits of juice or pomegranate extracts have been attributed to the polyphenol content and composition of this fruit (3). The main polyphenols proven to have antioxidant and anti-inflammatory bioactivities in pomegranate include the ellagitannins and anthocyanins, which are concentrated in the peel and piths of the fruit (2). Pure punicalagin, total pomegranate tannin extract, or pomegranate juice have been shown to inhibit, in a dose-dependent manner, TNF-α-induced cyclo-oxygenase 2 (COX-2) expression in HT-29 human colon cancer cells (3). In addition, a whole pomegranate methanol extract has been shown to inhibit TNF-α expression and release in microglial cells activated with lipopolysaccharides (4). A recent study suggested that a pomegranate extract could be particularly promising in the dietary prevention of intestinal inflammation since it was able to inhibit NF-κB activity and to decrease inflammatory cytokine (IL-8) and PGE2 in human intestinal Caco-2 cells stimulated by cytokines (5).

In this context, a question often raised is whether the concentration of a plant or fruit extract constituent compound that has been used in vitro experiments would be realistic or achievable in vivo. In a majority of the cases, this has to be denied because of the poor bioavailability of most constituents of plant or fruit extracts (6,7). In addition, the formation of bioactive compounds from molecules present in the original extract might be issued from intestinal and hepatic metabolism or from a too-often neglected source, namely the intestinal bacterial metabolism (8). The human intestine harbours a...
complex microbial ecosystem comprising a considerable metabolic versatility, using biological pathways that humans have not evolved. This capacity of the gut microbiome, encoded by the collective genomes of the gut microbiota or gut metagenome, includes the metabolism of indigestible polyphenols derived from fruit and vegetables. This last consideration is of particular interest knowing that the gut microbiota is increasingly considered as a symbiotic partner for the maintenance of health. Several data suggest that the activity of the gut microbiota is a factor to be taken into account when assessing the risk factors related to obesity, and associated disorders, such as dyslipidaemia, inflammation, insulin resistance and diabetes. Indeed, alterations in the composition of the gut microbiota – known as dysbiosis – have been proposed to contribute to the development of obesity, thereby supporting the potential interest of nutrients acting on the gut microbiota to produce beneficial effects on host energy metabolism.

The purpose of the present study was to determine whether the oral administration of a polyphenol-rich extract of pomegranate peel to mice could modulate the gut microbiota composition and offset increases in body weight, cholesterol profile, glucose intolerance, lipid storage and inflammation occurring in mice fed a high-fat (HF) diet. We decided to perform this in vivo study on Balb/c mice since it was demonstrated that (1) a HF diet induces hepatic lipid accumulation at a higher extent in Balb/c mice than in C57BL6J mice and (2) Balb/c mice exhibit a higher capacity to respond to inflammatory stimulus (higher increase in plasma and hepatic levels of cytokines/chemokines upon caecal ligation and puncture inducing septic peritonitis) than C57BL6J mice.

Experimental methods

Animals and diet

A total of eighteen male Balb/c mice (9 weeks old at the beginning of the experiment, Charles River Laboratories) were housed in groups of three per cage in a controlled environment (12 h daylight cycle, lights off at 18.00 hours) with free access to food and water. After 1 week of acclimatisation, the mice were divided into three groups (six per group): a control (CT) group, fed a control diet (AO4, SAFE), a group fed a HF diet and a group fed the same HF diet and receiving pomegranate peel extract (HF-PPE) at a dose of 0.2% in tap water (resulting in average consumption of 6 mg/d per mouse). The full composition of both the HF diet (D12492, Research Diets) and the AO4 standard diet is given in Table S1, available online. Extract (OXYLENT GR®, Steriron S.A.) used in the study was derived from pomegranate peel in which polyphenol content reached 30% (Folin–Ciocalteu method, equivalent gallic acid) and the concentrations of punicalagin and ellagic acid were 8 and 5% (ultra-performance liquid chromatography method with diode array detection), respectively. Food intake was recorded taking into account spillage twice a week during 4 weeks. After 4 weeks and a 6 h period of fasting, mice were anaesthetised (ketamine/xylazine intraperitoneal, 100 and 10 mg/kg, respectively) and blood samples were harvested for further analysis. Liver, adipose tissues, caecal content and intestinal tissues (proximal colon and caecum) were carefully dissected and immersed in liquid N2 before storage at −80°C. The animal experiments were approved by the local ethics committee and housing conditions were as specified by the Belgian Law of 6 April 2010 on the protection of laboratory animals (agreement no. LA 1230514).

Oral glucose tolerance test

After 3 weeks of treatment, an oral glucose tolerance test (OGTT) was performed on 6 h-fasted mice. Glucose was administered orally (3 g/kg body weight, 66% glucose solution) and blood glucose levels were determined using a glucose meter (Roche Diagnostics) on 3.5 μl of blood collected from the tail of the tip vein both before (−30 min and 0 min) and after glucose administration (15, 30, 60, 90 and 120 min).

Microbial analysis of the caecal contents

At the end of the experiment, the total caecum content was collected and weighed before storage at −80°C. Quantitative PCR for total bacteria, Bifidobacterium spp., Lactobacillus spp., Bacteroides–Prevotella spp. and Roseburia spp. were performed as reported by Neyrinck et al. using qPCR (Eurogentec). Real-time PCR were performed with the StepOnePlus™ real-time PCR system and software (Applied Biosystems). The cycle threshold of each sample was then compared with a standard curve (performed in triplicate) made by diluting genomic DNA (5-fold serial dilution of Bifidobacterium animalis for Bifidobacterium spp., Bacteroides fragilis for Bacteroides–Prevotella spp., Lactobacillus acidophilus for Lactobacillus spp. and total bacteria) (BCCM/LMG, Ghent, Belgium). Cell counts before DNA extraction were determined by culture.

Blood parameters

Plasma insulin concentrations were determined using an ultrasensitive ELISA kit (Alpco™ immunoassay, Alere Healthcare). The insulin resistance index was calculated by multiplying the AUC for glucose, and the AUC for insulin, calculated from −30 min until 15 min after glucose challenge. Concentrations of IL-1α, IL-1β, IL-6, monocyte chemoattractant protein 1 (MCP-1), TNFα, IL-10 and IL-13 were determined in 15 μl of plasma using a multiplex immunoassay kit (BioxPlex Cytokine Assay, Bio-Rad) and measured using Luminex® technology (BioPlex®, Bio-Rad).

Plasma TAG, cholesterol and NEFA concentrations were measured using kits coupling enzymatic reaction and spectrophotometric detection of reaction end-products (Diays Diagnostic and Systems). HDL-cholesterol concentration was measured enzymatically after VLDL, chylomicron and LDL-cholesterol antibody precipitation (Diays Diagnostic and Systems). LDL was estimated by the Friedewald formula.
Lipid analysis in the liver

TAG and cholesterol were measured in the liver tissue after extraction with chloroform–methanol, as described by Neyrinck et al. (19). Expression of selected genes in tissues

Total RNA was isolated using the TriPure isolation reagent kit (Roche Diagnostics Belgium). Complementary DNA was prepared by reverse transcription of 1 µg total RNA using the Kit Reverse transcription System (Promega). Real-time PCR were performed with the StepOnePlus™ real-time PCR system and software (Applied Biosystems) using SYBR Green for detection according to the manufacturer’s instructions. RPL19 RNA was chosen as the housekeeping gene. Primer sequences for the targeted mouse genes are available on request (audrey.neyrinck@uclouvain.be). All samples were run in duplicate in a single ninety-six-well reaction plate and the data were analysed according to the 2^(-ΔΔCt) method. The identity and purity of the amplified product were checked through analysis of the melting curve carried out at the end of amplification.

Statistical analysis

Results are presented as means with their standard errors. Statistical analysis was performed by the Mann–Whitney test (GraphPad Software). P < 0.05 was considered as statistically significant. A two-way analysis on repeated measures was performed for the evolution of body weight and the evolution of glycaemia upon OGTT.

Fig. 1. (a) Weight of caecal content, (b) caecal content of total bacteria, (c) caecal content of Bifidobacterium spp., (d) caecal content of Lactobacillus spp., (e) caecal content of Bacteroides–Prevotella spp. and (f) caecal content of Roseburia spp. Mice were fed a control (CT) diet, a high-fat (HF) diet or a HF diet with pomegranate peel extract (HF-PPE) in tap water for 4 weeks. * Values were significantly different from those of CT (P < 0.05). † Values were significantly different from those of HF (P < 0.05). ‡ Values were nearly significantly different from those of HF (P = 0.07).

Results

Oral administration of pomegranate peel extract promotes the growth of Bifidobacterium spp. in the caecal content of mice upon high-fat feeding

HF feeding decreased the weight of caecal content as compared to the control condition (Fig. 1(a)). Quantitative analysis performed in caecal content showed that HF diet decreased the number of total bacteria, the Gram-positive lactobacilli and Roseburia spp. as well as the Gram-negative bacteria such as Bacteroides–Prevotella spp. without effect on bifidobacteria content (Fig. 1). Surprisingly, the PPE treatment significantly increased the weight of caecal content and the caecal pool of Bifidobacterium spp. without modifying significantly other bacteria as compared to HF-fed mice. Of note, the increase of Bacteroides–Prevotella spp. due to PPE supplementation v. the HF-fed group was nearly of significance (P = 0.07).

The oral administration of pomegranate peel extract does not modify the high-fat-induced body weight gain, adiposity and glucose intolerance

A drop in body weight occurred within the first 3 d of treatment in both groups receiving the HF diet, signalling an adaptation to the dietary changes (Fig. 2(a)). The body weight evolution did not reveal any significance about time, treatment and interaction (time × treatment) after a repeated-measures analysis (two-way ANOVA). However, the HF diet significantly increased the body weight gain of mice when considering the difference between day 3 and day 30 (end of the experiment) (Fig. 2(b)). This effect was accompanied by fat accumulation as shown by the weight of the adipose tissues.
Oral administration of pomegranate peel extract decreases high-fat-induced expression of inflammatory markers in the colon and the visceral adipose tissue

In contrast to what happens in the liver, a higher expression of inflammatory markers was observed both in the colon and the visceral adipose tissue in mice fed a HF diet vs. control mice with a significant \( P \) value for IL-1\( \beta \) (Fig. 4). PPE administration significantly down-regulated COX-2 induction in colonic and adipose tissues but not in the liver. Moreover, PPE decreased the mRNA levels of IL-6 and IL-1\( \beta \), with a significant \( P \) value for IL-6 mRNA in the colon and IL-1\( \beta \) mRNA in the visceral adipose tissue. Several inflammatory markers measured in the serum, including of IL-6 and IL-1\( \beta \), were neither significantly affected by the HF diet nor by PPE administration (Data S1, available online).

**Discussion**

The health-promoting effect of plant constituents and extracts is subject to interesting developments, a phenomenon explaining that their consumption is on the rise in the Western world\(^{1,2,25}\). The health benefits of pomegranate fruit and/or juice consumption have recently received considerable scientific focus. PPE used in this study is a source of polyphenol (30%), with punicalagin concentration reaching 8%. When added in the tap water of HF-fed mice during 4 weeks, PPE did not significantly modify body weight, adiposity and glucose tolerance. However, it decreased the HF-induced inflammatory tone not only in the gastrointestinal tract but also in the adipose tissue. It has been proposed that the anti-inflammatory effect of the pomegranate extract was dependent on...
both the inhibition of p38-mitogen-activated protein kinase (MAPK) pathway and inhibition of the activation of transcription factor NF-κB, as demonstrated in vivo in mouse skin exposed to 12-O-tetradecanoylphorbol-13-acetate, and in vitro in human chondrocytes (26,27). The activation of p38-MAPK and NF-κB is intimately associated with the increased gene expression of TNF-α, IL-1β, IL-6, monocyte chemoattractant protein 1 (MCP-1), inducible NO synthase (iNOS) and COX-2 that are critical mediators of inflammation and the pathogenesis of inflammatory diseases (28,29). In particular, COX-2-mediated inflammation in visceral fat plays a pivotal role in the development of metabolic disorders associated with obesity induced by HF feeding (30). In the present study, we have shown that PPE consumption down-regulated IL-1β, IL-6 and COX-2 in the colon and the visceral adipose tissue as compared to HF-fed mice without effect on the expression of those markers in the liver. These data demonstrate that bioavailable constituents of PPE or metabolites

Table 1. Lipid contents in the serum and the liver‡

<table>
<thead>
<tr>
<th></th>
<th>CT</th>
<th>HF</th>
<th>HF-PPE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean SEM</td>
<td>Mean SEM</td>
<td>Mean SEM</td>
</tr>
<tr>
<td>Serum lipids (mM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAG</td>
<td>0.98 0.13</td>
<td>0.59* 0.06</td>
<td>0.53* 0.03</td>
</tr>
<tr>
<td>NEFA</td>
<td>0.67 0.07</td>
<td>0.47 0.11</td>
<td>0.35 0.06</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>1.51 0.07</td>
<td>2.26* 0.07</td>
<td>1.92† 0.03</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>0.28 0.05</td>
<td>0.79* 0.06</td>
<td>0.44† 0.08</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>0.79 0.02</td>
<td>1.20* 0.07</td>
<td>1.23* 0.06</td>
</tr>
<tr>
<td>Liver lipid content (nmol/mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAG</td>
<td>283 29</td>
<td>357 45</td>
<td>378 35</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>207 21</td>
<td>210 54</td>
<td>176 22</td>
</tr>
</tbody>
</table>

CT, control; HF, high-fat; PPE, pomegranate peel extract.
* Values were significantly different from those of CT (P < 0.05).
† Values were significantly different from those of HF (P < 0.05).
‡ Mice were fed a CT diet, a HF diet or a HF diet with PPE in tap water for 4 weeks.

Fig. 3. Oral glucose tolerance test performed in mice fed a control (CT; (a) –△–, (b – d) ▲) diet, a high-fat (HF; (a) –●–, (b – d) ■) diet or a HF diet with pomegranate peel extract (HF-PPE; (a) –○–, (b – d) ▼) in tap water for 3 weeks. (a) Plasma glucose levels after the oral glucose load, (b) area under the curve (AUC) of the glucose excursion, (c) plasma insulin levels 30 min before and 15 min after the oral glucose load, (d) insulin resistance index. * Values were significantly different from those of CT (P < 0.05). † Values were significantly different from those of CT (−30 min) (P < 0.05).
Results obtained by Gonzalez-Sarrias propose that urolithin A and B, which are metabolised by the gut microbiota and produce urolithins. When the ellagitannins and/or ellagic acid reach the distal small intestine and are absorbed, the metabolites are further metabolised into urolithins. The metabolites are absorbed, glucuronidation occurs in the intestinal cells and glucuronides are the main metabolites found in the plasma of rats after oral administration. It is known that the pomegranate by-product, which is rich in oligomers of total bacteria, in particular Bifidobacterium spp., as well as glucose in different combinations, enhances the growth of total bacteria and glucose intolerance, adipose tissue inflammation and lipopolysaccharide level. Interestingly, the number of bifidobacteria was inversely correlated with the development of fat mass, glucose intolerance, adipose tissue inflammation and lipopolysaccharide level. Our data support that the gut microbiota may be an important actor in the health beneficial action of PPE. Indeed, we have demonstrated that PPE increased the caecal pool of bifidobacteria and this increases the response of Caco-2 cells to urolithins. Therefore, it suggests that the gut microbiota could contribute to the anti-inflammatory effects of PPE through their capacity to metabolise polyphenols.

Fig. 4. mRNA levels of inflammatory markers in (a) the colon, (b) the visceral adipose tissue and (c) the liver of mice fed a control (CT) diet, a high-fat (HF) diet or a HF diet with pomegranate peel extract (HF-PPE) in tap water for 4 weeks. Values are expressed relative to CT group (set at 1). * Values were significantly different from those of CT (P<0.05). † Values were significantly different from those of HF (P<0.05).

Health benefits of pomegranate extracts
batch culture fermentation systems reflective of the distal region of the human large intestine in the fermentation medium\(^{(43)}\). It is worth noting that punicalagins did not affect the growth of bacteria in this in vitro system.

In addition to changes in the gut microbiota, the most important effect of PPE supplementation was to blunt HF-induced hypercholesterolaemia (total and LDL-cholesterol). Recently, we and other authors provided evidence that modulation of the gut microbiota-host metabolic interrelationship by dietary intervention has the potential to improve cholesterol homeostasis, which has relevance for cardiovascular health\(^{(44,45)}\). Martinez et al.\(^{(44)}\) have highlighted through correlation analysis that the *Bifidobacterium/Coriobacteriaceae* equilibrium was important for plasma cholesterol levels in hamsters, with bifidobacteria being beneficial and coriobacteria being detrimental. In fact, the gut microbiota may affect host cholesterol metabolism through the modulation of the enterohepatic circulation of bile acids\(^{(46)}\). We postulate that the modulation of the gut microbiota induced by PPE supplementation observed in the present study can be involved in its hypocholesterolaemic effects.

In conclusion, the present study has shown that oral administration of a PPE rich in polyphenols is able to modulate the gut microbiota in favour of bifidobacteria. This prebiotic effect was accompanied by a lower expression of key inflammatory expression in the colon and the visceral adipose tissue. The gut microbiota changes due to the PPE treatment were also accompanied by an improvement of atherogenic markers such as LDL-cholesterol in HF diet-induced obesity. Although mechanistic studies are needed in order to determine which bioactive constituent(s) or metabolite(s) coming from the gut bacteria were responsible for these effects, our results suggest that PPE can confer positive health impacts associated with gut microbiota modulation and may be a natural alternative in the prevention of obesity and CVD.

Acknowledgements

L. B. B. is a research fellow and P. D. C. is a research associate from the FRS-FNRS (Fonds de la Recherche Scientifique) in Belgium. N. M. D. and P. D. C. are recipients for grant from the FRS-FNRS. Financial support was provided by a grant from the Walloon Region (WalNut 20 Project, convention 5459). A. M. N. and N. M. D. conceived and designed the experiments, and wrote the paper. A. M. N. and V. F. V. H. analysed the data. A. M. N., V. F. V. H. and F. D. B. performed the *in vivo* experiments and biochemical analysis. V. F. V. H. and F. D. B. performed RNA extraction in tissues and measured mRNA levels by quantitative PCR. L. B. B. performed and interpreted gut microbiota analysis (quantitative PCR). P. D. C., N. M. D. and L. B. B. provided intellectual input on the paper and reviewed the paper. N. M. D. planned and supervised all experiments and the manuscript preparation. The authors declare that there are no conflicts of interest in relation to this study.

Supplementary table S1 and data are available online at http://www.journals.cambridge.org/bjn

References

Health benefits of pomegranate extracts


