Apple flavonols and n-3 polyunsaturated fatty acid–rich fish oil lowers blood C-reactive protein in rats with hypercholesterolemia and acute inflammation

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ABSTRACT

Both quercetin glycosides and omega-3 polyunsaturated fatty acids (n-3 PUFAs) are well established for their individual health benefits in ameliorating metabolic disease. However, their combined effects are not well documented. It was hypothesized that the beneficial properties of quercetin glycosides can be enhanced when provided in combination with n-3 PUFAs. Therefore, the aim of the present study was to investigate the effects of apple flavonols (AF) and fish oil (FO), alone and in combination, on proinflammatory biomarkers and lipid profiles in rats fed a high-fat diet. Sixty male Wistar rats were randomly divided into 5 groups (n = 12) and fed a high-fat diet for 4 weeks. One of the 5 groups of rats was used as the high-fat control (HFL), and the other 4 groups of rats were injected with lipopolysaccharide (LPS) (5 mg/kg body weight) intraperitoneally, 5 hours before euthanization. One of these 4 groups was used as the hypercholesterolemic and inflammatory control (HFL + LPS), and the other 3 received AF (HFL + 25 mg/kg per day AF), FO (HFL + 1 g/kg per day FO), or the combination (HFL + AF + FO). Compared to the HFL group, the AF, FO, and AF + FO groups showed lower serum concentrations of interleukin-6 and C-reactive protein (CRP) levels. The AF, FO, and AF + FO also had lowered serum triacylglycerol and non–high-density lipoprotein cholesterol (HDL-C) concentrations, but higher HDL-C levels relative to the HFL group. An additive effect was observed on serum CRP in the AF + FO group as compared with the AF or FO groups. The results demonstrated that AF and FO inhibited the production of proinflammatory mediators and showed an improved efficacy to lower serum CRP when administered in combination. They significantly improved blood lipid profiles in rats with diet-induced hyperlipidemia and LPS-induced acute inflammation.

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Abbreviations: AF, apple flavonols; ANOVA, analysis of variance; CE, cholesteryl ester; CRP, C-reactive protein; CVD, cardiovascular disease; ELISA, enzyme-linked immunosorbant assay; FC, free cholesterol; FO, fish oil; HDL-C, high-density lipoprotein cholesterol; HFC, high-fat control; HFL, high-fat with lipopolysaccharide; IFN-γ, interferon-γ; IL, interleukin; LDL-C, low-density lipoprotein cholesterol; LPS, lipopolysaccharide; NF-κB, nuclear factor-kappa B; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; TAG, triacylglycerols; TC, total cholesterol; TNF-α, tumor necrosis factor α.

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1. Introduction

The prevalence of obesity in Western populations has been postulated to be the major cause of metabolic syndrome, including insulin resistance, type 2 diabetes, hyperlipidemia, and hypertension [1]. Accumulating evidence suggests that a long-standing high-fat diet might have contributed to metabolic endotoxemia and chronic inflammation [2,3]. Hyperlipidemia is commonly characterized by elevated circulating levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triacylglycerol (TAG) and reduced high-density lipoprotein cholesterol (HDL-C) [4]. At the initial stage of most metabolic disorders, the circulating levels of C-reactive protein (CRP), tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), and adiponectin are altered, thus increasing risk for developing cardiovascular disease (CVD) and insulin resistance [5].

A high-fat diet is lipogenic and induces hyperlipidemia in rats [6,7] and excessive body weight, which serves as an experimental model of diet-induced obesity [8]. In addition to a healthy lifestyle, nutritional intervention has long been recommended for maintaining healthy lipid profiles [9]. Polyphenols have been shown to decrease the risk of degenerative diseases such as CVD and cancer [10]. Apples, especially the peel (skin), are a great source of dietary polyphenols [11,12]. It is reported that apple flavonoids possess anti-inflammatory and lipid-lowering properties [13,14]. The major flavonols in apple peel are quercetin glycosides, which contribute approximately 70% of all flavonols in the North American diet [15]. Quercetin is well documented for its antioxidant and anti-inflammatory effects [16]. In addition to flavonoids, omega-3 polyunsaturated fatty acids (n-3 PUFA) are another class of naturally occurring biomolecules that are recognized for their anti-inflammatory and hypotriglyceridemic benefits [9,17–20]. However, n-3 PUFA is highly susceptible to lipid oxidation due to a high degree of unsaturation. Flavonoids (as antioxidants) may inhibit oxidation, thus protecting n-3 PUFA [21] and enhancing quercetin efficacy in vivo [22].

The potential benefits of combining flavonoids and n-3 PUFA have not been well described. Herein, we hypothesized that the lipid-lowering and anti-inflammatory properties of apple quercetin glycosides can be enhanced by supplementing a diet with n-3 PUFA. Accordingly, the objective of this study was to determine the effect of apple flavonols (AF) and fish oil (FO), alone and in combination, on proinflammatory markers and lipid profiles in rats with diet-induced hyperlipidemia and lipopolysaccharide (LPS)–induced acute inflammation.

2. Methods and materials

2.1. Chemicals and apparatus

All dietary ingredients for animal feed, except AF and FO, were purchased from Dyets Inc. (Bethlehem, PA, USA). The eicosapentaenoic acid–rich FO that was provided by DSM (formerly Ocean Nutrition Canada, Dartmouth, NS, Canada) contained 75.7% total PUFA (n-3 and n-6), and 70.6% were n-3 PUFA (82% eicosapentaenoic acid and 14% docosahexaenoic acid). Rat cytokine flex kits and CRP enzyme-linked immunosorbant assay (ELISA) kit was purchased from BD Biosciences (Mississauga, ON, Canada), and rat ELISA kits (IL-6 and adiponectin) were obtained from Invitrogen (Burlington, ON, Canada). Reagents for liver and serum lipid reagents were obtained from BioVision (Milpitas, CA, USA) and BioPacific Diagnostic Inc. (North Vancouver, BC, Canada). Lipopolysaccharide lyophilized powder (from Escherichia coli) was purchased from Sigma-Aldrich (Oakville, ON, Canada).

2.2. Ultraperformance liquid chromatography–tandem mass spectrometry analysis of apple peel phenolic compounds

The phenolic compounds were extracted and fractionated using reversed-phase chromatography, as previously described [21]. The fractions containing high amounts of quercetin-3-O-glycosides were pooled and freeze-dried to form AF concentrate. Analyses of the major individual phenolic compounds present in apple peel extract were performed according to the method previously reported [23]. All analyses were conducted using ultrapressure liquid chromatography (Waters, Milford, MA, USA), coupled with Micromass Quattro micro API MS/MS system, and controlled with Mass lynx V4.0 data analysis system (Micromass, Cary, NC, USA).

2.3. Animal study

Six-week-old male Wistar rats (Charles River, Montreal, QC, Canada), weighing 200 to 250 g, were housed individually in cages with a 12-hour dark/light cycle, and free access to regular rodent chow and water. After a week of adaptation, the rats were weighed and randomly divided into five groups (n = 12 per group), and fed their respective diets for four additional weeks. One group was fed the AIN-93G diet, modified to contain 23% fat, 1.9% cholesterol, and 0.5% cholic acid (Table 1) to induce hyperlipidemia and other obesity-related characteristics (high-fat control [HFC]). Among the 4 high-fat with lipopolysaccharide (HFL) groups, 1 group was used as an obese and inflammatory control (HFL + FO, and HFL + AF + FO groups were injected intraperitoneally with 5 mg/kg body weight of LPS to induce acute inflammation). The animal use and experimental procedures were approved by the Joint Animal Care and Research Ethics Committee of the National Research Council Canada–Institute for Nutrisciences and Health and the University of Prince Edward Island, Canada. The study was conducted in accordance with the guidelines of the Canadian Council on Animal Care.

2.4. Collection and storage of blood and tissue samples

At the end of the study, the animals were anesthetized with isoflurane inhalation and killed by exsanguination. Blood was...
collected by cardiac puncture into serum tubes (BD Vacutainers, Franklin Lakes, NJ, USA). The serum was separated by centrifugation at 650 \( g \) for 15 min using an Allegra 25R centrifuge (Beckman Coulter, Mississauga, ON, Canada), and stored in a \(-80^\circ C\) freezer for later analyses. Liver, spleen, and adipose tissue were dissected, washed with saline solution, frozen in liquid nitrogen, and stored at \(-80^\circ C\).

2.5. Analysis of serum cytokines

The serum concentration of cytokines IL-10, TNF-\( \alpha \), and interferon-\( \gamma \) (IFN-\( \gamma \)) were quantified using cytometric bead array rat flex kits (BD Biosciences) following the manufacturer's instructions [25]. The samples were analyzed on a BD FACSAArray Bioanalyzer, and the data analysis was performed using the FCAP Array software (BD Biosciences). The concentration of each cytokine was presented as picogram per milliliter.

2.6. Determination of serum CRP

The serum CRP was quantified using a solid-phase sandwich ELISA kit (BD Biosciences) following the supplier's instructions [26]. The samples were read for absorbance at 460 nm on a BioTek Powerwave microplate reader (Winooski, VT, USA).

2.7. Determination of serum IL-6 and adiponectin

The serum IL-6 and adiponectin concentrations were measured using ELISA kits supplied by Invitrogen, according to the manufacturer's instructions [26]. The optical density was read at 450 nm on a Varioskan Flash plate reader (Thermofisher Scientific, Nepean, ON, Canada).

2.8. Lipid profile analysis in rat serum

The serum TC, HDL-C, and TAG were analyzed on a Pointe-180 chemistry analyzer (Pointe Scientific Inc., Canton, MI, USA), with all reagents provided by the manufacturer. The HDL-C was measured after the precipitation of apolipoprotein B containing lipoproteins with dextran sulfate and magnesium chloride. Non-HDL cholesterol was calculated by subtracting HDL-C from TC [24].

2.9. Analysis of liver lipids

The liver cholesterol was extracted by homogenizing 100 mg of liver in 1 mL of chloroform/isopropanol/nonidet P-40 (octylphenoxypolyethoxyethanol) (7:11:01, v/v/v). After centrifugation, 200 \( \mu L \) of supernatant was dried under nitrogen. For liver TAG analysis, 100 mg of liver was homogenized in 5% nonidet P-40 in water, heated for 5 minutes in the water bath at 90°C, and cooled down to room temperature. The liver TC,

### Table 1 – Ingredient composition of the diet fed to rats

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Composition (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>229.0</td>
</tr>
<tr>
<td>Corn starch</td>
<td>269.8</td>
</tr>
<tr>
<td>Sucrose</td>
<td>121.4</td>
</tr>
<tr>
<td>Fat a</td>
<td>229.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>63.5</td>
</tr>
<tr>
<td>DL-(-)ethionine</td>
<td>3.8</td>
</tr>
<tr>
<td>Mineral mix b</td>
<td>44.3</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.5</td>
</tr>
<tr>
<td>Butylated hydroxytoluene</td>
<td>0.2</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>19.1</td>
</tr>
<tr>
<td>Cholic acid</td>
<td>4.7</td>
</tr>
</tbody>
</table>

* Composed of 96% of lard and 4% of sunflower oil.

b Mineral mix contained the following: 5000 mg Ca, 1561 mg P, 3600 mg K, 1019 mg Na, 1571 mg Cl, 300 mg S, 507 mg Mg, 35 mg Fe, 6 mg Cu, 10 mg Mn, 30 mg Zn, 1 mg Cr, 0.2 mg I, 0.15 mg Se, 1 mg F, 0.5 mg B, 0.15 mg Si, 0.5 mg Ni, 0.1 mg F, and 0.1 mg V per kilogram of the mineral mix.

### Table 2 – Concentrations of phenolic compounds of Northern Spy AF concentrate prepared using reverse-phase chromatography

<table>
<thead>
<tr>
<th>Phenolic compounds</th>
<th>Concentration ( a ) (mg/g DW)</th>
<th>Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin-3-O-galactoside</td>
<td>104.6 ± 21.1</td>
<td>29.8</td>
</tr>
<tr>
<td>Quercetin-3-O-rhamnoside</td>
<td>99.98 ± 20.0</td>
<td>28.5</td>
</tr>
<tr>
<td>Quercetin-3-O-glucoside</td>
<td>10.89 ± 1.80</td>
<td>31.0</td>
</tr>
<tr>
<td>Quercetin-3-O-rutinoside</td>
<td>3.66 ± 1.18</td>
<td>1.04</td>
</tr>
<tr>
<td>Quercetin</td>
<td>2.88 ± 0.70</td>
<td>0.82</td>
</tr>
<tr>
<td>Phloridzin</td>
<td>66.78 ± 9.09</td>
<td>19.0</td>
</tr>
<tr>
<td>Phloretin</td>
<td>0.69 ± 0.07</td>
<td>0.19</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>16.55 ± 4.58</td>
<td>4.71</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>0.77 ± 0.08</td>
<td>0.21</td>
</tr>
<tr>
<td>Catechin</td>
<td>6.12 ± 0.99</td>
<td>1.74</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>37.86 ± 8.75</td>
<td>10.7</td>
</tr>
</tbody>
</table>

The polyphenolics extracted in absolute ethanol were fractioned using 20 to 100% ethanol gradient by reverse-phase chromatography. The fractions were analyzed using UPLC-MS/MS and the fractions rich in flavonols were concentrated and dried into powder. Abbreviation: UPLC-MS/MS, ultra-performance liquid chromatography–tandem mass spectrometry.

* Data presented as mean (SEM), \( n = 3 \).
free cholesterol (FC), cholesteryl esters (CEs), and TAG were determined using the commercial kits (BioVision), as reported previously [24].

2.10. Statistical analyses

All data analyses were performed using SAS software version 8 (Cary, NC, USA). The normality of the residuals was tested using the Anderson-Darling test, and the data were log-transformed for serum CRP, TC, and TAG. Treatment effects were determined using one-way analysis of variance (ANOVA). When a significant treatment effect was detected, the differences among the groups were determined using the Duncan test. Results are presented as mean (SEM) (n = 12). The significance level was set at P < .05.

3. Results

3.1. Composition of flavonol-rich apple peel concentrate

The total polyphenols content in the freeze-dried apple peel concentrate was 350 mg/g. Quercetin-3-glycosides were the predominant component, at 63%, that is, 221.9 mg/g (Table 2).

3.2. Food intake, body weight, and liver and spleen weights

There were no significant differences in the weekly and final body weights (Table 3) among the 5 groups, after 4 weeks of treatment. Similarly, the treatments had no effect on food intake. No effect was observed on the liver and spleen weights among the 5 groups (Table 3). Previous studies demonstrated that after 4 weeks of high-fat diet in rats, they developed characteristics of obesity [7,8]. To minimize the use of rats, a normal diet control group was not used as advised by the Animal Care and Research Ethics Committee.

3.3. Serum inflammatory biomarkers

The serum adiponectin levels did not show any difference among all groups (Fig. 1A). There was no significant difference in the serum CRP concentrations between HFC and HFL. However, significant reductions were observed following the 4-week treatment with AF, FO, and AF + FO (11, 27, and 29%, respectively), which was lower than the HFL group (Fig. 1B). The serum TNF-α concentrations of the HFL group were increased to 24-fold of the HFC group. Nevertheless, there were not differences between the HFL and each of the three treatment groups (Fig. 1C). The serum concentration of IFN-γ in the HFL group was increased to 99-fold of the HFC group. The supplementation of FO lowered serum concentrations of IFN-γ by 45%; however, AF or AF + FO did not show a significant effect (Fig. 1D). The serum IL-6 concentration was raised by nearly twofold after LPS injection in the HFL groups, compared with the HFC group. As compared with the HFL group, the supplementation of AF, FO, and AF + FO reduced IL-6 serum concentrations by 21, 25, and 35%, respectively (Fig. 1E). The IL-10 concentration in the HFL group was 18-fold of the HFC group (P < .05). The IL-10 concentration was further elevated with AF and AF + FO treatment (Fig. 1F).

3.4. Serum and liver lipids

The effects of the treatments on serum lipids are summarized in Fig. 2. There were no differences between the HFC and HFL groups in all parameters measured (Fig. 2A-D). The AF, FO, or AF + FO decreased the serum TAG by 35, 39, and 36%, respectively, as compared with the HFL group (Fig. 2A). Similarly, the three treatments decreased the serum non-HDL-C levels. Rats of the AF, FO, and AF + FO groups showed 53, 51, and 49% lower levels of serum non-HDL-C than the HFL group, respectively (Fig. 2D). In contrast, the AF, FO, and AF + FO increased serum HDL-C by 56, 46, and 43% relative to the HFL group (Fig. 2C). There were no differences among the 5 groups in the serum TC.

The concentrations of liver TAG (Fig. 3A) and CEs (Fig. 3D) did not differ among the 5 groups. In comparison to the HFL group, the AF and AF + FO groups reduced liver TC by 15 and 18%, respectively (Fig. 3B). The liver FC concentrations were decreased by 37, 38, and 37%, respectively, as compared with the HFL group (Fig. 3C).

4. Discussion

A moderate dose of LPS is known to trigger signal transduction associated with inflammation, mimicking the natural response of the human body under inflammatory conditions. High-fat and cholesterol diet–induced hyperlipidemia, in combination with LPS-induced acute inflammation, produces characteristics that are shared by metabolic diseases, such as CVD and insulin resistance [2]. There is a significantly increased public interest in seeking products derived from natural resources [23]. The results of the current study demonstrated that dietary supplementation of flavonols and
n-3 PUFA significantly improved blood lipid profiles and inflammatory biomarkers in rats of hyperlipidemia and acute inflammation. The results are in agreement with previous studies where quercetin-rich diets alleviated hyperlipidemia in obese Wistar rats after 4 weeks of treatment at a dose range of 19 to 35 mg/kg per day [27,28], and where omega-3 PUFA has demonstrated lower blood lipids at a daily intake of 1-4 g per day in humans [29].

Numerous studies in the past decade demonstrated that high blood TAG, low HDL-C levels, and high LDL-C levels are associated with increased blood levels of inflammatory cytokines, such as CRP, TNF-α, and IL-6, and that they form a cascade for endothelial dysfunction [30,31]. In hypercholesterolemic rats, intraperitoneal injection of LPS significantly increased circulating levels of proinflammatory cytokines, TNF-α, IFN-γ, and IL-6. This observation was in accordance with previous reports [32,33]. The insignificant change of serum TAG after the LPS injection was also in agreement with a previous study showing that serum lipid profiles were not altered after 18 hours of LPS injection in rats [33]. It may be suggested that chronic, rather than acute inflammation, increases blood TAG and cholesterol levels [32].

Fig. 1 – Serum adiponectin (A), CRP (B), TNF-α (C), IFN-γ (D), IL-6 (E), and IL-10 (F) in 5 groups of high-fat diet-fed rats. Except control 1 (HFC), LPS (5 mg/kg) was injected in control 2 (HFL) along with 4 treatment groups, 5 hours before euthanization. Results are expressed as mean (SEM) (n = 12). Data were analyzed by ANOVA, and statistical significance by Duncan test using the general linear model. The values with different letters are significantly different, *P < .05. NS, not significant.
The proinflammatory cytokines measured in the present study are considered to initiate a variety of pathophysiological processes and cause acute inflammation [31]. A body of evidence demonstrates that consumption of n-3 PUFA reduces blood TAG while having an inconsistent effect on cholesterol [34,35]. It is reported that n-3 PUFA lowers TAG levels by increasing mitochondrial fatty acid oxidation, which may increase the leakage of reactive oxygen species (ROS) from the electron transport chain [36]. The ROS causes oxidative stress, which is associated with chronic inflammation [31]. Antioxidants, such as flavonols, could counteract the negative effects of fatty acid oxidation by scavenging ROS [21]. Although the beneficial effects of n-3 PUFA and polyphenols on hyperlipidemia and inflammation are reported, little is known if a better effect could be achieved when they are provided in combination. Among the proinflammatory cytokines tested in this study, TNF-α and IL-6 play important roles in inducing an acute phase reaction. Elevation of the circulating IL-6 and TNF-α up-regulates the expression of CRP in the liver [31], resulting in an increase of CRP concentration in the blood stream. In return, CRP amplifies the production of proinflammatory cytokines and induces endothelial dysfunction [37]. Thus, the serum concentration of CRP is a crucial biomarker of systemic and chronic inflammation and a risk factor for CVD. Because there was no significant difference in serum CRP between the HFC and HFL groups, this may suggest that the 4-week feeding of the high-fat high-cholesterol diet might have induced obesity-related subclinical inflammation in rats; and thus, there was no further increase after the LPS injection. It is possible that a longer period is required to elevate CRP gene and protein expression after LPS injection through other cytokines such as IL-6 and TNF-α. This further supports the previous observation that obesity itself is an inflammatory condition [38]. It has been reported that flavonoid intake is inversely associated with serum CRP concentrations in human adults [39]. In line with this, in this study, dietary supplementation of AF significantly reduced the serum concentration of CRP. Moreover, a synergistic effect among multipolyphenols may be involved in lowering CRP production [40]. Fish oil showed a similar effect on CRP production, and interestingly, when they were combined, an additive effect was observed. Both the AF and FO lowered the serum levels of IL-6, but no additive effect was seen when supplemented together. Flavonols and n-3 PUFA possibly share a common molecular mechanism to down-regulate the production of CRP and IL-6 by inhibiting the expression of nuclear factor-κB (NF-κB) [41–43]. It is well known that NF-κB is the primary transcriptional regulator of proinflammatory mediator expression, including IL-6, TNF-α, cyclooxygenase-2, phospholipase, and inducible nitric oxide synthase [44,45].

Numerous in vitro and in vivo studies have shown the anti-inflammatory [32,46] and antioxidant effects [21,47] of apple

Fig. 2 – Serum TAG (A), serum TC (B), HDL-C, (C) and non–HDL-C (D) levels in 5 groups of high-fat diet-fed rats. Except control 1 (HFC), LPS (5 mg/kg) was injected in control 2 (HFL) and the 3 treatment groups, 5 hours before euthanization. Results are expressed as mean (SEM) (n = 12). Data were analyzed by ANOVA, and statistical significance by Duncan test using the general linear model. The values with different letters are significantly different, *P < .05. NS, not significant.
peel extract. Quercetin glycosides are a major group of flavonols in apple peel and are reported to suppress NF-\(\kappa\)B expression and function [48]. Apple peel also contains a high concentration of phloridzin, which has been shown to have similar biological properties as flavonols [49]. An in vitro study showed the anti-inflammatory effects of an apple extract fraction containing phloridzin, quercetin glycosides, and epicatechin [32]. A decrease in the circulating concentrations of adiponectin and a high TNF-\(\alpha\) level are considered to be predictive markers of CVD and diabetes and are strongly correlated with visceral fat accumulation [5,50]. The AF, FO, and AF + FO did not show any significant effect on adiponectin and TNF-\(\alpha\) concentrations, contrary to previous reports [42,51]. The significant decreases in other proinflammatory cytokines in the present study might suggest that TNF-\(\alpha\) expression is regulated by a pathway that is different from the NF-\(\kappa\)B–dependent signaling [33,52]. Fish oil significantly lowered the serum IFN-\(\gamma\) concentrations. The AF and AF + FO showed a trend to reduce IFN-\(\gamma\) but did not reach the significance level. The stimulation of IL-10 expression and production by LPS and quercetin was reported earlier [53,54]. Lipopolysaccharide induces both Th1- and Th2-mediated inflammations [55]. The serum concentration of IL-10 was increased after LPS injection, and it was further elevated by the supplementation of AF and marginally by the AF + FO. Interleukin-10 exerts an array of host defence responses by inducing the expression of a wide range of anti-inflammation genes [56].

Fish oil and AF lowered the serum TAG and elevated HDL-C levels. Feeding n-3 PUFA lowers TAG by reducing hepatic synthesis of TAG-rich very low-density lipoprotein and removing TAG from the circulating lipoproteins because of a higher activity of hormone-sensitive lipase [20,43]. The increase of serum HDL-C concentrations by FO is in line with a previous report [57]. Non–HDL-C predicts the risk of CVD as equally effective as LDL-C or even better than LDL-C [58]. The guidelines and recommendations for the treatment of hyperlipidemia consider non–HDL-C as the secondary target if TAG levels remain elevated after treating LDL-C levels [59]. Clinical studies have reported that n-3 PUFA, combined with statins, reduce the risk of coronary events without any significant changes in LDL-C and HDL-C, suggesting other mechanisms than LDL-C might be involved [59,60]. The hepatic cholesterol-lowering effect of apple flavonoids might be attributed to the modulation of hepatic cholesterol 7\(\alpha\)-hydroxylase, thus leading to the conversion of cholesterol into bile acids and the increase of fecal excretion of bile acids and neutral steroids [14,61]. By suppressing the sterol regulatory element-binding protein-1 mRNA expression, n-3 PUFA could encounter the hepatic lipid synthesis [62].

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**Fig. 3** – Liver TC (A), TAG (B), FC, (C) and CE (D) levels in 5 groups of high-fat diet–fed rats. Except control 1 (HFC), LPS (5 mg/kg) was injected in control 2 (HFL) and the 3 treatment groups, 5 hours before euthanization. Results are expressed as mean (SEM) (n = 12). Data were analyzed by ANOVA, and statistical significance by Duncan test using the general linear model. The values with different letters are significantly different, *\(P < .05\). NS, not significant.
The results of this study partially support the hypothesis that AF and FO could have complementary effects on the production of CRP, which can further influence the development of inflammation and hyperlipidemia. However, these results did not show that acute inflammation influences lipid profile under hyperlipidemia. One of the limitations of the present study in rats is the period of treatment after the induction of acute inflammation by LPS injection. The anti-inflammatory effects of AF and FO observed are more relevant to the prevention of acute inflammation rather than the treatment of chronic inflammation. If a longer time had been given after the induction of acute inflammation, an increase of serum CRP and significant treatment effects might have been achieved in rats. Another limitation is the lack of normal fat control and treatments of such rats to determine whether the observed beneficial effects are applicable to lower fat levels in rats. We were also limited since the potential molecular mechanism underlying the anti-inflammatory ability of AF and FO remains unclear. Finally, future research into lipid metabolism and related enzymes would be helpful to understand the hypolipidemic effects of AF and FO.

In conclusion, LPS injection markedly increased the serum concentration of several proinflammatory biomarkers, such as IL-6, IFN-γ, and TNF-α, as well as anti-inflammatory biomarker IL-10, whereas no effect was noted on CRP in rats fed a high-fat diet. The supplementation of AF, FO, or AF + FO decreased or reversed the serum concentration of these proinflammatory cytokines, and by contrast, increased the anti-inflammatory cytokine. The AF, FO, and/or AF + FO group of rats had lower serum TAG and non-HDL-C, while increasing HDL-C levels and lower liver TC and FC contents. When AF and FO were provided in combination to rats, they showed an improved effect on serum CRP. The combination therapies of two or more drugs, especially those that involve different mechanisms, have been increasingly used in treating metabolic diseases. Apple flavonoids and n-3 PUFA–rich FO should be further investigated for their complementary effects on proinflammatory cytokines and lipids, particularly in a metabolic syndrome model with chronic inflammation.

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