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3rd International Immunonutrition Workshop

Session 1: Antioxidants and the immune system Flavonoids as anti-inflammatory agents

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Epidemiological evidence suggests that a high intake of plant foods is associated with lower risk of chronic diseases. However, the mechanism of action and the components involved in this effect have not been identified clearly. In recent years, the scientific community has agreed to focus its attention on a class of secondary metabolites extensively present in a wide range of plant foods: the flavonoids, suggested as having different biological roles. The anti-inflammatory actions of flavonoids *in vitro* or in cellular models involve the inhibition of the synthesis and activities of different pro-inflammatory mediators such as eicosanoids, cytokines, adhesion molecules and C-reactive protein. Molecular activities of flavonoids include inhibition of transcription factors such as NF-κB and activating protein-1 (AP-1), as well as activation of nuclear factor-erythroid 2-related factor 2 (Nrf2). However, the *in vitro* evidence might be somehow of limited impact due to the non-physiological concentrations utilized and to the fact that *in vivo* flavonoids are extensively metabolized to molecules with different chemical structures and activities compared with the ones originally present in the food. Human studies investigating the effect of flavonoids on markers of inflammation are insufficient, and are mainly focused on flavonoid-rich foods but not on pure molecules. Most of the studies lack assessment of flavonoid absorption or fail to associate an effect on inflammation with a change in circulating levels of flavonoids. Human trials with appropriate placebo and pure flavonoid molecules are needed to clarify if flavonoids represent ancillary ingredients or key molecules involved in the anti-inflammatory properties of plant foods.

Flavonoids: Inflammation: Human subjects: Plant foods

It is widely recognized that lifestyle factors, particularly diet, play a paramount role in the development or prevention of degenerative diseases[1,2]. Thus, there is growing interest worldwide in the prospect that overall diet as well as particular foods can promote and help maintain a good health status. Evidence has been provided that suggests that dietary patterns rich in foods of plant origin, such as fruits and vegetables, play a key role in disease prevention through a multi-factorial action involving a modulation of the immune system and the inflammatory response[3,4]. If the inflammatory response is not properly controlled, excess inflammatory stress may be induced, becoming a key modulator of endothelial damage playing a role in the pathogenesis of risk factors for CVD including obesity, hyperglycaemia and dyslipidaemia[1]. Inflammatory response mediated by acute-phase proteins such as C-reactive protein (CRP) and cytokines such as IL-6 may directly influence plaque vulnerability and rupture[5]. TNFα promotes the inflammatory cascade within the arterial wall, by inducing endothelial cell injury, as well as regulating leucocyte activation, maturation, cytokine and chemokine release, and production of reactive oxygen and nitrogen intermediates[6]. Soluble forms of cellular adhesion molecules, such as intercellular adhesion molecule-1 and

Abbreviation: AP-1, activating protein-1; CRP, C-reactive protein; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase.

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vascular cell adhesion molecule-1, are considered to be markers of endothelial activation[7] elevated in patients suffering from CVD[8].

Polyphenols are secondary metabolites of plants involved in pigmentation, reproduction and protection against pathogens[9]. Currently, there are more than 8000 known polyphenols sharing a common chemical structure (hydroxyl group on aromatic ring) with different constituents. Flavonoids are the most abundant polyphenols present in the human diet and represent a class of molecules characterized by a C6–C3–C6 backbone structure[9]. Flavonoids can be divided into several subclasses according to different constituents such as flavanones, flavone, flavanols and flavonols. They can be found in almost all foods of vegetable origin and are present in high amounts in apples, onions, red wine, grapes, citrus fruits, tea, berries and olive oil. Among the different flavonols, myricetin, kaempferol and quercetin are the most representative. Catechins are the most abundant flavanols and are mainly contained in tea leaves. Flavanones are mainly represented by taxifolin, naringinin and hesperitin. The main sources of flavanones are citrus fruits. Flavones, luteolin, wogonin and apigenin are less common and identified in sweet red pepper and celery. In addition to these flavonoids, other subclasses are present such as proanthocyanidins and their oligomers present in cocoa products.

A growing number of observational epidemiological studies have examined the association between the intake of foods rich in polyphenols (onions, apples, tea, cocoa and red wine) as well as of individual dietary flavonoids (mainly flavonols, flavones and catechins) and chronic diseases[10]. Overall, the epidemiological evidence generally shows that a higher flavonoid intake is associated with lower CVD[11] and cancer risk[12,13]. However, intervention trials in human subjects using pure compounds are scarce and studies with flavonoid-rich foods provided contrasting findings, failing to identify the flavonoids as the molecules responsible for the observed effect[14–16]. However, it must be considered that the biological activities of flavonoid-rich foods are critically determined by their bioavailability. The most abundant flavonoids in the diet are not always those able to reach the highest levels in human circulation. We showed[17] that, in healthy subjects, plasma concentrations of caffeic acid (34 μg/l basal, 63 μg/l at 3 h), *p*-coumaric acid (46 μg/l basal, 85 μg/l at 3 h) and quercetin (46 μg/l basal, 66 μg/l at 3 h) after ingestion of 250 g lettuce do not reflect their content in food (31·7 mg caffeic acid, 7·3 mg *p*-coumaric acid and 12·7 mg quercetin). Flavonoids can be absorbed in the stomach and at small intestine level by passive diffusion or active transport[18,19]. Once absorbed, metabolism of flavonoids in humans involves a biotransformation through enzymic conjugation with sulphate, methyl or glucuronide groups both in the small intestine epithelial cells and liver[19]. Variable amounts of flavonoids, not absorbed in the upper gastrointestinal tract, reach the colon where they are subject to the action of the colon microflora, resulting in cleavage of glycosidic linkages and the breakdown of the flavonoid heterocycle into phenolic acids and aldehydes[20–24]. These microbial catabolites are absorbed into the circulatory system from the large intestine[25]. Upon absorption, polyphenols are readily metabolized in intestinal cells to form glucuronide and sulphate conjugates that appear in the portal blood.

The present paper will review the more recent evidence regarding the role of flavonoids as dietary modulators of the cascade of events associated with inflammatory responses.

Anti-inflammatory properties of flavonoids: evidence from *in vitro* and cellular models

The molecular mechanisms involved in the anti-inflammatory activities of flavonoids have been suggested to include: inhibition of pro-inflammatory enzymes, such as cyclooxygenase-2, lipoxygenase and inducible NO synthase, inhibition of NF-κB and activating protein-1 (AP-1) and activation of phase II antioxidant detoxifying enzymes, mitogen-activated protein kinase (MAPK), protein kinase C and nuclear factor-erythroid 2-related factor 2[26–28]. Cyclooxygenase-2 is inhibited by quercetin and kaempferol in rat peritoneal macrophages[29]. Catechin weakly inhibits cyclooxygenase-2 but at a very high concentration (100 μM) with respect to the serum concentrations found following the ingestion of flavonoid-rich foods[30]. Flavonols such as kaemferol, quercetin, morin and myricetin were found to be better lipoxygenase inhibitors than flavones. Flavanones such as naringenin were ineffective. In lipopolysaccharide (LPS)/cytokine-treated macrophages or macrophage cell lines, quercetin, apigenin and luteolin were found to inhibit NO production[31]. Using LPS-treated RAW cell lines, it was found that catechins and flavanones were not active in reducing NO production below a concentration of 100 μM[32]. Further evidence[33] showed that the inhibitory effect of apigenin, genistein and kaempferol on NO production is mediated by an effect on inducible NO synthase down-regulation.

Inflammatory cytokines produced and regulated at the transcriptional level can either enhance or inhibit the inflammatory process. It has been observed that several flavonoids are able to decrease the expression of different pro-inflammatory cytokines/chemokines, including TNFα, IL-1β, IL-6, IL-8 and monocyte-chemoattractant protein-1, in different cell types such as RAW macrophages, Jurkat T-cells and peripheral blood mononuclear cells[26]. Quercetin and catechins coupled their inhibitory action on TNFα and IL-1β to an enhanced release of the anti-inflammatory cytokine IL-10[26]. Molecular mechanisms involved in their cytokine-modulating activity, including polyphenol-mediated inhibition of transcription factors NF-κB and AP-1 and reduction of MAPK activity, have been suggested as relevant anti-inflammatory pathways[26,34]. Genistein has been reported to inhibit IL-1β, IL-6 and TNFα production in LPS-induced human blood monocytes[35]. Genistein and silybin were shown to inhibit TNFα production from LPS-treated RAW cells[36]. Quercetin has been shown to affect inducible NO synthase and TNFα expression from LPS-induced RAW cells by inhibiting MAPK and AP-1 DNA binding[37,38]. Quercetin was shown to also affect NF-κB activation by extracellular

signal-regulated kinase and p38 kinase inhibition[39]. The effect on NF-κB was shown also for genistein, apigenin, kaempferol and epigallocatechin 3-gallate in LPS-stimulated macrophages. Evidence suggests that flavonoids are able to inhibit the expression of inflammation-related enzymes/proteins, partly by suppressing the activation of NF-κB, AP-1 and MAPK[26]. Specifically, quercetin showed an inhibitory effect on extracellular signal-regulated kinase and c-Jun N-terminal kinase, while catechin inhibited p38 and c-Jun N-terminal kinase[26]. The AP-1 transcription factors and AP-1 factor-associated signal transduction, implicated in inflammatory response, are important targets of flavonoid action[34,40]. In addition, induction of nuclear factor-erythroid 2-related factor, the transcription factor responsible for both constitutive and inducible expressions of the antioxidant responsive element-regulated genes[41], suppressed MCP-1 and vascular cell adhesion molecule-1 expression, monocyte adhesion to endothelial cells and transmigration, activation of p38 MAPK and inhibited atherosclerotic lesion formation in mice and rabbit[42]. The body of evidence suggests that dietary flavonoids can modulate inflammatory responses also through an activation of pathways inducing antioxidant transcription and detoxification defence systems, such as glutathione peroxidase, haem oxygenases, γ-glutamylcysteine synthetase, superoxide dismutase and glutathione reductase, through anti-oxidant responsive element[43–46].

Anti-inflammatory properties of flavonoids: human studies

Dietary intervention trials investigating the effect of flavonoids on markers of inflammation in human subjects are scarce and are focused on a limited number of foods of plant origin such as black and green teas, fruit juices, grape extract and red wine, as described and summarized in Table 1. Four-week administration of black tea, green tea and green tea extracts had no effect on the inflammatory markers IL-6, IL-1β, TNFα, CRP and fibrinogen[47]. However, the same period of time was enough to show a reduction of P-selectin levels concomitantly with an increase of urinary 4-*O*-methyl gallic acid, following black tea supplementation[48]. In agreement with this evidence, Murphy *et al.*[15] showed an effect on P-selectin plasma levels together with an increase of plasma catechins after 4 weeks of cocoa tablet administration[15]. Widlansky *et al.*[49] observed, in patients with coronary artery disease, an increase in plasma catechins concentration after 4 weeks of daily ingestion of 900 ml black tea without any effect on CRP. In diabetic subjects, CRP and IL-6, were unaffected by green tea (900 ml) administration[50] as were fibrinogen, TNFα, intercellular adhesion molecule and vascular cell adhesion molecule after 6 months of daily consumption of black tea in diabetic subjects[51]. Steptoe *et al.*[52] showed a reduction in CRP levels after 6 weeks of black tea consumption; however, no evidence was provided regarding the extent of flavonoid absorption.

Studies with alternative sources of dietary flavonoids such as grape juice and red wine were also contradictory. Watzl *et al.* showed that both acute[53] and chronic[54] administration of red wine, de-alcoholized red wine and red grape juice had no effect on cytokine production, phagocytic activity of neutrophils and monocytes, lymphocyte proliferation and lytic activity of natural killer cells. However, Estruch *et al.*[55] found reduced plasma levels of fibrinogen, IL-1α, CRP, vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 and an increase in plasma levels of epigallocatechin, after 4 weeks of red wine consumption. Chronic supplementation with red grape juice and grape extract reduced neutrophil NADPH oxidase activity[56] and TNFα in postmenopausal women[57]. Polyphenol-rich juices enhanced immune function as showed by increases in lymphocyte proliferation, IL-2 secretion and lytic activity of natural killer cells[58]. In all these studies, neither plasma nor urinary flavonoid levels were measured.

Long-term intervention studies conducted using soya as a source of bioactive molecules showed reduction in levels of vascular cell adhesion molecule-1 and CRP[59,60], but also a lack of effect on CRP[61–63], TNFα[61,63] and IL-6[60,62]. Increases in total isoflavones, genistein and daidzein[59] and genistein levels[63] induced by long-term soya consumption were not associated with CRP reductions[59,63].

When pure molecules were utilized, CRP and IL-8 were lowered by quercetin supplementation in runners[64] and bikers[65], while quercetin plasma levels increased. Other cytokines, such as IL-6[64–66] and TNFα[65,66], were unaffected by quercetin supplementation, also when consumed in combination with vitamin C[66].

Conclusions

Although existing evidence indicates that flavonoids potentially display a multitargeting anti-inflammatory action, a clear conclusion on their effects in human subjects cannot be drawn. The large body of evidence *in vitro* and on cellular models might be somehow biased by the non-physiological concentrations (in the range of 5–100 μM) utilized. Human intervention studies have shown that absorption of flavonoids in the gastrointestinal tract is typically between 1 and 5 % of the ingested dose, with the overall result of reaching plasma concentrations not higher than 1 μM following ingestion of flavonoid-rich food[9]. Moreover, *in vivo*, flavonoids are extensively metabolized and transformed in molecules with different chemical structures and activities compared with the ones originally present in the food. The large majority of *in vitro* and cellular experiments have not been performed with the metabolites present in body fluids, thereby increasing the chance of misinterpretation of the results.

The experimental evidence in human subjects suggests a direct role for plant foods in modulating the inflammatory response *in vivo*. However, the mechanism and the molecules responsible for this effect have not been identified. The assumption that flavonoids might be responsible for the anti-inflammatory effect of plant food is not fully justified on the basis of the current *in vivo* evidence. Studies investigating the effect of flavonoids on markers of

Table 1. Overview of human intervention studies on the anti-inflammatory effects of plant foods and flavonoids

Treatment	Days	*n*	Biomarkers affected	Biomarkers not affected	PP levels	Reference
BT	28	21	↓ P-selectin	E-selectin, ICAM-1, VCAM-1	↑ urinary 4-*O*-methyl gallic acid	Hodgson *et al.*[48]
BT	28	66		CRP	Unchanged: ECG, EGCG, EGC ↑ EC, Total	Widlansky *et al.*[49]
BT	180	28		Fibrinogen, CRP, IL-6, TNFα, ICAM, VCAM	↑ urinary 4-*O*-methyl gallic acid	Mukamal *et al.*[51]
BT	42	75	↓ CRP	P-Selectin	Not measured	Steptoe *et al.*[52]
BT, GT, GTE	28	64		IL-6, IL-1β, TNFα, CRP fibrinogen	Not measured	de Maat *et al.*[47]
GT	28	55		CRP and IL-6	Not measured	Ryu *et al.*[50]
Cocoa tablets	28	32	↓ P-selectin		↑ EC, catechin	Murphy *et al.*[15]
RGJ, vitamin E or both	14	32	↓ neutrophil NADPH oxidase activity ↓ ICAM-1		Not measured	Castilla *et al.*[56]
RW, DRW, RGJ	1	6		TNFα, IL-2, IL-4, phagocytic activity, lymphocyte proliferation, and lytic activity of NK cells	Not measured	Watzl *et al.*[53]
RW, DRW, RGJ	14	24		TNFα, IL-2, IL-4, TGF-β, phagocytic activity, lymphocyte proliferation, lytic activity of NK cells	Not measured	Watzl *et al.*[54]
RW, gin	28	40	↓ fibrinogen, IL-1α, CRP, VCAM-1, ICAM-1		↑ EGC	Estruch *et al.*[55]
GE	28	44	↓ TNFα		Not measured	Castilla *et al.*[56]
PRJ	14	27	↑ lymphocyte proliferation, IL-2 and lytic activity of NK cells		↑ urinary PP	Bub *et al.*[58]
Soy	30	41	↑ IL-6 in women	CRP, amyloid A, TNFα	Not measured	Jenkins *et al.*[61]
Soy	56	25		CRP	↑ genistein, daidzein, total isoflavones	Fanti *et al.*[59]
Soy	112	52		IFN-γ, IL-2, TNFα, CRP	↑ genistein	Ryan-Borchers *et al.*[63]
Soy	56	60	↓ VCAM-1, CRP	ICAM-1, IL-6	Not measured	Nasca *et al.*[60]
Soy	90	24		IL-6, CRP	Not measured	Maskarinec *et al.*[62]
Quercetin	21	39	↓ CRP, IL-8 mRNA ↑ IL-10 mRNA	IL-6 mRNA	↑ quercetin	Nieman *et al.*[64]
Quercetin	21	40	↓ IL-8, TNFα ↓ IL-8 and IL-10 mRNA	NF-κB, cytokine mRNA (IL-6, IL-8, IL-1β and TNFα), COX-2	↑ quercetin	Nieman *et al.*[65]
Quercetin and vitamin C	28	20		TNFα, IL-1β, IL-6, CRP	Not measured	Bae *et al.*[66]

PP, polyphenol; *n*, number of total subjects; ↓, decrease; ↑, increase; BT, black tea; GT, green tea; GTE, green tea extract; RGJ, red grape juice; RW, red wine; DRW, de-alcoholized red wine; GE, grape extract; PRJ, polyphenol-rich juices; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; CRP, C-reactive protein; NK, natural killer; TGF-β, transforming growth factor-β; IFN-γ, interferon-γ; COX-2, cyclooxygenase 2; ECG, epicatechin gallate; EGCG, epigallocatechin gallate; EGC, epigallocatechin; EC, epicatechin.

inflammation have been mainly focused on flavonoid-rich foods and not on pure molecules. Moreover, most of the studies lack assessment of flavonoid absorption or failed to associate the anti-inflammatory effect following ingestion of plant foods with changes in circulating levels of flavonoids. Plant foods are rich in flavonoids as well as other components such as antioxidants, vitamins, fibre and nutrients that may be involved in their biological activity. Specifically, planned human trials with proper placebo and pure molecules are needed to clarify whether flavonoids represent ancillary ingredients or key molecules involved in the anti-inflammatory properties of plant foods.

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