FLAVONOIDS AND CARDIOVASCULAR RISK FACTORS: A REVIEW

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SUMMARY

Several modifiable and non-modifiable risk factors are associated with incidence of cardiovascular diseases, the leading cause of death worldwide. A healthy diet, such as the Mediterranean diet, lowers the incidence of cardiovascular disease. Intake of flavonoids, a class of plant-derived polyphenols widely distributed in fruits, vegetables, tea, coffee, and wine, is the hallmark of the Mediterranean diet that has been associated with reduced cardiovascular risk factors. Several mechanisms underpin this beneficial activity: a direct vasodilatory effect, prevention of endothelial dysfunction, inhibition of platelet aggregation, and smooth muscle cell proliferation along with an anti-oxidant, anti-inflammatory, anti-obesity, anti-diabetic, and anti-atherosclerotic effects. This review provides an updated overview of the mechanisms by which flavonoids ameliorate cardiovascular risk factors, thus retarding cardiovascular disease progression.

Key words

*Cardiovascular diseases;
flavonoids; endothelial cells;
hypertension; obesity.*

Abbreviations

BMI: body mass index; COX: cyclooxygenase; eNOS: endothelial NO synthase; HDL: high-density lipoprotein; ICAM-1: intercellular adhesion molecule I; IP3: inositol 1,4,5-trisphosphate; KLF-2: Krüppel-like factor-2; LDL: low-density lipoprotein; L-NAME: N(ω)-nitro-L-arginine methyl ester; NrF2: nuclear factor erythroid-2 related factor 2; NF-kB: nuclear factor kappa-light-chain enhancer of activated B cell; PPAR- γ : peroxisome proliferator-activated receptor gamma; ROS: reactive oxygen species; SHR: spontaneously hypertensive rat; SOD: superoxide dismutase; TGFBR1: transforming growth factor- β (TGF- β) receptor 1; TNF- α : tumor necrosis factor- α ; TXA2: thromboxane A2; VCAM-1: vascular cell adhesion molecule 1; VLDL: very low-density lipoprotein; VSMC: vascular smooth muscle cell.

INTRODUCTION

Cardiovascular diseases, a group of disorders that affect blood vessels and heart, include coronary heart disease, myocardial infarction, and stroke. They represent the leading cause of death worldwide resulting in 17.9 million deaths every year (1). Several risk factors are associated with the incidence of cardiovascular diseases. These include non-modifiable (age, gender, ethnicity, and family history) and modifiable risk

factors (hypertension, hyperglycemia, obesity, oxidative stress, physical inactivity, smoking, inappropriate diet, mental stress, and others) that are not independent but often occur simultaneously (2, 3). In this scenario, diet plays a critical role: excessive intake of saturated fat, processed red meat, sugar, sodium, along with low intake of fruits, vegetables, whole grain, fibers, and legumes are associated with a high risk of cardiovascular diseases (4). On the other

hand, plant-based diets are seemed to prevent the occurrence of cardiovascular diseases (5), due to the high content of vegetable proteins, mono- and poly-unsaturated fat, fibers, and bioactive compounds, the latter endowed with several biological functions such as antioxidant and anti-inflammatory activities (6).

Flavonoids are a class of plant-derived bioactive compounds synthesized from the amino acid phenylalanine through the shikimic acid pathway (7). Flavonoids were first discovered in 1930 by Albert Szent-Gyorgyi, a Hungarian scientist, who termed them as vitamin P. However, interest in the pharmacological and nutraceutical features of these bioactive compounds was boosted only during the 90s, after the publication of the French paradox, an epidemiological study highlighting the low incidence of coronary heart disease in the French population despite its high daily intake of saturated fats. This paradox, ascribed to the consumption of red wine and polyphenols therein (8), stimulated researchers all over the world to investigate flavonoid beneficial effects at cellular, animal, and human levels. Nowadays, flavonoids are recognized as pleiotropic compounds exhibiting a wide array of biological and pharmacological effects such as antioxidant, antitumor, antidiabetic, antimicrobial, cardio-protective, and neuroprotective activities (9, 10). More importantly, several clinical trials and epidemiological studies have correlated flavonoid intake with a low incidence of cancer (11, 12), cardiovascular diseases (13, 14), diabetes (15), obesity (16), Alzheimer's disease (17), and Parkinson disease (18). This review briefly discusses structural features and occurrence of flavonoids, and provides an overview of the mechanisms underpinning the beneficial effects towards cardiovascular diseases.

FLAVONOIDS: CHEMISTRY AND OCCURRENCE

Flavonoids are characterized by a diphenylpropane skeleton consisting of 15 carbon atoms arranged into two 6-carbon rings (named as

A- and B-ring) linked via an oxygen-containing, heterocyclic benzopyran C-ring (19). Based on oxidation and saturation of the C ring, or position of the B-ring, flavonoids are classified into eight classes: flavone, flavonol, flavanone, flavanoneol, isoflavone, flavan-3-ol, anthocyanidin, and chalcone (**figure 1**). Flavones have a double bond between C-2 and C-3 and a ketone group at position 4 of the C-ring. Also, flavonols have a double bond between C-2 and C-3, a ketone at C-4, and, in addition, an OH group at C-3. Unlike flavones, isoflavones have the B-ring attached to C-3 instead of C-2. Flavanones, also called dihydroflavones, have a saturated bond between C-2 and C-3 and a ketone group. Flavanoneols, also called dihydroflavonols, have a saturated bond between C-2 and C-3 and an OH group at C-3 along with the ketone group. Flavan-3-ols, also called catechins, have an OH group at C-3 but lack both the ketone group and the double bond between C-2 and C-3. Chalcones present the C-ring open at C-2. The last class anthocyanidins lacks the ketone group but have two double bonds between C-1 and C-2, and C-3 and C-4 (20).

Flavonoids can be further diversified according to the substitutions on A- and B-rings, namely methylation, acylation, prenylation and, most commonly, by hydroxylation and glycosylation, which markedly influence their solubility, stability, and reactivity (21, 22).

Flavonoids are widely distributed in all plant parts including roots, shoots, leaves, flowers, and fruits (23). Dietary flavonoids can be found in fruits, vegetables, tea, coffee, and wine (24), different classes being characteristic of different plants. Celery, parsley, red peppers, chamomile, spinach, and ginkgo biloba, for example, are rich of flavones (25). Flavonols are abundantly present in many vegetables, fruits, and beverages such as lettuce, onions, kale, tomatoes, berries, apples, grapes, tea, and red wine (26). Flavanones are found in grapes and *Citrus* fruits such as oranges and lemons where they are responsible for the bitter taste of the juice and peel (27). Isoflavones, also known as phytoestrogens, are abundant in soybeans

Figure 1. Structures and classification of the most common flavonoids.

and leguminous plants (28). Tea, bananas, apples, blueberries, peaches, and pears are rich of flavan-3-ols or catechins (29). Flavanonols are limited in their distribution to *Citrus* fruits and *Glycosmis* species (30). Finally, anthocyanidins are predominantly present in flowers and in the outer layers of fruits such as cranberries, black currants, grapes, and berries: they confer beautiful colors that depend on pH and chemical modifications of A- and B-rings (31).

FLAVONIDS AND CARDIOVASCULAR DISEASES

Accumulating evidence obtained from *in vitro*, as well as *in vivo* clinical and epidemiological studies have highlighted the potential of flavonoids as beneficial agents capable of im-

proving cardiovascular health and ameliorating risk factors associated with cardiovascular diseases (32). This effect has been generally ascribed to the direct antioxidant activity exhibited by this class of polyphenols. Flavonoids, however, may also improve vessel vasodilation via endothelium-dependent and/or -independent mechanisms, reduce the smooth muscle cell proliferation, inhibit platelet aggregation, ameliorate oxidative stress and inflammation, and exert an anti-obesity, anti-diabetic, and anti-hypertensive effect (33) (**figure 2**).

FLAVONIDS PROTECT AGAINST ENDOTHELIAL DYSFUNCTION

Endothelial dysfunction is an independent predictor and a risk factor of cardiovascular diseases-

Figure 2. Summary of the mechanisms involved in the cardiovascular protective effect of flavonoids.

es. Its hallmarks are impaired endothelium-dependent vasodilation, due to reduced synthesis and/or increased degradation of NO, and increased expression of chemokines and adhesion molecules, leading to a pro-inflammatory state of blood vessels (34). Dietary flavonoids improve endothelial dysfunction by stimulating NO synthesis through up-regulation of endothelial NO synthase (eNOS) expression, and/or enhancing its activity (35, 36). For instance, luteolin and cynaroside from artichoke (*Cynara scolymus* L.) upregulate eNOS in human umbilical vein endothelial cells (EA.hy 926 and HUVECs) and potentiate the vasorelaxant response to acetylcholine in rat aortic rings (37). Similarly, the red wine polyphenol cyanidin and the tea catechins epicatechin gallate and epigallocatechin gallate stimulate eNOS expression in EA.hy926 endothelial cells (38). Several flavonoids activate eNOS in a Ca^{2+} -independent manner through the induction of

Ser-1179 phosphorylation and/or Thr-495 dephosphorylation [e.g., epigallocatechin gallate (39), quercetin (40), luteolin (41), hesperetin (42), hesperidin and naringin (43)] or through a Ca^{2+} -dependent mechanism [e.g., quercetin (44) and (-)-epicatechin (45)]. Furthermore, flavonoids down-regulate cavelin-1 (a negative regulator of eNOS) expression or dissociate it from eNOS (45, 46), activate the chaperone protein HSP90 that is involved in eNOS activation (47), reduce the level of the eNOS inhibitor asymmetrical dimethylarginine (48, 49), or inhibit arginase to increase the level of the eNOS substrate arginine (50, 51), thus stimulating eNOS activity, and enhancing NO production. Moreover, quercetin, naringenin, and fisetin activate sirtuin-1 that, in turn, deacetylates eNOS, thus improving endothelial function (52). Also, flavonoids up-regulate eNOS transcription factor Krüppel-like factor-2 (KLF-2) to increase NO production (53).

Flavonoids protect NO from inactivation and prevent eNOS uncoupling by: 1) directly scavenging superoxide anion and preventing peroxynitrite formation (54); 2) inhibiting reactive oxygen species (ROS)-producing enzymes such as NADPH oxidase, 5-lipoxygenase, cyclooxygenases (COX), myeloperoxidase, and xanthine oxidase; 3) stimulating antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (36, 55); 4) increasing the activity of nuclear factor erythroid-2 related factor 2 (Nrf2) and up-regulating antioxidant enzymes such as NADPH:quinone oxidoreductase 1, hemeoxygenase-1, glutathione-S-transferase, and γ -glutamylcysteine ligase (56, 57). In this context, several *in vitro* studies demonstrated the endothelium-dependency of flavonoid-induced relaxation of different vascular preparations (58).

Furthermore, flavonoids protect against endothelial dysfunction by hampering the synthesis of prostaglandins (59), stimulating prostacyclin production (60), lowering the expression and release of endothelin-1 (53), limiting the cellular damage caused by either angiotensin II (61) or glucose (62), and down-regulating pro-inflammatory chemokines and adhesion molecules (63). Taken together, these studies suggest that flavonoids improve endothelial function by increasing NO production and inhibiting its degradation, by modulating prostaglandins, prostacyclin and chemokines synthesis, and by decreasing endothelial damage induced by pathological stimuli. However, numerous flavonoids identified to-date have not been assessed for this activity, hence future works should test the effect of yet unexplored flavonoids against endothelial dysfunction, characterize the structure-activity relationship, and validate the mechanism of action in an attempt to identify the scaffold responsible for this trait that, in turn, can be used as pharmacophore to synthesize more potent drugs. Additionally, the assessment models of endothelial dysfunction (cell lines, animal models or humans) should be strictly standardized to limit the discrepancies arising from the different

stimuli employed and/or the level of endothelial dysfunction. Moreover, it is of great interest to clarify whether flavonoids protect endothelium from becoming dysfunctional, or reverse established endothelial dysfunction or both.

FLAVONOIDS IMPROVE VASCULAR SMOOTH MUSCLE CELL FUNCTION

Vascular smooth muscle cells (VSMCs), the structural and functional unit of blood vessels, provide the tonic contraction that allows blood flow (64). Flavonoids relax a plethora of conduit and resistance arteries and veins (58). Mechanistically, this vasodilation can be mediated by: 1) blockade of Ca^{2+} channels (65, 66); 2) opening of K^{+} channels (67, 68); 3) inhibition of Ca^{2+} release from (69) or stimulation of Ca^{2+} uptake to intracellular stores (70); 4) inhibition of protein kinase C (71), Rho-kinase (72, 73), and phosphodiesterases (74, 75); 5) stimulation of soluble guanylyl cyclase (76), protein kinase A (76, 77), and protein kinase G (78); and/or 6) stimulation of G-protein coupled receptors like β adreno-receptor (79). Notably, the same flavonoid can induce vasodilation through one or more of these pathways. Altogether, flavonoids induce VSMC relaxation and/or inhibit their contraction. However, these studies are limited to the most common flavonoids and performed mainly on large conduit arteries. Therefore, future studies should focus on small resistance arteries that are responsible for blood pressure regulation. A molecular approach supported by computational analysis should complement the so far used classical pharmacological approach to precisely identify the molecular targets behind their vasodilatory effect. These two aspects are expected to identify the pharmacophore(s) responsible for the vasoactivity that can then be used to improve potency, efficacy and selectivity.

VSMCs are dynamic cells capable of changing their phenotype according to environmental cues. Under certain stimuli or pathological conditions such as hypertension, asthma, and atherosclerosis, VSMCs can transform from

quiescent contractile into synthetic non-contractile phenotype capable of proliferation, migration, and vascular remodeling (80). Luteolin inhibits proliferation and migration of rat (A7r5) and human aortic vascular smooth muscle cells. This effect is mediated by inhibition of transforming growth factor- β (TGF- β) receptor 1 (TGFBR1) signaling because: 1) phosphorylation of TGFBR1 and its downstream player Smad 2/3 was decreased and 2) overexpression of TGFBR1 relieved the inhibitory effect of luteolin on VSMC proliferation. Furthermore, Molecular simulation demonstrated the direct binding of luteolin to TGFBR1 (81). A similar effect has been reported for several flavonoids including quercetin (82), morin (83), cardamonin (84), xanthohumol (85), baicalin (86), kaempferol (87), apigenin (88), and genistein (89), etc. Various mechanisms underpin this activity including reduced activation of mitogen-activated protein kinase (82), modulation of p27KIP1, Akt, and MMP-9 functions (83), down-regulation of p38 MAPK, Akt, and extracellular regulated protein kinase phosphorylation (84), activation of maternally expressed gene 3/p53 signaling pathway (86), modulation of microRNA expression (87), and/or suppression of oxidative stress (90). In summary, flavonoids maintain non-synthetic, contractile phenotype of VSMC by inhibiting their proliferation, migration, and remodeling. However, the mechanisms underpinning this effect are not conclusive and deserve further investigation.

FLAVONIDS INHIBIT PLATELET AGGREGATION

Platelet aggregation plays a pivotal role in the pathogenesis of acute and chronic cardiovascular diseases such as *angina pectoris*, stroke, and myocardial infarction (91). Platelet activation is a complex phenomenon involving multiple players, which fundamentally depends on cytosolic Ca^{2+} concentration. Several agents stimulate phospholipase C and Ca^{2+} release from intracellular stores via inositol 1,4,5-tris-

phosphate (IP3): Ca^{2+} activates phospholipase A to release arachidonic acid that, in turn, is metabolized into thromboxane A2 (TXA2) by COX-1, thus triggering and amplifying platelet aggregation (92).

Flavonoids, either as flavonoid-rich beverage (tea, coffee, and wine) or as pure compounds, have shown an excellent anti-platelet aggregation activity in various experimental settings. Purple grape juice, for example, inhibits platelet aggregation both *in vitro* and *in vivo*; simultaneously increasing platelet derived NO production (93). Ingestion of 200 ml of coffee reduces *ex vivo* platelet aggregation induced by collagen and arachidonic acid: this effect is caffeine-independent (94). The *Citrus* flavonoids naringin and naringenin show anti-aggregation activity, the former being more potent than the latter in peripheral blood platelets isolated from healthy human (95). Also, quercetin diminishes collagen-, thrombin- and ADP-dependent platelet aggregation in a concentration dependent manner: this effect is mediated by the inhibition of ATP release, P-selectin expression, intracellular Ca^{2+} mobilization, platelet integrin $\alpha\text{IIb}\beta 3$ activation, as well as by an increase in the levels of cAMP and vasodilator-stimulated phosphoprotein (96). A similar effect has been reported for 3,6-dihydroxyflavone (97), apigenin (98), isorhamnetin, and tamarixetin (99), among others. Ravishankar and coworkers (2018) found that OH groups in the A-ring are key structural determinants for optimum platelet inhibitory activity, which decreases when OH is replaced with CHO; conversely, C = O substitution with C = S or B-ring modification seems less critical (100).

Flavonoid inhibition of platelet aggregation occurs preferentially through the blockade of signaling pathways initiated by activators such as ADP (97), collagen (101), TXA2 (102, 103), and thrombin (104) (**figure 3**). Furthermore, these natural products inhibit COX-1 (105) or phospholipase A and C activity (105, 106), reduce Ca^{2+} mobilization from the sarcoplasmic reticulum, increase cyclic nucleotide

Figure 3. Mechanisms underpinning flavonoid-induced inhibition of platelet aggregation.

ADP: adenosine diphosphate; COX-1/2: cyclooxygenase-1/2; IP3: inositol 1,4,5-triphosphate; PLA₂: phospholipase A₂; PLC: phospholipase C; TXA₂: thromboxane A₂.

levels through phosphodiesterase inhibition, inhibit protein disulfide isomerase involved in the activation of platelet integrin αIIbβ3 (107), stimulate NO production, ameliorate oxidative stress, and inhibit the expression of endothelial cell adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and E-selectin (108, 109). Taken together, these observations indicate that flavonoids counteract platelet aggregation exploiting several mechanisms including inhibition of the arachidonic acid pathway, and platelet degranulation. Future studies should analyze the anti-platelet aggregation activity of several flavonoids under standardized experimental conditions, to highlight

the structural determinants, and validate the mechanism of action underpinning this activity. On the other hand, well controlled clinical trials involving large cohort of human subjects will be essential for a better understanding of the interaction between flavonoids and platelet function and to develop a clinical approach counteracting cardiovascular ailments.

FLAVONOIDS AMELIORATE OXIDATIVE STRESS AND VASCULAR INFLAMMATION

Oxidative stress is caused by an imbalance between production and scavenging of radicals or, more generally, oxidants. It plays a central

role in the progression of cardiovascular diseases from various cardiovascular risk factors such as hypertension, obesity and vascular inflammation (110); therefore, it presents an important therapeutic target (111).

Flavonoids can prevent oxidative stress and alleviate vascular inflammation through a plethora of mechanisms (112). First, these polyphenols act as direct scavengers of oxidants such as superoxide anion, H_2O_2 , hydroxyl radical, etc. The antioxidant activity of flavonoids is attributed to the presence of the double bond at position C2-C3, the OH group at position 3 on C-ring, the polyhydroxylated B-ring, and the OH group at position 5 on A-ring (113). However, they cannot be regarded as direct scavengers of free radicals *in vivo* as the reported antioxidant activity is usually detected at micromolar concentrations, which are hardly attainable in the blood stream due to their poor absorption, extensive metabolism, and rapid excretion. Hence, other mechanisms may account for oxidative stress amelioration, such as regulation of defense and repair systems along with the maintenance of a nucleophilic tone (*i.e.* protection against electrophiles, including radicals and oxidants (114)). Second, flavonoids decrease the expression and/or directly inhibit ROS-generating enzymes such as NADPH oxidase, xanthine oxidase, myeloperoxidase, lipoxygenase, COXs, and inducible NOS as well as limit ROS production at the mitochondrial electron transport chain (115, 116). Third, some flavonoids modulate antioxidant enzymes like catalase, SOD, and glutathione peroxidase, thus favoring ROS elimination (54, 117). Fourth, they activate the transcription factor Nrf2, which regulates the expression of about 250 genes coding for antioxidant proteins, detoxifying enzymes, and cytoprotective proteins (57, 118). Under normal conditions, Nrf2 is located in the cytoplasm in association with its negative regulator Keap1, responsible for its ubiquitination and proteasomal degradation. Under oxidative stress conditions, Nrf2 disassociates from Keap1, translocates to the nucleus and binds to the promoter region of

antioxidant response elements, thus initiating the transcription of antioxidant enzymes (119). Fifth, flavonoids inhibit nuclear factor kappa-light-chain enhancer of activated B cells (NF- κ B), thus alleviating the associated cardiovascular inflammation (120, 121). NF- κ B is a family of inducible transcription factors that play a pivotal role in the expression of pro-inflammatory mediators and cell cycle regulators. It is normally sequestered in the cytoplasm by proteins known as I κ B family, but once stimulated, enters the nucleus and binds to the NF- κ B response site located on DNA to initiate the transcription of a plethora of genes coding for inflammatory cytokines, chemokines, adhesion molecules, anti-apoptotic factors, and cell cycle regulators, which contribute to inflammation, endothelial dysfunction, angiogenesis, VSMC proliferation and migration, and platelet adhesion (122, 123). Sixth, flavonoids chelate transition metal ions such as iron and copper that may contribute to the oxidative stress (114). Noticeably, flavonoids, like many antioxidants, show also pro-oxidant effects by: 1) direct production of hydroxyl radical *via* Fenton reaction, 2) oxidation of flavonoid phenoxyl radicals (the endproduct of ROS scavenging reaction by flavonoids), 3) inhibition of mitochondrial respiration, 4) oxidation of low molecular antioxidants, and 5) direct oxidation by peroxidases (113). Altogether, flavonoids protect the vascular system from oxidative stress due to their antioxidant activity and/or shifting the redox signaling balance towards a more antioxidant one. However, these mechanisms, drawn from studies conducted on cell lines, rodent animal models, and human studies, are actually inconclusive. Therefore, the link between flavonoid consumption, oxidative stress amelioration, and improvement of vascular diseases has still to be challenged. This problem may have arisen from the fact that oxidative stress cannot be reliably quantified in the biological system and the presence of complex food matrix containing flavonoids and extensive metabolism characterizing this class of natural compounds further complicate

this issue. Hence, future studies should focus on improving the reliability of oxidative stress biomarkers, and standardizing the experimental conditions for flavonoid administration to rodents and humans.

FLAVONOIDS AMELIORATE DYSLIPIDEMIA/HYPERLIPIDEMIA

Dyslipidemia is defined as a persistent elevation of cholesterol, triglyceride, and low-density lipoprotein (LDL) levels along with prolonged decrease of high-density lipoprotein (HDL). Dyslipidemia is the major risk factor for atherosclerosis and coronary heart disease (124).

The lipid-lowering effect of flavonoids has been evaluated in different dyslipidemic animal models (125) and in humans (126). Quercetin, for example, markedly reduces serum cholesterol and triglycerides in rabbits fed high-fat diet for 12 weeks (127). Similarly, administration of this flavonoid with a high-cholesterol diet to rats results in a significant reduction of liver triglycerides (- 24%), liver and serum cholesterol (- 22% and - 20%, respectively), and serum LDL (- 31%) while doubling serum HDL cholesterol as compared to control animals (128).

Naringenin normalizes the levels of cholesterol, triglycerides, free fatty acids, very low-density lipoprotein (VLDL), and LDL, increases plasma HDL, lipoprotein lipase, and lecithin cholesterol acyltransferase, and reduces the expression of β -hydroxy β -methylglutaryl-CoA reductase in rats fed 20% ethanol for two months (129). Chrysin, orally administered to triton WR-1339-induced hyperlipidemic female C57BL/6 mice, significantly decreases total cholesterol, LDL cholesterol, and triglyceride levels (130). Apigenin supplementation to C57BL/6J mice fed high-fat diet for 16 weeks significantly decreases plasma levels of free fatty acids, total cholesterol, and apolipoprotein B (131).

The black tea flavonoids theaflavin and thearubigins markedly reduce cholesterol, LDL, and triglyceride levels while increasing

HDL level in rat fed high-cholesterol diet, theaflavin-based drink being more effective than thearubigins alone or theaflavin and thearubigins combination (132). The lemon fruit flavonoid eriocitrin does not affect hepatic total cholesterol or triglycerides though significantly decreasing total serum cholesterol, VLDL, LDL, triglyceride, and phospholipid levels in rat fed high-fat diet (133). A similar effect has also been demonstrated for epigallocatechin gallate (134), and rutin (135) in different dyslipidemic rat models.

Studies concerning the anti-dyslipidemic effect of flavonoids in humans are scarce. For example, quercetin supplementation gives rise to contrasting results either decreasing triglycerides and LDL or being ineffective (136, 137). A meta-analysis of five randomized control trials performed on this flavonoid evidenced only a reduction of total triglycerides at doses > 50 mg/day (138).

The mechanisms underlying the lipid-lowering effect of flavonoids are numerous and include: 1) decreased absorption of cholesterol and lipids; 2) up-regulation of genes involved in fatty acid oxidation, Krebs's cycle, oxidative phosphorylation, and electron transport chain; 3) down-regulation of lipolytic and lipogenic genes; 4) inhibition of enzymes involved in triglyceride and cholesterol biosynthesis; 5) enhanced cholesterol excretion in bile acid and inhibition of its extrahepatic circulation; and 6) enhanced lipid clearance by increasing LDL receptor expression on hepatocytes or by reducing its recycling (131, 139). These studies suggest that flavonoids exert an excellent lipid-lowering effect in different dyslipidemic animal models by reducing the levels of total cholesterol, LDL, free fatty acids, and triglycerides as well as by increasing HDL level. Future studies are needed to test more flavonoids on dyslipidemic and healthy control animals, to further characterize the mechanism of action. Furthermore, clinical trials involving large number of human subjects are needed to establish the link between flavonoid consumption and improvement of lipid profile.

FLAVONOIDS IMPROVE HYPERTENSION

Hypertension represents the main risk factor for several cardiovascular diseases including coronary heart disease, stroke, and cardiac arrhythmia (140). The anti-hypertensive effect of flavonoids has been claimed based on data obtained in *in vitro* blood vessel assays, animal models, and subsequently confirmed by human intervention and population studies (141). *In vitro* studies performed on blood vessels obtained from different animal species highlighted an excellent vasodilating activity of quercetin, naringenin, galangin, chrysin, apigenin, fisetin, tamarixetin, eupatorin, morin, kaempferol, genistein, and other flavonoids (58). In animal models, quercetin lowers high blood pressure of spontaneously and salt-induced hypertensive rats (142, 143). Interestingly, the glucuronidated form of this flavonoid also decreases blood pressure of spontaneously hypertensive rat (SHR) after deconjugation at the target tissue by β -glucuronidase (144). Similarly, chrysin shows an excellent anti-hypertensive effect in SHR and N(ω)-nitro-L-arginine methyl ester (L-NAME)-induced hypertensive rats (145, 146). This effect has also been reported for catechin (147), epigallocatechin gallate (148), hesperidin (149), and other flavonoids (150) in various animal models of hypertension. Noticeably, these studies demonstrate that flavonoids do not modify blood pressure in normotensive rats.

Human data are limited and sometimes conflicting. Quercetin, for example, does not affect blood pressure in healthy men and women (151) or in rheumatoid arthritis patients (152), but reduces it in women with type-2 diabetes (153), in hypertensive patients (154), in obese individuals with metabolic syndrome traits (155), in healthy male smokers (136), and in healthy male carrying apolipoprotein E genotype (137). A meta-analysis of these seven clinical studies (enrolling 587 patients) shows that quercetin significantly reduces both systolic and diastolic blood pressure, though only at doses > 500 mg/day (156). Recently,

quercetin-3-O-glucoside does not affect blood pressure in healthy volunteers (157).

In a cross-over clinical trial, supplementation with a high-flavonoid sweetie juice (rich in naringin and narirutin) followed by a low-flavonoid sweetie juice (25% of naringin and 30% of narirutin as compared the former), each for 5 weeks, significantly decreased both diastolic and systolic blood pressure in twelve stage-1 hypertensive patients. The high-flavonoid juice was particularly effective in reducing diastolic blood pressure (158). Furthermore, the prolonged consumption or even a single intake of a hesperidin-enriched orange juice reduced systolic blood pressure in a randomized clinical trial involving pre- and stage-1 hypertensive individuals (159). Altogether, flavonoids exhibit an anti-hypertensive effect when administered to hypertensive models though only at high concentrations and without affecting the normotensive ones. This effect seems to be mediated by their excellent endothelium-dependent and -independent vasorelaxant activity as well as by alleviation of oxidative stress (160). In the future, clinical trials involving large number of human subjects with established hypertension and healthy controls should be conducted to examine the anti-hypertensive effect of flavonoids other than quercetin. Additionally, since flavonoids do not modify blood pressure in normotensive subjects, it would be interesting to assess if flavonoids protect against development of hypertension, thus supporting the epidemiological studies already associating flavonoid consumption to a low incidence of the disease (161, 162).

FLAVONOIDS ALLEVIATE OBESITY

Obesity, an accumulation of excessive fat giving rise to body mass index (BMI) ≥ 30 , affects human health and reduces the quality of life. Its occurrence increases the incidence of hypertension and type-2 diabetes, and is an independent risk factor for cardiovascular diseases such as coronary heart disease, and myocardial infarction (163). Several cellular,

animal models and human intervention studies demonstrate the anti-obesity activity of flavonoids (164, 165). At cellular level, the *Citrus* flavonoid hesperetin reduces lipid accumulation in 3T3-L1 cells by 63% at 25 μ M and decreases ROS generation compared to control (166). Similarly, kaempferol inhibits adipogenesis in pre- and mature adipocytes. In 3T3-L1 cells, this effect is mediated by down-regulation of CCAAT enhancer binding protein alpha (Cebpa) mRNA (encoding for a transcription factor involved in the modulation of proteins that regulate cell cycle and body weight homeostasis) and up-regulation of *Pnpla2* and *Lipe* genes expression (both genes encode for enzymes involved in triglyceride hydrolysis) (167). A similar activity has been described also for quercetin (168), chrysin (169), and apigenin (170), among others.

The flavonol myricetin administered to obese C57BL/6 mice fed high-fat diet significantly reduces body weight and ameliorates several obesity-associated parameters such as serum glucose, triglyceride, and cholesterol levels. It improves also oxidative stress markers such as glutathione peroxidase activity, total antioxidant capacity, malondialdehyde level, as well as inflammatory markers [e.g., tumor necrosis factor- α (TNF- α)] (171). Galangin reduces body weight, energy intake, liver weight, and parametrial adipose tissue in obese female albino rats fed cafeteria diet for 6 weeks. It also restores the level of serum lipids, counteracts lipid peroxidation and limits accumulation of hepatic triglycerides (172). Chrysin significantly reduces body weight, BMI, abdominal circumference-thoracic circumference ratio, calorie intake while increasing locomotor activity and fecal cholesterol excretion in obese rats fed high-fructose diet compared to control (173). More interestingly, apigenin administered to high fat-diet induced obese mice, reduces both body weight and visceral adipose tissues, by binding to non-phosphorylated STAT3 in visceral adipose tissues, thus reducing its transcriptional activity. This, in turn, decreases the expression of cluster of differentiation 36

(CD36) that, under physiological conditions, controls the level of peroxisome proliferator-activated receptor gamma (PPAR- γ), a critical nuclear factor for adipogenesis (174).

So far, clinical trials assessing flavonoids in humans have produced conflicting results (175). Akhlaghi and coworkers (2018) meta-analyzed 58 studies published until 2018 concluding that flavanols have the potential for reducing BMI and waist circumference in the overall population. Stratification analysis shows that flavanols decrease BMI also in the Asian and in subjects under 50 year-olds, or in those consuming doses > 500 mg/day. Similarly, isoflavones reduce the BMI in non-Asian populations or in those consuming doses $\geq$ 75 mg/day. However, neither the BMI nor the waist circumference is modified by flavonols, flavanones, and anthocyanins (163).

Several mechanisms underlying the anti-obesity effect of flavonoids were hypothesized:

1. reduction of appetite and food intake via modulation of neuropeptide and leptin, which control the hunger/satiety center (176);
2. reduction in carbohydrate and lipid digestion and/or absorption through the decreased activity of digestive enzymes such as pancreatic lipase (177), α -amylase (178), and α -glucosidase (179);
3. modulation of lipid metabolism in favor of suppressed adipocyte differentiation and/or adipogenesis and enhanced lipolysis, β -oxidation, and apoptosis, through a complex hormonal and biochemical signaling involving CCAAT-enhancer binding protein (C/EBP α , β , δ), and PPAR- γ (162);
4. induction of adipose tissue browning through the modulation of, for example, AMPK and/or sirtuin-1 signaling pathways that, in turn, induce mitochondrial biogenesis and uncoupling protein-1 expression and, hence, energy expenditure, as demonstrated for rutin (180), pentamethylquercetin (181), quercetin (182), and other flavonoids (183);
5. normalization of obesity-associated gut dysbiosis, as reported for quercetin, rutin,

catechin, procyanidin, epigallocatechin gallate, and other flavonoids (184, 185).

In summary, flavonoids exhibit a powerful anti-adipogenic activity at cellular and organism level, particularly under obesity conditions. This effect involves a wide array of mechanisms: inhibition of lipid digestion and absorption, modulation of metabolism, and induction of adipose tissue browning. Controlled clinical trials are recommended to assess the anti-obesity effect of single flavonoids in large cohorts of patients.

FLAVONIDS IMPROVE HYPERGLYCEMIA AND DIABETES

Type-2 diabetes is not only a metabolic disorder characterized by persistent hyperglycemia but also an independent risk factor for cardiovascular diseases, the latter representing the most frequent cause of morbidity and mortality in diabetic patients (186). Flavonoids seem to exert antidiabetic effects as demonstrated in several animal models and clinical trials (187). In particular, diosmin administered orally for 4 weeks to streptozotocin-induced diabetic rats significantly restores blood sugar level, improves lipid profile, reduces malondialdehyde level, and increases glutathione level and SOD activity (188). Similarly, apigenin improves diabetes-associated nephropathy in streptozotocin-induced diabetic rats as compared to control animals and prevents renal dysfunction, oxidative stress, inflammation, collagen deposition, glomerulosclerosis, and fibrosis (189). A promising anti-diabetic effect characterizes also other flavonoids like quercetin (190), morin (191), hesperetin (192), and kaempferol (193) as demonstrated in various diabetic animal models.

Unlike studies on animal models, human clinical trials are limited to few flavonoid-rich food or flavonoid mixtures: these studies show sometimes inconsistent results. For instance, flavonoids intake was not associated with a lower risk of type-2 diabetes in post-meno-

pausal women (194), whereas in a large cohort study involving 340,234 participants from 8 European countries, flavanols (monomer, dimer or proanthocyanidin with low degree of polymerization) and flavonols (in particular myricetin) were associated with a lower risk of type-2 diabetes (195). A similar result was observed with quercetin in the Chinese population (196). However, the consumption of flavonols, flavones, quercetin, apigenin, myricetin, kaempferol, and luteolin was not associated with type-2 diabetes incidence (197).

In a cross-sectional study performed on 1997 women aged 18-76 years, a high intake of anthocyanins and flavones was associated with low peripheral insulin resistance and inflammation biomarkers; the other flavonoid subclasses assessed were ineffective (198). Similarly, a follow-up study of 200,000 US women and men demonstrated that high anthocyanin consumption lower the risk of type-2 diabetes; this beneficial effect seems to be specifically correlated to this class of flavonoids as no association was detected with the total flavonoid intake (199). However, a subsequent meta-analysis considering four studies involving 284,806 participants found that total intake of flavonoids is indeed associated with a lower risk of type-2 diabetes (200). The same conclusion was reached by an independent meta-analysis of nine prospective cohort studies, involving 172,058 participants, for flavonols, flavan-3-ols, and isoflavones (201).

Xu *et al.* (2018) demonstrated in a meta-analysis of eight prospective studies involving 312,015 participants that, beyond total flavonoids, anthocyanidins, flavan-3-ols, flavonols, and isoflavones are inversely correlated with type-2 diabetes risk (15). In a meta-analysis of 18 studies, involving 457,922 participants, a high intake of coffee, decaffeinated coffee, or tea was associated with a low risk of type-2 diabetes (202), in line with two studies that took into account tea consumption (203, 204). In a previous study, however, tea consumption shows only a borderline effect (197).

Various mechanisms underpin the anti-diabetic effect of these natural compounds (as depicted in **figure 4**): 1) modulation of digestive enzymes and, hence, lipid and carbohydrate absorption; 2) increased expression and translocation of glucose transporter-4; 3) modulation of hepatic enzymes involved in glucose metabolism, enhancing glucose uptake and storage; 4) protection of β -cells from apoptosis and enhancement of insulin secretion; 5) increased expression and activation of PPAR- γ , thus improving glucose uptake and insulin sensitivity; 6) activation of the AMP-kinase pathways; 7) inhibition of tyrosine kinase activity; and 8) down-regulation of the NF- κ B pathway (205-207). Altogether, flavonoids seem to have a promising anti-diabetic effect in particular in animal models of diabetes. In humans, this ef-

fect appears to be conferred by flavonoid-rich foods rather than by individual flavonoids. Future studies should address the anti-diabetic effect of individual flavonoids in clinical trials and elucidate the mechanism of action, in an attempt to develop these pleiotropic compounds as anti-diabetic drugs.

FLAVONOIDS ATTENUATE ATHEROSCLEROSIS DEVELOPMENT

Atherosclerosis is the major direct cause of cardiovascular diseases such as myocardial infarction, stroke, and heart failure. It initiates with fat deposition on vessel wall, which develops forming plaques that, in turn, either restrict blood flow or induce thrombosis (208). Several flavonoids hamper atherosclerosis pathogene-

Figure 4. Mechanisms involved in flavonoid anti-diabetic effects.

cAMP: cyclic adenosine monophosphate; Glut-4: glucose transporter 4; HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A; IRS: insulin receptor substrate; PI3K: phosphatidylinositol-3-kinase; PKA: protein kinase A; PKC: protein kinase C; PLC: phospholipase C; ROS: reactive oxygen species; SREBP-1c: sterol regulatory element-binding protein; TG: triglycerides; VLDL: very low-density lipoprotein.

sis in various animal models (209). In particular, in apolipoprotein E knockout mice fed high-fat diet, quercetin ameliorates atherosclerosis-associated alterations by enhancing autophagy, up-regulating p21 and p53 expression, and down-regulating that of TNF- α , IL-1 β , IL-18, and mTOR (210). Interestingly, apigenin shows an anti-atherosclerotic effect both in RAW264.7 macrophages and apolipoprotein E knockout mice due to the upregulation of ATP Binding Cassette Subfamily A 1 (ABCA1) expression via miR-33 repression that, in turn, increases cholesterol efflux and decreases foam cells formation, and the down-regulation of inflammation-associated markers like TLR-4, MyD88, p-IkB- α , and nuclear NF- κ B p65 subunit (211). Also fisetin shows a promising anti-atherosclerotic effect in apolipoprotein E knockout mice fed high-fat diet: in fact, it reduces atherosclerotic plaque formation and lipid accumulation in the aortic sinus. This effect is mediated by down-regulation of PCSK9, LOX-1, p53, p21, and p16 expression (212). Kaempferol improves the inflammatory status in atherosclerotic rabbits fed high cholesterol-diet. This effect is mediated by down-regulation of inflammatory markers such as TNF- α , IL-1 β , malondialdehyde, E-selectin, ICAM-1, VCAM-1, and monocyte chemoattractant protein-1 as well as enhancement of serum SOD activity (213).

Studies examining the effect of flavonoids on human atherosclerosis are limited and usually confined to flavonoid-rich food rather than single molecules. For instance, tea but not coffee, caffeine or caffeinated coffee consumption is inversely associated to coronary artery plaque progression (214). In Danish population, a daily intake of flavonoids reduces by 14% the risk of atherosclerosis, particularly in smokers and alcohol consumers (215).

The main mechanism responsible for the anti-atherosclerotic effect of flavonoids is inhibition of LDL oxidation and, hence, foam cell formation, which is considered the hallmark of atherosclerosis progression. This is achieved by, for example, quercetin enhancement of

cholesterol efflux from foam THP-1 cells treated with oxidized LDL, an effect mediated by the up-regulation of PPAR- γ and ABCA1 expression (216). Quercetin enhances also the viability of RAW264.7 macrophages treated with oxidized LDL, and decreases intracellular accumulation of cholesterol and lipids. Up-regulation of ABCA1, ABCG1, and liver X receptor- α (LXR- α) along with down-regulation of PCSK9, p53, p21, and p16 seems involved in this activity (217). Similarly, chrysin inhibits foam cell formation by both increasing efflux and decreasing influx of cholesterol. This effect is mediated by up-regulation of PPAR- γ , LXR- α , ABCA1, and ABCG1, as well as down-regulation of scavenger receptor A1 (SR-A1) and SR-A2 (218). A similar effect has been reported also for baicalin (219), kaempferol (220), and fisetin (221).

Other mechanisms participate in the anti-atherosclerotic effect of flavonoids: regulation of lipid metabolism in favor of enhanced cholesterol excretion (222); inhibition of endothelial cell senescence induced by oxidized LDL (223); enhanced autophagic activity of foam cells (224); and reduced plaque rupture by metalloproteinase inhibition (225). In conclusion, flavonoids exhibit, at least in animal models, a promising anti-atherosclerotic effect, which is mediated by reduced lipid oxidation, lipid accumulation, and foam cell formation. Clinical and epidemiological studies are now necessary to discern whether flavonoid consumption is associated with low risk of atherosclerosis development in human population.

CONCLUSIONS AND LIMITATIONS

Flavonoids are endowed with a plethora of biological and pharmacological activities, which seem responsible for the low incidence of cardiovascular diseases. The cardioprotective function of flavonoids is mediated through a paradigm of mechanisms that collectively ameliorate cardiovascular risk factors and retard the development of diseases. Evidence supporting their claimed cardioprotective ef-

fect is based on studies carried out mainly on cellular and animal models, that are supported by a few human clinical intervention and epidemiological studies. There is still a need to carry out large clinical cohort studies, involving humans from different ethnics, using single pure or standardized mixtures of flavonoids, to precisely identify the link between flavonoid consumption and beneficial cardiovascular activity. However, flavonoid-based drugs development still suffers many limitations and challenges, including purification high cost and low yield, low solubility and stability, extensive metabolism in small intestine and liver, low bioavailability, interaction with intestinal microflora, and high promiscuity of interaction with concomitant therapies. More importantly, human clinical trials, either prospective observational or prospective interventional, still represent a hurdle due to the small and slow effects produced by flavonoids

in vivo. Furthermore, appropriate, standardized, and reliable biomarkers to trace these effects are required to reach a universally recognized conclusion on this still open issue: "flavonoids: friends or foes?".

Although the physiological effects on biomarkers for cardiovascular disease are much lower than those expected by drugs used for the same purpose, flavonoids can easily be incorporated into the daily diet and usually present no adverse effects.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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