Pomegranate juice flavonoids inhibit low-density lipoprotein oxidation and cardiovascular diseases: Studies in atherosclerotic mice and in humans

POMEGRANATE JUICE FLAVONOIDS INHIBIT LOW-DENSITY LIPOPROTEIN OXIDATION AND CARDIOVASCULAR DISEASES: STUDIES INATHEROSCLEROTIC MICE AND IN HUMANS


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Summary: The beneficial health effects attributed to the consumption of fruit and vegetables are related, at least in part, to their antioxidant activity. Of special interest is the inverse relationship between the intake of dietary nutrients rich in polyphenols and cardiovascular diseases. This effect is attributed to polyphenols' ability to inhibit low-density lipoprotein (LDL) oxidation, macrophage foam cell formation and atherosclerosis. Pomegranate polyphenols can protect LDL against cell-mediated oxidation via two pathways, including either direct interaction of the polyphenols with the lipoprotein and/or an indirect effect through accumulation of polyphenols in arterial macrophages. Pomegranate polyphenols were shown to reduce the capacity of macrophages to oxidatively modify LDL, due to their interaction with LDL to inhibit its oxidation by scavenging reactive oxygen species and reactive nitrogen species and also due to accumulation of polyphenols in arterial macrophages; hence, the inhibition of macrophage lipid peroxidation and the formation of lipid peroxide-rich macrophages. Furthermore, pomegranate polyphenols increase serum paraoxonase activity, resulting in the hydrolysis of lipid peroxides in oxidized lipoproteins and in atherosclerotic lesions. These antioxidative and antatherogenic effects of pomegranate polyphenols were demonstrated in vitro, as well as in vivo in humans and in atherosclerotic apolipoprotein E-deficient mice. Dietary supplementation of polyphenol-rich pomegranate juice to atherosclerotic mice significantly inhibited the development of atherosclerotic lesions and this may be attributed to the protection of LDL against oxidation.

Atherogenic modifications of low-density lipoprotein (LDL) and atherosclerosis

Atherosclerosis is the leading cause of morbidity and mortality among individuals with a western lifestyle. The early atherosclerotic lesion is characterized by the accumulation of arterial foam cells derived mainly from cholesterol-loaded macrophages (1, 2). Most of the accumulated cholesterol in foam cells originates from plasma LDL, which is internalized into the cells via the LDL receptor. Native LDL, however, does not induce cellular cholesterol accumulation, because the LDL receptor activity is downregulated by the cellular cholesterol content (3, 4). LDL has to undergo some modifications, such as aggregation or

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oxidation, in order to be taken up by macrophages at an enhanced rate via the macrophage scavenger receptors pathway which, unlike the LDL receptor, are not subjected to downregulation by cellular cholesterol (5-7). The underlying mechanisms leading to the formation of atherosclerotic lesions are complicated and are the result of multiple interactive processes (8-10).

Atherosclerosis is related to inflammatory conditions, which are initiated by lipoprotein invasion into the artery wall (11). Elevated plasma levels of LDL, a major risk factor for cardiovascular disease, are associated with increased adherence of circulating monocytes to arterial endothelial cells and to an increased rate of LDL infiltration into the intima. When LDL particles are entrapped in the artery, they can undergo progressive oxidation followed by a rapid internalization via the macrophage scavenger receptors, leading to foam cell formation. The atherogenicity of LDL in the arterial wall depends on the LDL content, which is locally retained in the intima. The "response to retention" hypothesis of atherosclerosis supports subendothelial retention of atherogenic lipoproteins as the central pathological process in atherogenesis (12-14). This theory points to the retention of LDL as a prerequisite step to oxidative modification of the lipoprotein.

Lipoprotein retention in the artery wall was suggested to be more important as a risk factor for atherosclerosis than the rate of LDL transport into the artery wall (15, 16). Retention of LDL in the intima involves its binding to arterial proteoglycans and the association of LDL with such proteoglycans depends on structural properties of LDL, such as its size and density (12, 17, 18). Proteoglycan-bound LDL forms aggregates (18) and aggregated LDL is avidly taken up by macrophages, as well as by smooth muscle cells (19), leading to foam cell formation (20-22). Retention and aggregation of LDL in the arterial wall are key events in atherogenesis and aggregated LDL exists in atherosclerotic lesions (23). In vitro, LDL aggregation can be induced by phospholipase C, by sphingomyelinase, or even by mechanical vortexing (24, 25). Aggregation of LDL stimulates its uptake by macrophages independently of the LDL receptor pathway, thus converting macrophages into foam cells (26). Recently, extensive oxidation of LDL has been demonstrated to lead to its aggregation (27-29). On the other hand, adherence of LDL to arterial proteoglycans increases the susceptibility of LDL to oxidation (18, 30).

The "oxidative modification of lipoproteins" hypothesis of atherosclerosis proposes that LDL oxidation plays a pivotal role in early atherogenesis (31-40). This hypothesis is supported by evidence that LDL oxidation occurs in vivo (36, 41) and contributes to the clinical manifestation of atherosclerosis. The uptake of oxidized LDL (Ox-LDL) by scavenger receptors promotes cholesterol accumulation and foam cell formation (5, 7, 39, 42). In addition, Ox-LDL atherogenicity is related to the recruitment of monocytes into the intima (43), to stimulation of monocyte adhesion to the endothelium (44) and to Ox-LDL cytotoxicity toward arterial cells (45, 46).

Oxidation of LDL involves free radical attack on lipoprotein components including cholesterol, phospholipids, fatty acids and apolipoprotein B-100.

LDL oxidation results first in the consumption of its antioxidants (mainly vitamin E and carotenoids) and in a substantial loss of polyunsaturated fatty acids and cholesterol, which is converted to oxysterols. A predominant oxysterol formed at early stages of oxidation is 7-hydroxycholesterol and at later stages, 7-ketocholesterol is formed (47). Both of these oxysterols are formed as a result of oxygenation at position 7. The polyunsaturated groups of the esterified cholesterol esters and of phospholipids are also major targets for oxidation. The primary products formed are hydroperoxides, which can undergo subsequent reduction to hydroxy acids and aldehydes. Nonenzymatic peroxidation of arachidonic acid results in the formation of isoprostanes and epoxyisoprostanes (48). In the presence of transition metal ions, acyl hydroperoxides also
undergo carbon-carbon bond cleavage to form reactive short-chain aldehydes.

During oxidation of LDL, apolipoprotein B-100 also undergoes direct and indirect modifications. Direct attack of oxidants can oxidize amino acid side-chains and fragment the polypeptide backbone. Reactive lipid peroxidation products, such as short-chain aldehydes, can form stable adducts with amino acid residues in the apolipoprotein B-100 (49), which can then lead to intermolecular cross-linking and to aggregation of lipoprotein particles (50). Enrichment of LDL with lipid hydroperoxides appears to be an important first step in LDL oxidation. After depletion of LDL antioxidants, transition metal ions catalyze propagation reactions, the breakdown of lipid hydroperoxides and the formation of reactive products, such as malondialdehyde and hydroxyynonenal, which are responsible for apolipoprotein B-100 modification. All these reactions result in changes in the LDL structure. The oxidatively modified LDL can no longer bind to the LDL receptor, but interacts with the macrophage scavenger receptors, leading to the accumulation of cholesterol and oxidized lipids and to foam cell formation.

Macrophage-mediated oxidation of LDL

The process of LDL oxidation is unlikely to occur in plasma because of high plasma concentrations of antioxidants and of metal ion chelators. It is more likely to occur within the artery wall, an environment depleted of antioxidants and where the LDL is therefore exposed to oxidative stress. The identity of the cells responsible for the oxidation of LDL along atherogenesis in the arterial wall is uncertain. Monocyte-derived macrophages are likely candidates to induce the oxidation of LDL during early atherogenesis, because they are prominent in atherosclerotic lesions and because they generate reactive oxygen and nitrogen (51, 52).

Macrophage-mediated oxidation of LDL is considerably affected by the oxidative state in the cells, which depends on the balance between cellular antioxidants and macrophage-associated antioxidants (53). Macrophage binding of LDL to the LDL receptor initiates the activation of cellular oxidases (54, 55). LDL oxidation by arterial wall cells was suggested to involve the activation of macrophage 15-lipoxygenase and of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (56, 57). When NADPH oxidase is activated, the cytosolic components of the NADPH oxidase complex, P 47 and P 67, translocate to the plasma membrane where, together with the membrane bound cytochrome b558, they form the active NADPH oxidase complex. Both phospholipase A2 and phospholipase D can induce macrophage NADPH oxidase-dependent oxidation of LDL (57). On the other hand, macrophage antioxidants also contribute to the extent of cell-mediated oxidation of LDL. Cellular reduced glutathione is a most potent antioxidant (58, 59) and an inverse relationship was shown between the extent of macrophage-mediated oxidation of LDL and the reduced cellular content of glutathione (59). Macrophage-mediated oxidation of LDL can also result from initial cellular lipid peroxidation. When cultured macrophages were exposed to luminous ions, cellular lipid peroxidation took place (60, 61). These "oxidized macrophages" could easily oxidize the LDL lipids, even in the absence of any additional transition metal ions. LDL oxidation by oxidized macrophages can also result from the transfer of peroxidized lipids from the cell membranes to LDL particles.

Paraoxonase and LDL oxidation

Human serum paraoxonase (PON1) is an esterase, which is physically associated with high-density lipoprotein (HDL) and is also distributed in tissues such as liver, kidney and intestine (62, 63). The activ-
ities of serum PON1, which are routinely measured, include hydrolysis of organophosphates, such as paraoxon (the active metabolite of the insecticide parathion), hydrolysis of esters, such as phenyl acetate, as well as lactonase activities. Human serum paraoxonase activity has been shown to be inversely related to the risk of cardiovascular disease (64-66). As shown in atherosclerotic, hypercholesterolemic and diabetic patients (67-69). HDL-associated PON1 has recently been shown to protect LDL, as well as the HDL particle itself, against oxidation induced by either copper ions or by free radical generators (70, 71) and this effect could be related to the hydrolysis of specific lipoproteins’ oxidized lipids such as cholesterol linoleate hydroperoxides of some specific oxidized phospholipids. Protection of HDL from oxidation by PON1 was shown to preserve the antiatherogenic effect of HDL in reverse cholesterol transport, as shown by its beneficial effect on HDL-mediated macrophage cholesterol efflux (70). These effects of PON1 may be relevant to its beneficial properties against cardiovascular disease (63-65). Antioxidants were shown to preserve the activity of PON1 as they decrease the formation of lipid peroxides, which can inactivate PON1 (72).

Flavonoids and cardiovascular disease

Dietary consumption of flavonoids was shown to be inversely related to morbidity and mortality from coronary heart disease (73-75). Flavonoids compose the largest and most studied group of plant phenols. Over 4,000 different flavonoids have been identified to date. They are usually found in plants such as glycosides and large compositional differences exist between different types of plants and even between different parts of the same plant. Flavonoids are grouped into anthocyanins and flavonoids. Anthocyanins are glycosides of anthocyanidin and are the most important group of water soluble plant pigments, responsible for the red, blue and purple colors of flowers and fruits. Anthocyanins are colorless or colored white-to-yellow and include flavonols, flavanols, flavones, flavans and isoflavones.

Flavonoids are powerful antioxidants and their activity is related to their chemical structures (76-78). Plant flavonoids can act as potent inhibitors of LDL oxidation via several mechanisms, including scavenging of free radicals, chelation of transition metal ions, or preservation of serum PON1 activity (and as a result hydrolysis of LDL-associated lipid peroxides). Flavonoids are also quite suitable for protecting cell membranes from free radical-induced oxidation, since they are both lipophilic and hydrophilic, thus resulting in reduced cell-mediated oxidation of LDL. Being partly inside and partly outside of the cell’s plasma membrane, flavonoids can scavenge free radicals, which are generated within the cells, as well as free radicals that attack the cell from the outside. We have indeed demonstrated that enrichment of macrophages with the isoflavon glycodrin protected the cells from lipid peroxidation under oxidative stress (79).

Pomegranate, LDL oxidation and atherosclerosis

The pomegranate tree, which is said to have flourished in the Garden of Eden, has been extensively used as a folk medicine in many cultures (80, 81). Edible parts of pomegranate fruits (about 50% of total fruit weight) comprise 80% juice and 20% seeds. Fresh juice contains 95% moisture, 10% total sugars, 1.5% pectin, ascorbic acid, polyphenols and flavonoids. Pomegranate seeds are a rich source of crude fibers, pectin and sugars, and pomegranate peel was shown to contain phenols from the condensed and hydrolyzable tannin class (82-84). Dried pomegranate seeds contain the steroidal estrogen estrone (85, 86), the isoflavonic phytoestrogens genistein and daidzein, and the phytoestrogen coumestrol (87).
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The soluble polyphenol content in pomegranate juice is between 0.2% and 1%, depending on variety and includes mainly anthocyanins (such as cyanidin-3-glycoside, cyanidin-3, 3-diglycoside and delphinidin-3-glucosid, and anthoxanthins such as catechins, ellagic tannins and gallic and ellagic acids (85-87). Fermented pomegranate juice and cold-pressed pomegranate seeds possess antioxidant activity and can reduce prostaglandins and leukotrienes formation by inhibiting cyclooxygenases and lipoxigenases, respectively (88).

Pomegranate juice was shown to possess an antioxidant activity that was three times higher than the antioxidant activity of red wine or of green tea (84). The antioxidant activity was higher in juice extracted from whole pomegranates than in juice obtained from arils only, suggesting that the processing extracts some of the hydrolyzable tannins present in the fruit peel.

We have demonstrated that pomegranate juice contained a higher concentration of total polyphenols than red wine or other fruit juices (Fig. 1A). In parallel, pomegranate juice exhibited a very low IC_{50} (the concentration needed to inhibit LDL oxidation by 50%) in protection of LDL against oxidation compared with other examined beverages (Fig. 1B).

The possible antiatherogenic effects of pomegranate flavonoids are illustrated in Figure 2.

![Graph](image)

**Fig. 1** Total polyphenols concentration of several common fruit juices, white and red wine (A) and juice-induced inhibition of LDL oxidation (B) expressed as IC_{50}, which is the concentration needed to inhibit LDL oxidation by 50%.
Fig. 2. Antiatherogenic effects of pomegranate complexed polyphenols (hydrolyzable tannins). Dietary consumption of nutrients rich in polyphenols inhibits low-density lipoprotein (LDL) oxidation, foam cell formation and the development of aortic atherosclerotic lesions. Major pomegranate polyphenols are shown along with their chemical structure. CE = cholesterol ester; Ox-LDL = oxidized LDL; UC = unesterified cholesterol.

The effect of pomegranate juice on LDL atherogenic modifications (oxidation, aggregation and retention) was studied in vitro and ex vivo in humans and in atherosclerotic apolipoprotein E deficient (E') mice (89).

In vitro studies. In vitro studies demonstrated significant dose-dependent antioxidant capability of pomegranate juice against LDL oxidation (Fig. 3A). The mechanisms for the antioxidative effects of pomegranate juice against lipoprotein oxidation could be related to its capacity to scavenge free radicals. The water-soluble fractions of the pomegranate's inner and outer peels, but not that of the seeds, were even stronger antioxidants against LDL oxidation than the juice (89).

The atherogenicity of LDL is attributed not only to its oxidative modification but also to its aggregation (27). It was previously shown that LDL oxidation leads to its subsequent aggregation (28, 29). The addition of increasing concentrations of pomegranate juice to LDL decreased its susceptibility to aggregation (induced by vortexing) in a dose-dependent fashion (Fig. 3B). Pomegranate juice also reduced an additional related modification of LDL, i.e., its retention to matrix proteoglycans (as analyzed by LDL binding to chondroitin sulfate). Extracellular matrix proteoglycans can bind LDL through their glycosaminoglycan moieties and such interaction leads to entrapment of the LDL particle in the arterial wall, a phenomenon called “LDL retention” (12). Addition of increasing concentrations of pomegranate juice (0.3-5 µmol of polyphenols/l) to LDL (200 µg of lipoprotein protein/ml) induced a substantial dose-dependent reduction in the capacity of LDL to bind chondroitin sulfate (100 µg/ml). LDL binding to chondroitin sulfate decreased by up to 75% following its incubation with 3.5 µmol/l of pomegranate juice polyphenols (Fig. 3C).

In vivo studies. LDL derived from healthy human volunteers after consumption of pomegranate juice (“Wonderful” cultivation, 50 ml/day of concentrated...
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![Graphs](image)

**Fig. 3** A. LDL oxidation: LDL (100 μg of protein/ml) was incubated without (control) or with increasing concentrations of pomegranate juice (PJ). 0-3.5 μmol/l of polyphenols. LDL oxidation was induced by its incubation with 5 μM of CuSO₄, and was measured as conjugated diene formation that was kinetically monitored at 234 nm. B. LDL aggregation: LDL (100 μg of protein/ml) was incubated without (control) or with pomegranate juice (7 μmol/l or 24 μmol/l of polyphenols) for 10 min at 25°C. LDL aggregation induced by vortexing was then kinetically monitored at 600 nm. C. LDL retention: LDL (100 μg of protein/ml) was incubated with increasing concentrations of pomegranate juice (0-3.5 μmol/l of polyphenols) for 1 h at 37°C, followed by the addition of chondroitin sulfate (100 μg/ml) and a further incubation for 30 additional minutes at 25°C. LDL was then precipitated and the LDL-associated glycosaminoglycan content was analyzed in the precipitate. The results are presented as the means ± SD from three separate experiments.

Pomegranate juice, equivalent to 1.5 millmoles of total polyphenols/day, for 2 weeks, was found to be more resistant to copper ion-induced oxidation than LDL obtained prior to pomegranate juice supplementation (89). This effect was demonstrated by a 43% prolongation of the LDL oxidation lag time after 2 weeks of pomegranate juice consumption, in comparison to LDL obtained prior to juice consumption (Fig. 4A); this latter effect was accompanied by a significant 10% increment in plasma total antioxidant status (Fig. 4B). Pomegranate juice consumption by healthy humans also resulted in an increased serum paraoxonase activity (Fig. 4C).

We have extended our studies on the antioxidative effects of pomegranate juice consumption to hypertensive patients (75, 90). The effect of consumption of this juice for 2 weeks by 10 hypertensive patients on their blood pressure was small but significant. In seven out of 10 of the hypertensive patients studied, serum angiotensin-converting enzyme (ACE) activity was significantly decreased by 36%. Pomegranate juice was shown to directly exhibit a dose-dependent inhibitory effect on serum ACE activity. Because ACE inhibitors are metabolized by cytochrome P-450 enzymes, serum ACE activity can be significantly reduced by modulating P-450 enzyme activity (91). Therefore, we next analyzed the effect of pomegranate juice on cytochrome P-450 enzymes (90). Pomegranate juice decreased the activities of cytochrome P-450 3A4, 2D6, 2E1 and 2B6 by 40%,
30%, 20% and 60%, respectively. In hypertensive patients treated with ACE inhibitors, the inhibitory effect of pomegranate juice consumption on P-450 enzymes can possibly decrease P-450 mediated drug (the ACE inhibitor) breakdown and hence serum ACE activity may be further decreased in these patients. In three hypertensive patients treated with the ACE inhibitor losartan (20 mg/day for 1 month) we observed that their serum ACE activity decreased after 2 weeks of pomegranate juice consumption (50 ml of juice containing 1.5 mmol/L of total polyphenols/day) by 26%. Taken together, the results on the inhibitory effect of pomegranate juice on serum ACE activity on the one hand and on cytochrome P-450 enzymes on the other suggest that pomegranate juice may also affect ACE activity indirectly, secondary to its inhibitory effect on cytochrome P-450 enzymes.

In vivo studies in athrogenic mice: A mechanistic approach. Pomegranate juice supplementation to E' mice reduced the size of their atherosclerotic lesions and the number of foam cells in the lesions (89) compared with control placebo-treated E' mice that were supplemented with water (Fig. 5A). Furthermore, pomegranate juice supplementation to L' mice with already advanced atherosclerosis was still able to reduce the size of murine atherosclerotic lesions by 17% compared with those of age-matched placebo-treated mice (92).

Consumption of pomegranate juice by L' mice has also been shown to have considerable antioxidative
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**Fig. 5** Pomegranate juice consumption by atherosclerotic E1 mice reduces atherosclerotic lesion area and LDL oxidation. A. Lesion size. The lesion area derived from E1 mice that consumed placebo or pomegranate juice (PJ; 12.5 ml/kg/day) for 2 months was analyzed for lesion size. The results are expressed as the mean of the lesion area in square micrometers. *p < 0.05 (vs. placebo). B. CuSO4-induced LDL oxidation. LDL (100 µg of protein/ml) isolated from E1 mice that consumed placebo or 12.5 µl of pomegranate juice (equivalent to 0.35 µl of total polyphenols) was incubated with 5 µM of CuSO4, and LDL oxidation was measured by the thiobarbituric acid-reactive substances (TBARS) assay. The results are expressed as the mean ± SD of three separate determinations. *p < 0.01 (vs. placebo). C. Cell-mediated LDL oxidation. Mouse peritoneal macrophages (MφM) were isolated from the peritoneal fluid of control E1 mice or from mice that consumed 12.5 µl of pomegranate juice/mouse/day for a period of 2 months. The mouse peritoneal macrophages were incubated for 4 h at 37 °C with LDL (100 µg of protein/ml) under oxidative stress in the presence of 2 µM of CuSO4. LDL oxidation was measured directly in the medium by the TBARS assay. The results are expressed as mean ± SD (n = 3). *p < 0.01 (vs. placebo).

and antiatherogenic properties (89). Pomegranate juice consumption substantially reduced the propensity of E1 mice-derived LDL to copper ion-induced oxidation (Fig. 5B) and to macrophage-mediated oxidation of LDL (Fig. 5C) by reducing the oxidative capacity of the cells. The mechanism responsible for this effect was associated with inhibition of translocation to the macrophage plasma membrane of p-47 (the NADPH oxidase cytosolic factor) and hence inhibition of NADPH oxidase activation. As a result, a 49% reduction in superoxide anion release from the macrophages (Fig. 6A) and a 25% elevation in cellular glutathione content (Fig. 6B) were observed. These effects could also be related to reduced levels of macrophage-associated lipid peroxide after pomegranate juice consumption compared with macrophages isolated from control E1 mice that consumed placebo (Fig. 6C).

Our current view on the major pathways by which pomegranate polyphenols protect LDL against oxidative modifications, and thereby reduce macrophage foam cell formation and the development of advanced atherosclerosis (83-96), are summarized in Figure 7.

References

Fig. 7 Major pathways by which pomegranate polyphenols inhibit LDL cholesterol oxidation and atherosclerosis. Polyphenols affect LDL directly by their interaction with the lipoprotein and inhibition of LDL oxidation. Polyphenols can also protect LDL indirectly, by their accumulation in arterial cells and protection of arterial macrophages against oxidant stress. This latter effect is associated with inhibition of the formation of "toxic macrophages" and reduction in the capacity of macrophages to oxidize LDL. In addition, polyphenols pressure or even increase paracrine or activity, thereby increasing hydrolysis of lipid peroxides in lipoproteins or in atherosclerotic lesion, leading to attenuation in the progression of atherosclerosis. DHDL — oxidized LDL, SMC — smooth muscle cells, CII — cholesteryl ester, UC — unidentified cholesterol, SH — scavenger receptor.

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