Amlodipine attenuates oxidative stress in the heart and blood of high-cholesterol diet rabbits

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Summary

Introduction: Oxidative stress is a key component of atherosclerosis. It has been suggested that amlodipine inhibits oxidative stress. In this study, we evaluated the effects of amlodipine on the total antioxidant capacity of heart tissue and blood in 36 control and cholesterol-fed male New Zealand white rabbits.

Methods: The rabbits were divided into four groups (n = 9). Group 1 rabbits were fed a regular diet, group 2 were fed a diet with 2% cholesterol, group 3 were fed a regular diet plus 5 mg/kg/day oral amlodipine, and group 4 were fed 2% cholesterol diet plus amlodipine 5 mg/kg/day. At the end of eight weeks, blood samples were drawn and at the same time heart tissue was isolated and frozen in liquid nitrogen. After homogenisation, the solution was centrifuged and the light supernatant was stored at –80˚C. This was used for determination of glutathione peroxidase (GPX), superoxide dismutase (SOD) and (MDA) levels.

Results: Eight weeks of amlodipine treatment significantly reduced the levels of total cholesterol, low-density lipoprotein cholesterol and triglycerides in the group on the hypercholesterolaemic diet (p < 0.05). In the blood, the level of thiobarbituric acid-reactive substances increased in the group on the hypercholesterolaemic diet (p < 0.05). Lipid peroxidation in the heart tissue was similar to that in the blood, except in the amlodipine-only group (group 3). In the blood, the activity of total SOD (tSOD) decreased in the group on the 2% cholesterol diet (group 2) and 2% cholesterol-plus-amlodipine diet (group 4) and decreased in the amlodipine-only group (group 3) (p < 0.05).

Conclusion: Amlodipine decreased oxidative stress in the heart and blood and improved the lipid profile in cholesterol-fed rabbits. Therefore, it may be considered a useful tool for the reduction of oxidative stress and improvement of lipid profiles in diseases related to atherosclerosis.

Keywords: oxidation stress, cholesterol-fed rabbits, lipid peroxidation, amlodipine

Oxidative stress, an imbalance between the production of reactive oxygen species (ROS) and their detoxification by antioxidants, is involved in cardiovascular diseases such as atherosclerosis, hypertension and heart disease. ROS cause oxidation of membrane phospholipids, proteins and DNA, and result in contractile failure and apoptosis in cardiomyocytes. But short-term oxidative stress may also be important in the prevention of aging by the induction of a process called mitohormesis. ROS can also be beneficial as they are used by the immune system to attack and kill pathogens.

Atherosclerosis represents a state of heightened oxidative stress characterised by lipid oxidation in the vascular wall. Overproduction of ROS under pathophysiological conditions is an important part of atherosclerosis. Therefore oxidative stress is considered to play a key role in the pathogenesis of atherosclerosis.

It has been proposed that oxidative stress plays an important role in the inflammatory processes that are key components of atherosclerosis. Under physiological conditions, the toxic effects of ROS can be reduced by enzymatic and non-enzymatic antioxidants. Superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) provide the first line of enzymatic antioxidant defence against ROS-mediated cardiac injury.

To improve the prognosis of patients with heart disease and injury, novel therapeutic strategies have focused on regulating oxidative stress in the cardiovascular system. Since both oxidative stress and inflammation need the participation of calcium ions (Ca++) to cause atherosclerosis, calcium channel blockers (CCBs) are widely used in the cardiovascular field for the control of angina and hypertension and as an alternative to β-blockers in patients with heart failure.

Amlodipine, a third-generation dihydropyridine CCB, has a high affinity for the lipid constituents of the cellular membrane. There is much basic and clinical data indicating that amlodipine, in addition to having haemodynamic properties, exerts non-calcium channel-related modulation in the vasculature, such as antioxidant activity. Since the antioxidant activity of amlodipine, particularly in the heart, has not yet been well examined, we aimed to investigate the role of amlodipine in the heart and blood of rabbits.

Methods

Thirty-six male New Zealand white rabbits (± 1.4 kg) were obtained from the laboratory animal house of Tabriz University of Medical Sciences. They were housed in an animal room at 22–24˚C and given free access to commercial rat chow and tap water. All the experimental procedures used, as well as rabbit care
and handling were in accordance with guidelines provided by the experimental animal laboratory and approved by the Animal Care Committee of the Tabriz University of Medical Sciences.

The rabbits were equally divided (n = 9) into four groups: group 1 rabbits were fed a regular diet, group 2 were fed a diet containing 2% cholesterol, group 3 had a regular diet plus 5 mg/kg/day oral amlodipine, and group 4 had a diet with 2% cholesterol plus amlodipine 5 mg/kg/day, for eight weeks. Cholesterol powder and amlodipine powder were provided from Merck and Aria companies, respectively. Cholesterol powder was mixed into the feed. Amlodipine was dissolved in distilled water and was given with a special gavage tube at 09:00 daily for eight weeks.

The study protocol was designed in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, No. 86-23, revised 1996) and approved by the Ethics Committee for the Use of Animals in Research at Tabriz University of Medical Sciences.

At the end of the experiments, all animals were fasted for eight hours and then anesthetised by injecting ketamine (25 mg/kg, intravenously) via the ear vein. Blood samples were drawn from the inferior vena cava and were stored in tubes for determination of serum lipid profiles and blood oxidative stress.

After decapitation, the heart was quickly removed, washed in ice-cold saline and the atria and great blood vessels were trimmed away. The ventricles were weighed and quickly frozen in liquid nitrogen.

For analysis of oxidative stress, cardiac and blood homogenates were prepared at 0–4°C as described by Rothermel et al.14 In brief, 50 mg of ventricle muscle were homogenised on ice in 1 ml of ice-cold lyses buffer (10 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol, pH 7.4). The homogenates were centrifuged at 4 500 g for 1 min at 4°C (Avanti J 25 USA). The supernatant containing the cytoplasmic protein fraction was collected and a protease inhibitor cocktail (104 mM AEBSF, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64) (P8340, Sigma-Aldrich, St Louis, MO) was added, and it was stored at –80°C until use. Protein concentration of the supernatant was estimated using the Bradford technique.15

Blood samples were drawn from the inferior vena cava and stored in tubes for an hour. The serum was prepared and used for the determination of serum lipid profiles, including total cholesterol and triglycerides. These were determined by enzymatic methods using an automatic analyser (Abbott, Alecyon Falcort, USA).

Lipid peroxidation was analysed by measuring thiobarbituric acid-reactive substances (TBARs) in the homogenates, as previously described by Draper and Hadley.16 Briefly, the samples were mixed with 1 ml 10% trichloroacetic acid (TCA) and 1 ml 0.67% thiobarbituric acid. The samples were heated in a boiling water bath for 15 min, and butanol (2:1 v:v) was added to the solution. After centrifugation at 800 g for 5 min (Avanti J 25 USA), TBAR levels were determined from the absorbance at 535 nm.

SOD activity was determined using a RANSOD kit (Randox labs. Crumlin, UK), according to Delmas-Beauvieux et al.17 SOD activity was measured in the supernatant on a spectrophotometer (Stat Fax, 2100, Awareness, USA) at 505 nm. In this method, xanthin and xanthin oxidase were used to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitropheno)-5-phenyl tetrazolium chloride (ITN) to form a red formazan dye. Concentrations of substrates were 0.05 mmol/l for xanthin and 0.025 mmol/l for INT. SOD activity was measured by the degree of inhibition of this reaction. To assay the mitochondrial SOD (mtSOD) activity in the heart, the cytosolic SOD was inhibited with 1 mM KCN.

Glutathione peroxidase activity was determined using a RANSEL kit (Randox labs Crumlin, UK) according to the method of Paglia and Valentine.18 GPX catalyses the oxidation of glutathione (at a concentration of 4 mmol/l) by cumene hydroperoxide. In the presence of glutathione reductase (at a concentration ≥ 0.5 units/l) and 0.28 mmol/l of NADPH, the oxidised glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to NAD⁺. The decrease in absorbance at 340 nm was measured using a spectrophotometer.

Statistical analysis
All determinations were performed at least in duplicate. Data were expressed as mean ± SEM and were analysed by a one-way ANOVA using a standard computerised statistical program, SPSS13.0 for windows software (SPSS INC, Chicago, IL, USA). When a significant p-value was obtained, the Tukey post hoc test was used to determine the differences between groups. A level of p < 0.05 was selected to indicate statistical significance.

Results
Our results clearly demonstrate that eight weeks on a 2% high-cholesterol diet significantly increased serum levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG). These observations indicate that an atherogenic diet induced hypercholesterolaemia in our in vitro model. Although amlodipine treatment tended to enhance HDL-C:LDL-C and HDL-C:TC ratios in this group, these effects were not statistically significant. The significant increase observed in plasma levels of HDL-C and decrease in LDL-C, TG and TC is considered the main effect of amlodipine treatment on serum lipid profiles in the rabbits fed a regular diet (Table 1).

Lipid peroxidation
In the blood samples, the level of TBARs increased in the rabbits on the 2% cholesterol diet (group 2) and those on 2% cholesterol plus amlodipine (group 4), and decreased in the group on a regular diet plus amlodipine (group 3) (p < 0.05). In addition, in those on a regular diet plus amlodipine (group 3) and those on 2% cholesterol plus amlodipine (group 4), the level of TBARs was less than in the group on a diet of 2% cholesterol (group 2) (Fig. 1). Lipid peroxidation levels in all the heart samples showed a similar trend to that of the blood samples, except in the rabbits on a regular diet plus amlodipine (group 3), where the levels of TBARs did not diminish compared to the control group (Fig. 1).

Antioxidant enzymes
Fig. 2 shows that the activity of total SOD (tSOD) in the blood samples decreased in the rabbits on the 2% cholesterol diet (group 2) (p < 0.05), and markedly increased in those on a regular diet plus amlodipine (group 3) and those on 2% cholesterol
plus amlodipine (group 4), compared with the controls ($p < 0.05$). Moreover, the activity of mitochondrial SOD (mtSOD) in the heart samples was enhanced in the rabbits on a regular diet plus amlodipine (group 3) and those on 2% cholesterol plus amlodipine (group 4), compared to the controls (group 1) and the 2% cholesterol group (group 2) ($p < 0.05$).

The changes in GPX activity (Fig. 3) in the heart samples were similar to the changes in SOD activity, except for the rabbits on the 2% cholesterol diet (group 2), whose GPX activity decreased compared to the control group ($p < 0.05$).

**Discussion**

Our results indicate that eight weeks of a 2% high-cholesterol diet increased all serum cholesterol profile fractions and induced oxidative stress in the blood and heart tissue, since the level of TBARs (a marker of lipid peroxidation) significantly increased in all cholesterol-fed rabbits. The key finding of this study was that eight weeks of amlodipine treatment reduced oxidative stress in the blood and hearts of cholesterol-fed rabbits.

As an index of the anti-oxidative defence system, we measured the activities of SOD and GPX and the levels of MDA. A considerable number of studies have accumulated to suggest that most CCBs may be effective in preventing the development of atherosclerosis. The most important mechanisms involved may include antioxidant effects, and changes in Ca$^{2+}$ and cholesterol metabolism.

In this study, chronic amlodipine treatment increased HDL-C levels or the ratio of HDL-C to LDL-C, and reduced LDL-C and TG levels in the plasma. The importance of LDL-C, HDL-C and TC is well documented in the pathogenesis of atherosclerosis, but TG levels should not be ignored. In addition to the pivotal role that HDL-C plays in reverse cholesterol transport and cellular cholesterol efflux, it possesses a spectrum of anti-inflamma-

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**TABLE 1. COMPARISON OF THE SERUM LIPID PROFILE CHANGES (MG/DL)**

| Variable       | Group 1   | Group 2   | Group 3   | Group 4   |
|----------------|-----------|-----------|-----------|-----------|
| Total cholesterol | 49.13 ± 0.6 | 860.3 ± 0.6* | 40.3 ± 0.8 | 524.5 ± 5.8** |
| LDL            | 7.23 ± 1.39 | 722 ± 0.86* | 13.13 ± 0.20 | 451.43 ± 6.70** |
| HDL            | 14 ± 0.73 | 49 ± 0.63* | 19.83 ± 0.54* | 48.33 ± 0.95* |
| TG             | 95.50 ± 1.7 | 466.6 ± 2.5* | 81 ± 0.50* | 138.6 ± 1.8** |
| HDL/LDL        | 2.47 ± 0.60 | 0.07 ± 0.001* | 1.50 ± 0.05 | 0.11 ± 0.002* |
| HDL:TC         | 0.35 ± 0.02 | 0.06 ± 0.001* | 0.4 ± 0.007* | 0.09 ± 0.001* |

Rabbits were fed a regular diet (group 1), 2% cholesterol diet (group 2), regular diet plus amlodipine 5 mg/kg/day (group 3) and a 2% cholesterol-plus-amlodipine diet (group 4).

Data are expressed as mean ± SEM for each group ($n = 9$).

Differences of $p < 0.05$ were considered significant.

*Group 1 vs groups 2, 3 and 4; #group 2 vs group 4.
tory, antioxidative, anti-apoptotic, antithrombogenic, vasodilatory and anti-infectious properties, all of which potentially contribute to its atheroprotective nature. Accumulating evidence shows that HDL-C protects LDL-C from oxidation.

Epidemiological and observational studies have demonstrated that HDL-C level is a strong, independent predictor of risk of coronary heart disease (CHD), and raising HDL-C levels may afford clinical benefit. Since decreased HDL-C levels are associated with increased production of ROS, increases in HDL-C levels by amlodipine treatment may be a consequence of reduction in oxidative stress.

MDA is one of the most reliable and widely used indices of oxidative stress and, as a marker of oxidative damage, has been studied extensively. It is known that lipid peroxidation is the co-operative event involved in the development of atherosclerosis. Also a positive correlation has been found between MDA levels and coronary artery calcification scores, and it is more convincingly correlated with TG levels and inversely correlated with HDL-C levels.

In our study, as the level of MDA significantly increased in cholesterol-fed rabbits, amlodipine treatment reduced it in the blood and heart muscle. It has been suggested that highly lipophilic CCBs such as amlodipine, by inserting to a location in the membrane near conjugated double bonds, are capable of donating protons to lipid peroxide molecules, thereby blocking the peroxidation process. Since amlodipine is lipophilic, it easily enters the cells and prevents lipid peroxidation. In our results, increased MDA levels may have been attributed to a high production of free radicals by a high-cholesterol diet, and the ability of amlodipine to diminish lipid peroxidation in the rabbits fed a high-cholesterol diet may have been non-calcium dependent and more related to proton donation.

It has been proposed that a high-cholesterol diet induces free radical production and may result in oxidative stress, which plays an important role in hypercholesterolaemia-induced atherosclerosis. Our data showed that a high-cholesterol diet decreased antioxidant enzyme activity (SOD and GPX) in both blood and heart tissue, and also confirmed that amlodipine could decrease the activation of oxidative stress. Previous reports indicate that markers of oxidative stress, such as superoxide production, were increased in atherosclerotic lesions. This antioxidant activity of amlodipine has also been observed in various animal models, including non-human primates, which reveals the important anti-atherogenic effect of this compound.

Increased ROS production can initiate a cascade of signal transduction, which leads to endothelial dysfunction, changes in vascular tone, vascular remodelling and vascular inflammatory responses. Under normal conditions, the heart minimises oxidative stress-induced injuries by the enhancement of SOD and GPX activity. SOD converts superoxide radicals to H₂O₂ and GPX eliminates H₂O₂. Since hypercholesterolaemia significantly decreased GPX activity in the present study, this probably resulted in aggregation of H₂O₂ and other reactive oxygen species and may have caused lipid peroxidation. It has also been reported that amlodipine reduced oxidative stress by restoring copper/zinc-containing SOD activity in the heart in hypertensive rats.

Amlodipine with or without a high-cholesterol (2%) diet significantly increased SOD and GPX activity in the heart and blood, compared with control or high-cholesterol fed groups. Surprisingly the activity of these antioxidant enzymes was approximately equal in groups 3 and 4. This suggests that the high-cholesterol diet could not decrease the antioxidant enzyme activity in the presence of amlodipine. Reduction of oxidative stress protects not only lipids, but also DNA, which is crucial to eventual cell death.

Exactly how amlodipine exercises its antioxidant activity is unclear, although several possible mechanisms have been proposed, including antithrombogenic, anti-inflammatory and antioxidant properties of HDL-C, and decrease in plasma LDL-C levels. The anti-oxidative property of these L-type CCBs may stem from their chemical structure; they contain an aromatic ring, which attracts free radicals. Furthermore, the dihydropyridine ring in these CCBs is able to donate a proton, which stabilises the free electron.

Since CCBs primarily affect the cellular interaction of endothelial cells, smooth muscle cells, monocytes and thrombocytes, which play key roles in the early phases of the development of atherosclerosis, the amlodipine effect of inhibiting calcium influx is the main mechanism for attenuation of oxidative stress in atherosclerosis. Therefore one of the major pathways by which amlodipine exerts its antioxidant effect is prevention of calcium overload. There are some conflicting results in the literature that may be partly due to differences in in vitro models or the interventional drugs.

Conclusion
The present study indicated that the CCB amlodipine decreased oxidative stress in the heart and blood and improved lipid profiles in cholesterol-fed rabbits. It may therefore be beneficial for the reduction of oxidative stress and improvement of lipid profiles in patients with diseases related to hyperlipidaemia. Further clinical trials are needed to prove the importance of amlodipine and other CCBs in patients with atherosclerosis and similar diseases.

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