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In-vivo & In-vitro Antioxidant Potential of Amlodipine Used in the Management of Hypertension and Diabetes Mellitus

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Abstract
Amlodipine is an angiotensin II type 1 receptor blocker and partial agonist of peroxisome proliferator-activated receptor gamma. Here, we investigated the protective capacity of Amlodipine against high glucose (HG) elicited oxidative. The activity of lactate dehydrogenase (LDH), NADPH oxidase (NOX), superoxide dismutase (SOD), catalase (CAT) as well as the levels of malondialdehyde (MDA), glutathione (GSH), intracellular reactive oxygen species (ROS), and all other in-vitro & in-vivo antioxidant experimental models. Moreover, the direct antioxidant effect of amlodipine was determined by 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay and protein expression. Amlodipine exhibited antioxidant activity inthe ABTS assay with the IC_{50} value of 37.5 µM. Additionally, the cell viability, GSH level, SOD and CAT activity were notably elevated by amlodipine. These findings suggest that amlodipine has protective effects on Management of Hypertension and Diabetes Mellitus.
Keywords: Antioxidant; DPPH; SOD; LPO; Amlodipine.

Introduction

Oxidative stress and vascular inflammation are closely interrelated to endothelial dysfunction and vascular damage. Type 2 diabetes mellitus is characterized by a state of glycative and oxidative stress. Overproduction of the reactive oxygen species in diabetic patients may be due to chronic hyperglycaemia, hyperinsulinemia, elevated free fatty acids (FFAs), and dyslipidaemia, typical of this condition. Oxidative stress and mild chronic vascular inflammation also play a role in the pathophysiology of hypertension and atherosclerosis [1]. In the literature, many studies reported that the increased oxidized low-density lipoprotein (LDL) in type 2 diabetes mellitus is correlated with an increased risk of cardiovascular complications [2]. This increased susceptibility of LDL to oxidation is dependent on the antioxidant capacity of high-density lipoprotein (HDL)-associated paraoxonase-1 (PON-1). PON-1 is a glycoprotein expressed in several tissues, but it is mainly synthesized by the liver and circulates within HDL particles [3]. The pleiotropic effects of some well-known antihypertensive agents and statins on oxidative stress and inflammation have been reported in the literature. Among the antihypertensive agents, olmesartan has been reported to protect against oxidative stress in rats, via the induction of nuclear factor-erythroid-2-related factor 2 (Nrf2) signaling pathways [4]. Olmesartan medoxomil is a long-acting angiotensin II type I receptor (AT1R) antagonist approved for the treatment of mild to severe hypertension, alone or in combination with other agents. Olmesartan is therapeutically effective for the treatment of patients with heart failure by decreasing cytokines and oxidative stress through its anti-inflammatory

effects. Regarding calcium channel blockers, in vitro studies proved that calcium channel blockers, including amlodipine, exhibit inhibitory effects on PON-1 at low concentrations [5]. However, studies conducted in vivo in people with type 2 diabetes evaluating effects of antihypertensive agents on PON-1 are lacking. We already reported the effects of olmesartan/amlodipine combination on in hypertensive patients, but not in type 2 diabetic patients. Amlodipine is the third generation calcium antagonist, 1, 4-dihydropyridine (1, 4-DHP) compound with prolonged action. It has expressed properties of Ca^{2+} channel (especially L-type and T-type) blockers (CCB). Besides, it exerts multisided (pleiotropic, namely, when a drug has actions other than those for which the compound was specifically indicated or developed) effects on other metabolic processes. It is the basis for the beneficial therapeutic action at heart and vasculature diseases and delays the development of atherosclerosis [6]. This drug is well tolerated by patients. It has no significant side effects. It could be used in the monotherapy, as well in combination with other drugs-diuretics, angiotensine-converting enzyme (ACE) inhibitors, antagonists of the angiotensin II receptors, statins [7]. Amlodipine at nanomolar concentrations binds to the voltage-dependent L-type calcium channels. It has optimal lipophylicity, and it has a free amino group, which guarantees the good bioavailability. Amlodipine also influences the NO-dependent metabolic processes too; it stimulates NO synthesis and prolongs NO action duration [8]. Results of the studies of amlodipine pharmacological and clinical properties are summarized in several reviews [9]. This review summarizes outlook about experimental materials from the last decades concerning amlodipine pleiotropy in the action mechanism, as well sometimes controversial clinical observations about its cardio-protective and vazoprotective action.

Material & Methods

Collection of drug

Amlodipine was collected from Crinova Healthcare Pvt Ltd. All the chemicals were collected from the department of Pharmacy, Dr. APJ Abdul Kalam University Indore.

Selection of animals

Two groups of each six rats were used in the present investigation. The basal concentration of blood glucose level of all the animals was recorded and 6 animals were separated to serve as normal control. The remaining animals received a single injection of amlodipine in water for injection at a dose of 150-mg/kg bodyweight given by intra-peritoneal route.

In-vitro Antioxidant activity

DPPH radical scavenging assay

When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in color (from deep violet to light yellow) were measured at 517 nm on a UV/visible light spectrophotometer [10]. Extract solutions were prepared by dissolving 0.025 g of dry extract in 10 ml of methanol. The solution of DPPH in methanol (6x10.5 M) was prepared daily before UV measurements, 3 ml of this solution were mixed with 77 µl extract solution in 1 cm path length disposable micro cuvettes (final mass ratio of extracts with DPPH was approximately 3:1, 1.5:1, 0.75:1). Radical scavenging activity was calculated as follows:

$$\textbf{Scavenging rate} = [As\text{-}Ai/As] \times 100$$

Where as is the absorbance of pure DPPH, Ai is the absorbance of DPPH in the presence of various extracts. BHT and Vitamin C were used as references standards.

Superoxide scavenging activity

The assay was based on the capacity of the samples to inhibit blue formazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system. The reaction mixture contains 50 mM phosphate buffer, pH 7.6, 20µg riboflavin, 12 mM EDTA, O.1 mg/3 ml NBT, added in that sequence. The reaction was started by illumination the reaction mixture with different concentrations (5-100 µg/ml) of samples for 15, 30 and 45 min. immediately after illumination the absorbance was measured at 590 nm. Ascorbic acid was used as standard drug. Percentage inhibition and IC_{50} were calculated.

ABTS radical scavenging assay

The $ABTS^+$ solution was prepared by the reaction of 7 mM ABTS (5 mL) and 2.45mM (88 µL) potassium persulphate after incubation at room temperature in the dark for 16 h. It was then diluted with 80 % ethanol to obtain an absorbance of 0.700±0.005 at 734 nm. The $ABTS^+$ solution (2.7 mL) was thoroughly mixed with 0.3mL of the test sample. The reaction mixture was allowed to stand at 30 °C for 30 min and the absorbance at 734nm was immediately recorded. Samples of BHT and Vitamin C with the same concentrations were used as references. The level of radical scavenging was calculated using the aforementioned equation for DPPH.

Reducing power assay

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants. 1 ml extract is mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferricyanide (30 mM) and incubated at 50°C for 20 min. Thereafter, 2.5 ml of trichloroacetic acid (600 mM) is added to the reaction mixture, centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) is mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (6 mM) and absorbance is measured at 700 nm. Ascorbic acid and butylated hydroxytoluene (BHT) used as for comparative purpose.

Nitric oxide radical scavenging (NO) assay

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interact with oxygen to produce nitrite ions, which were measured using the Griess reaction reagent. 3.0 ml of 10 mM sodium nitroprusside in phosphate buffer is added to 2.0 ml of extract and reference compound in different concentrations (20 - 100 µg/ml). The resulting solutions are then incubated at 25°C for 60 min. A similar procedure is repeated with methanol as blank, which serves as control. To 5.0 ml of the incubated sample, 5.0 ml of Griess reagent (1 % sulphanilamide, 0.1 % naphthyethylene diamine dihydrochloride in 2% H_3PO_3) is added and absorbance of the chromophore formed is measured at 540 nm. Percent inhibition of the nitrite oxide generatedis measured by comparing the absorbance values of control and test preparations. Curcumin, caffeic acid, sodium nitrite, BHA, ascorbic acid, rutin, BHT or tocopherol can be used as a positive control [11].

Hydroxyl radical scavenging (HO) assay

Hydroxyl radical is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell [12]. The model used is ascorbic acid-iron- EDTA model of $HO^{\cdot}$ generating system. This is a totally aqueous system in which ascorbic acid, iron and EDTA conspire with each other to generate hydroxyl radicals. The reaction mixture (1.0 ml) consist of 100 µl of 2-deoxy-Dribose (28 mM in 20 mM KH2PO4 -KOH buffer, pH 7.4), 500 µl of the extract, 200 µl EDTA (1.04 mM) and 200 µM $FeCl_3$ (1:1 v/v), 100 µl of H_2O_2 (1.0 mM) and 100 µl ascorbic acid (1.0 mM) which is incubated at 37ºC for 1 h. 1.0 ml of thiobarbituric acid (1 %) and 1.0 ml of trichloroacetic acid (2.8 %) are added and incubated at 100ºC for 20 min. After cooling, absorbance is measured at 532 nm, against a blank sample. Gallic acid, mannitol, catechin, vitamin, quercetin, BHA, tocopherol, rutin or ascorbic acid can be used as a positive control.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay is based on the ability of antioxidants to reduce Fe3+ to Fe2+ in the presence of 2,4,6-tri(2-pyridyl)- s-triazine (TPTZ), forming an intense blue Fe2+-TPTZ complex with an absorption maximum at 593 nm. This reaction is pH-dependent (optimum pH 3.6). 0.2 ml of the extract is added to 3.8 ml of FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10.0 mM TPTZ solution and 1 part of 20.0 mM FeCl3. 6H2O solution) and the reaction mixture is incubated at 37°C for 30 min and the increase in absorbance at 593 nm is measured. FeSO4 is used for calibration. The antioxidant capacity based on the ability to reduce ferric ions of sample is calculated from the linear calibration curve and expressed as mmol $FeSO_4$ equivalents per gram of sample. BHT, BHA, ascorbic acid, quercetin, catechin or trolox can be used as a positive control [13].

Oxygen radical absorbance capacity (ORAC) assay

The capacity of a compound to scavenge peroxyl radicals, generated by spontaneous decomposition of 2, 2'- azo-bis, 2- amidinopropane dihydrochloride (AAPH), was estimated in terms of standard equivalents, using the ORAC assay. The reaction mixture (4.0 ml) consist of 0.5 ml extract in phosphate buffer (75 mM, pH 7.2) and 3.0 ml of fluorescein solution both are mixed and pre-incubated for 10 min at 37°C. Then, 0.5 ml of 2, 2'-azo-bis, 2- amidinopropane (AAPH) dihydrochloride solution is added and immediately the loss of fluorescence (FL) is observed at 1 min intervals for 35 min.

Peroxynitrite radical scavenging activity

Peroxynitrite is a cytotoxicant with strong oxidizing properties toward various cellular constituents, including sulfhydryls, lipids, amino acids and nucleotides and can cause cell death, lipid peroxidation, carcinogenesis and aging. It is generated in vivo by endothelial cells, Kupffer cells, neutrophils and macrophages. Peroxynitrite radical is a relatively stable species compared with other free radicals but once protonated gives highly reactive peroxynitrous acid (ONOOH), decomposing with a very short half-life (1.9 s) at 37 °C to form various cytotoxicants and that can induce the oxidation of thiol (−SH) groups on proteins, nitration of tyrosine, lipid peroxidation and also nitrosation reactions, affecting cell metabolism and signal transduction. It

can ultimately contribute to cellular and tissue injury with DNA strand breakage and apoptotic cell death, e.g. in thymocytes, cortical cells and HL-60 leukemia cells. Its excessive formation may also be involved in several human diseases such as Alzheimer's disease, rheumatoid arthritis, cancer and atherosclerosis. Due to the lack of endogenous enzymes responsible for inactivation, developing specific scavengers is of considerable importance. The method involves the use of a stock solution of dihydroxyrhodamine 123 (DHR 123, 5 mM) in dimethylformamide that is purged with nitrogen and stored at −80 °C. Working solution with DHR 123 (final concentration 5 μM) is diluted from the stock solution and is placed on ice in the dark immediately prior to the experiment. Buffer solution, 50 mM sodium phosphate (pH 7.4), containing 90 mM sodium chloride and 5 mM potassium chloride with 100 μM diethylene triamine penta acetic acid (DTPA) are purged with nitrogen and placed on ice before use. Scavenging activity of by the oxidation of DHR 123 is measured on a microplate fluorescence spectrophotometer with excitation and emission wavelengths of 485 nm and 530 nm at room temperature, respectively. The background and final fluorescent intensities are measured 5 min after treatment without 3-morpholino- sydnonimine (SIN-1) or authentic. Oxidation of DHR 123 by decomposition of SIN-1 gradually increased whereas authenti rapidly oxidized DHR 123 with its final fluorescent intensity being stable over time.

Phosphomolybdenum method

Total antioxidant capacity assay is a spectroscopic method for the quantitative determination of antioxidant capacity, through the formation of phosphomolybdenum complex. The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of a green phosphate Mo (V) complex at acidic pH. Total antioxidant capacity can be calculated by the method described by Prieto et al. (1999). 0.1 mL of sample (100 lg) solution is combined with 1 mL of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tube is capped and incubated in a boiling water bath at 95 ^{0}C for 90 min. After cooling the sample to room temperature, the absorbance of the aqueous solution is measured at 695 nm against blank in UV spectrophotometer. A typical blank solution contained 1 mL of reagent solution and the appropriate volume of the same solvent used for the sample and it is incubated under same conditions as rest of the sample. For samples of unknown composition, antioxidant capacity can be expressed as equivalents of a-tocopherol.

DMPD (N,N-dimethyl-p-phenylene diamine)

dihydrochloride method DMPD radical cation decolorization method has been developed for the measurement of the antioxidant activity in food and biological samples. This assay is based on the reduction of buffered solution of colored DMPD in acetate buffer and ferric chloride. The procedure involves measurement of decrease in absorbance of DMPD in the presence of scavengers at its absorption maximum of 505 nm. The activity was expressed as percentage reduction of DMPD. The radical by mixing 1 mL of DMPD solution (200 mM), 0.4 mL of ferric chloride (III) (0.05 M), and 100 mL of sodium acetate buffer solution at 0.1 M, modifying the pH to 5.25. The reactive mixture has to be kept in darkness, under refrigeration, and at a low temperature (4–5 ^{0}C) .The reaction takes place when 50 l L of the sample (a dilution of 1:10 in

water) is added to 950 l L of the DMPDÆ+ solution. Absorbance is measured after 10 min of continuous stirring, which is the time taken to reach constant decolorization values. The results are quantified in mM Troloxon the relevant calibration curve.

In-vivo Antioxidant activity

Ferric reducing ability of plasma

It is one of the most rapid test and very useful for routine analysis. The antioxidative activity is estimated by measuring the increase in absorbance caused by the formation of ferrous ions from FRAP reagent containing TPTZ (2,4,6-tripyridyl-s-triazine) and $FeCl_2$.6H2O. The absorbance is measured spectrophotometrically at 593 nm. The method illustrated by Benzie and Strain (1996) involves the use of blood samples that are collected from the rat retro orbital venous plexus into heparinized glass tubes at 0, 7 and 14 days of treatment. Three mL of freshly prepared and warm (37 ^{0}C) FRAP reagent 1 mL (10 mM) of 2,4,6 tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, 1 mL 20 mM $FeCl_2.6H_2O$, 10 mL of 0.3 M acetate buffer (pH 3.6) is mixed with 0.375 mL distilled water and 0.025 mL of test samples. The absorbance of developed color in organic layer is measured at 593 nm. The temperature is maintained at 37 ^{0}C. The readings at 180 s are selected for the calculation of FRAP values.

Reduced glutathione (GSH) estimation

GSH is an intra-cellular reductant and plays major role in catalysis, metabolism and transport. It protects cells against free radicals, peroxides and other toxic compounds. Deficiency of GSH in the lens leads to cataract formation. Glutathione also plays an important role in the kidney and takes part in a transport system involved in the reabsorption of amino acids. The method illustrated by Ellman (1959) can be used for determination of antioxidant activity. The tissue homogenate (in 0.1 M phosphate buffer pH 7.4) is taken and added with equal volume of 20 % trichloroacetic acid (TCA) containing 1 mM EDTA to precipitate the tissue proteins. The mixture is allowed to stand for 5 min prior to centrifugation for 10 min at 2000 rpm. The supernatant (200 lL) is then transferred to a new set of test tubes and added with 1.8 mL of the Ellman's reagent (5,50-dithiobis-2- nitrobenzoic acid (0.1 mM) prepared in 0.3 M phosphate buffer with 1% of sodium citrate solution). Then all the test tubesare made up to the volume of 2 mL. After completion of the total reaction, solutions are measured at 412 nm against blank. Absorbance values were compared with a standard curve generated from known GSH.

Glutathione peroxidase (GSHPx) estimation

GSHPX is a seleno-enzyme two third of which (in liver) is present in the cytosol and one third in the mitochondria. It catalyzes the reaction of hydroperoxides with reduced glutathione to form glutathione disulfide (GSSG) and the reduction product of hydroperoxide. GSHPx is found throughout the tissues, being present as four different isoenzymes, cellular glutathione peroxidase, extracellular glutathione peroxidase, phospholipid hydroperoxide glutathione peroxidase and gastrointestinal glutathione peroxidase. GSHPx measurement is considered in particular with patients who are under oxidative stress for any reason; low activity of this enzyme is one of the early consequences of a disturbance of the prooxidant/antioxidant balance. Cytosolic GPx is assayed via a 3-mL cuvette containing 2.0 mL of 75 mM/L phosphate buffer,

pH 7.0. The following solutions are then added: 50 lL of 60 mM/L glutathione reductase solution (30 U/mL), 50 lL of 0.12 M/L NaN3, 0.10 of 0.15 mM/L Na2EDTA, 100 lL of 3.0 mM/L NADPH, and 100 lL of cytosolic fraction obtained after centrifugation at 20,000 g for 25 min. Water is added to make a total volume of 2.9 mL. The reaction is started by the addition of 100 lL of 7.5 mM/L H2O2, and the conversion of NADPH to NADP is monitored by a continuous recording of the change of absorbance at 340 nm at 1 min interval for 5 min. Enzyme activity of GSHPx was expressed in terms of mg of proteins.

Glutathione-S-transferase (GSt)

Glutathione-S-transferase is thought to play a physiological role in initiating the detoxication of potential alkylating agents, including pharmacologically active compounds. These enzymes catalyze the reaction of such compounds with the - SH group of glutathione, thereby neutralizing their electrophilic sites and rendering the products more water-soluble. The reaction mixture (1 mL) consisted of 0.1 N potassium phosphate (pH 6.5), 1 nM/L GSt, 1 M/L l-chloro-2, 4-dinitrobenzene as substrate and a suitable amount of cytosol (6 mg protein/mL). The reaction mixture is incubated at 37 ^{0}C for 5 min and the reaction is initiated by the addition of the substrate. The increase in absorbance at 340 nm was measured spectrophotometrically.

Superoxide dismutase (SOD) method

It is estimated in the erythrocyte lysate prepared from the 5 % RBC suspension. To 50 lL of the lysate, 75 mM of Tris–HCl buffer (pH 8.2), 30 mM EDTA and 2 mM of pyrogallol are added. An increase in absorbance is recorded at 420 nm for 3 min by spectrophotometer. One unit of enzyme activity is 50 % inhibition of the rate of autooxidation of pyrogallol as determined by change in absorbance/min at 420 nm. The activity of SOD is expressed as units/mg protein.

Catalase (CAT)

Catalase activity can be determined in erythrocyte lysate using Aebi's method (Aebi, 1984). Fifty microliter of the lysate is added to a cuvette containing 2 mL of phosphate buffer (pH 7.0) and 1 mL of 30 mM H_2O_2. Catalase activity is measured at 240 nm for 1 min using spectrophotometer. The molar extinction coefficient of H2O2, 43.6M cm^{-1} was used to determine the catalase activity. One unit of activity is equal to 1 mmol of H2O2 degraded per minute and is expressed as units per milligram of protein.

c-Glutamyl transpeptidase activity (GGT) assay

According to Singhal et al. (1982), the serum sample is added to a substrate solution containing glycylglycine, $MgCl_2$ and g- Glutamyl-p-nitroanilide in 0.05 M tris (free base), pH 8.2. The mixture is incubated at 37 ^{0}C for 1 min and the absorbance read at 405 nm at 1 m interval for 5 m. The activity of GGT is calculated from the absorbance values.

Glutathione reductase (GR) assay

The ubiquitous tripeptide glutathione (GSH), which is the most abundant low molecular weight thiol in almost all cells, is involved in a wide range of enzymatic reactions. A major function of GSH is to serve as a reductant in oxidation–reduction processes; a function resulting in the formation of glutathione disulfide (GSSG). A heat labile system capable of reducing GSSG was discovered in liver. The enzyme directly involved in reduction of GSSG. Livers (about 400 g)

are obtained from killed rats (200–250 g). The livers are cut into small pieces and homogenized in 9 mL of 0.25 M ice-cold sucrose per g of rat liver in a blender. The homogenate is centrifuged for 45 min at 14,000 rpm. The pellets are suspended in a small volume of 0.25M sucrose and centrifuged. The supernatants are combined with the previous centrifugate. The pooled material is adjusted to pH 5.5 with cold 0.2 M acetic acid and centrifuged again for 45 min at 14,000 rpm. The rate of oxidation of NADPH by GSSG at 30 ^{0}C is used as a standard measure of enzymatic activity. The reaction system of 1 mL contained: 1.0 mM GSSG, 0.1 mM NADPH, 0.5 mM EDTA, 0.10M sodium phosphate buffer (pH 7.6), and a suitable amount of the glutathione reductase sample to give a change in absorbance of 0.05– 0.03/min. The oxidation of 1 lM of NADPH/min under these conditions is used as a unit of glutathione reductase activity. The specific activity is expressed as units per mg of protein.

Lipid peroxidation (LPO) assay

LPO is an autocatalytic process, which is a common consequence of cell death. This process may cause peroxidative tissue damage in inflammation, cancer and toxicity of xenobiotics and aging. Malondialdehyde (MDA) is one of the end products in the lipid peroxidation process. Malondialdehyde (MDA) is formed during oxidative degeneration as a product of free oxygen radicals, which is accepted as an indicator of lipid peroxidation. The tissues are homogenized in 0.1 M buffer pH 7.4 with a Teflonglass homogenizer. LPO in this homogenate is determined by measuring the amounts of malondialdehyde (MDA) produced primarily. Tissue homogenate (0.2 mL), 0.2 mL of 8.1 % sodium dodecyl sulfate (SDS), 1.5 mL of 20 % acetic acid and 1.5 mL of 8 % TBA are added. The volume of the mixture is made up to 4 mL with distilled water and then heated at 95 ^{0}C on a water bath for 60 min using glass balls as condenser. After incubation the tubes are cooled to room temperature and final volume was made to 5 mL in each tube. Five mL of butanol: pyridine (15:1) mixture is added and the contents are vortexed thoroughly for 2 min. After centrifugation at 3000 rpm for 10 min, the upper organic layer is taken and its OD is taken at 532 nm against an appropriate blank without the sample. The levels of lipid peroxides can be expressed as n moles of thiobarbituric acid reactive substances (TBARS)/mg protein using an extinction coefficient of 1.56 · 105 ML cm^{-1}.

LDL assay

The isolated LDL is washed and dialyzed against 150 mmol/L NaCl and 1 mmol/L Na2EDTA (pH 7.4) at 4 ^{0}C. The LDL is then sterilized by filtration (0.45 lM), kept under nitrogen in the dark at 4 ^{0}C. LDL (100 lg of protein/mL) is incubated for 10 min at room temperature with samples. Then, 5 lmol/ L of CuSO4 is added, and the tubes are incubated for 2 h at 37 ^{0}C. Cu2+-induced oxidation is terminated by the addition of butylated hydroxytoluene (BHT, 10 lM). At the end of the incubation, the extent of LDL oxidation is determined by measuring the generated amount of lipid peroxides and also by the thiobarbituric acid reactive substances (TBARS) assay at 532 nm, using malondialdehyde (MDA) for the standard curve.

Result & Discussion

In-vitro Antioxidant activity of Amlodipine

Table 1(A): Antioxidant activity (%) of the Amlodipine

Con.	DPPH		Nitric Oxide		Super Oxide		ABTS		ORAC		FRAP	
C (µg/ml)	Control	Test Drug	Control	Test Drug	Control	Test Drug	Control	Test Drug	Control	Test Drug	Control	Test Drug
5	8.6±1.0	46.8±0.2	6.45±1.5	13.1±0.5	21.9±0.4	23.2±0.1	22.2±0.4	6.45±0.3	19.8±0.5	20.4±0.2	21.1±0.5	6.4±0.2
10	16.8±0.2	77.8±0.7	11.5±0.8	56.6±0.6	23.1±0.3	34.3±0.2	33.4±0.6	33.23±0.2	20.9±0.4	30.5±0.4	30.3±0.2	32.50±0.1
50	33.4±0.1	91.8±0.2	41.6±0.1	96.4±0.1	25.2±0.1	66.2±0.1	45.9±0.8	52.56±0.5	21.6±0.7	58.3±0.4	41.8±0.3	51.21±0.4
100	61.5±0.6	95.7±0.2	70.4±0.3	97.3±0.1	31.5±0.3	81.5±0.2	57.4±1.0	94.1±0.3	30.7±0.4	80.5±0.2	57.3±0.9	92.15±0.2
500	85.1±0.5	96.9±0.1	97.3±0.1	98.7±0.1	71.3±0.1	94.3±0.3	70.9±2.0	96.49±0.1	71.6±0.2	93.2±0.4	88.2±0.8	98.49±0.2

All values are expressed as mean □ S.E.M (n=6)

Table 1(B): Antioxidant activity (%) of the Amlodipine

Con.	Hydroxyl radical		Reducing power		Phospho Molybdenum		Peroxynitrite redical		DMPD	
C (µg/ml	Control	Test Drug	Control	Test Drug	Control	Test Drug	Control	Test Drug	Control	Test Drug

)										
5	8.4±1.0	42.5±0.3	9.4±1.4	12.5±0.6	20.9±0.5	21.1±0.4	22.1±0.3	8.19±0.4	20.8±0.5	22.2±0.1
10	16.7±0.4	72.8±0.7	15.4±0.6	51.5±0.5	24.8±0.4	35.4±0.2	35.2±0.5	34.74±0.2	25.4±0.4	31.3±0.1
50	33.5±0.2	90.4±0.2	39.3±0.2	90.1±0.2	29.7±0.9	65.4±0.2	49.6±0.9	58.25±0.4	27.8±0.7	62.2±0.2
100	61.6±0.7	94.2±0.2	71.2±0.5	92.9±0.2	31.6±0.4	80.2±0.2	58.4±1.0	92.08±0.3	33.3±0.4	81.8±0.1
500	86.4±0.6	95.6±0.1	90.1±0.3	98.8±0.3	78.8±0.2	98.2±0.5	90.4±1.0	96.59±0.4	78.1±0.2	94.2±0.3

All values are expressed as mean □ S.E.M (n=6)

In-Vivo Antioxidant activity of Amlodipine

Table 2(A): Antioxidant activity (%) of the Amlodipine

Con.	Ferric reducing		GSH		GSHPx		GSt		SOD	
C (µg/ml)	Control	Test Drug	Control	Test Drug	Control	Test Drug	Control	Test Drug	Control	Test Drug
5	15.2±9.42	41.4±0.4	0.69±1.1	0.71±0.5	0.4±0.6	0.2±0.5	20.2±0.4	12.35±0.2	16.1±0.6	25.4±0.1
10	26.3±8.6	63.5±0.5	0.42±0.5	0.72±0.6	0.6±0.1	0.3±0.3	32.2±0.6	30.85±0.1	18.4±0.1	50.2±0.3

50	35.6±5.1	72.2±0.2	0.45±0.3	0.83±0.3	0.16±0.2	0.5±0.2	40.3±0.7	41.57±0.3	21.6±0.2	66.7±0.4
100	42.2±3.2	83.5±0.4	0.51±0.4	0.89±0.4	0.11±0.4	0.7±0.1	52.5±1.0	53.39±0.2	37.4±0.3	85.9±0.2
500	564±5.53	95.3±0.2	0.75±0.2	0.96±0.6	0.65±0.1	0.8±0.3	95.1±1.0	79.89±0.2	70.7±0.2	95.1±0.5

All values are expressed as mean □ S.E.M (n=6)

Table 2(A): Antioxidant activity (%) of the Amlodipine

Con.	**Catalase**		**GGT**		**Glutathione reductase**		**LPO**		**LDL**	
C (µg/ml)	**Control**	**Test Drug**	**Control**	**Test Drug**	**Control**	**Test Drug**	**Control**	**Test Drug**	**Control**	**Test Drug**
5	0.5±0.3	0.7±0.4	6.1±1.5	12.2±0.5	11.7±0.4	20.4±0.7	22.1±0.5	7.35±0.2	16.3±0.5	21.4±0.2
10	0.7±0.4	0.8±0.6	8.3±0.8	47.4±0.6	20.3±0.1	32.3±0.3	30.6±0.6	30.21±0.1	18.6±0.4	38.6±0.3
50	0.4±0.2	0.7±0.5	30.2±0.2	66.4±0.3	27.7±0.6	54.4±0.6	42.4±0.8	42.77±0.3	21.1±0.7	52.2±0.5
100	0.8±0.5	1.3±0.3	44.2±0.5	78.3±0.4	31.6±0.2	61.3±0.1	55.2±4.0	66.29±0.4	23.5±0.4	71.7±0.3
500	0.4±0.4	1.6±0.4	72.5±0.4	85.5±0.2	52.4±0.2	79.9±0.4	90.5±7.0	71.79±0.2	41.8±0.2	85.2±0.5

All values are expressed as mean □ S.E.M (n=6)

Antioxidants (free radical scavengers) are chemicals that interact with and neutralize free radicals, thus preventing them from causing cellular damage in the biological system [14]. The body makes some of the antioxidants it uses to neutralize free radicals. These antioxidants are called endogenous antioxidants. However, the body also relies on external (exogenous) sources, primarily the diet, to obtain the rest of the antioxidants it needs. These exogenous antioxidants are commonly called dietary antioxidants. Fruits, vegetables, and grains are rich sources of dietary antioxidants. The in vitro antioxidant potential of amlodipine was assayed using DPPH photometric assay, while the in vivo antioxidant potential was evaluated using serum superoxide dismutase, catalase activity, and malondialdehyde level assay etc. The in vitro antioxidant assay of amlodipine revealed that it has a potent antioxidant activity comparable to vitamin C which was used as a reference standard. 2, 2-diphenyl-1- picrylhydrazyl (DPPH) is a dark-coloured crystalline powder composed of stable free radical molecules. In laboratory, it is used to monitor chemical reactions involving radicals, most notably antioxidant assay. The antioxidant compounds neutralize the free radical character of DPPH by transferring either electrons or hydrogen atoms to DPPH, thereby changing the colour from purple to the yellow coloured stable diamagnetic molecule diphenylpicrylhydrazine. The degree of discoloration indicates the scavenging potential of the extract or antioxidant in terms of hydrogen donating ability [15].

The in vivo antioxidant assay showed that the extract increased the activity of serum superoxide dismutase (SOD) and catalase and decreased the serum level of TBARS. Catalase is a ubiquitous enzyme that catalyzes the decomposition of hydrogen peroxide, a reactive oxygen species, which is a toxic product of both normal aerobic metabolism and pathogenic ROS production. The SOD catalyzes the dismutation of superoxide to hydrogen peroxide and oxygen, thereby reducing the likelihood of superoxide anion reacting with nitric oxide to form reactive peroxynitrite. The increased serum activities of catalase and SOD as observed in this study suggest that the extract has an in vivo antioxidant activity and is capable of ameliorating the effect of ROS in biologic system. Also, ROS react with all biological substance; however, the most susceptible ones are polyunsaturated fatty acids. Reactions with these cell membrane constituents lead to lipid peroxidation (LPO) [16]. Increased LPO impairs membrane

function by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptor [16]. Thiobarbituric acid reactive substance (TBARS) levels were measured as a marker of LPO and malondialdehyde (MDA) production. Malondialdehyde is an endogenous genotoxic product of enzymatic and ROS-induced LPO whose adducts are known to exist in DNA isolated from healthy human being [17]. In our study, the level of TBARS in the extract treated groups decreased in a dose dependent manner when compared to control. This decrease in the TBARS levels may indicate increase in the activities of glutathione peroxidase and hence inactivation of LPO reactions [18]. Some of the phytochemical constituents of the extract may be responsible for the antioxidant activity as demonstrated in our study. Flavonoids or bioflavonoids are a ubiquitous group of polyphenolic substances which are present in most plants, concentrated in seeds, fruit skin or peel, bark, and flowers [19]. Numerous studies have

shown that flavonoids possess potent antioxidant activities capable of scavenging hydroxyl radicals, superoxide anions, and lipid peroxy radicals.

Conclusion

In conclusion, the demonstrated in-vitro and in-vivo antioxidant effects of amlodipine may be the rationale behind some of its medical uses and also may be responsible for some of its pharmacological effects.

References

1. Park Chang-Gyu. Pleiotropic effect of 3rd generation L-type calcium channel antagonist. Circulation (Kr). Workshop, Spring 2004: 1-11.
2. Wandikayi C.. Matowe, Maryam Khaja, Sahar Nasser. Pleiotropic Effects of Calcium Channel Blockers: A Systematic Review.
3. Omran M.O. Alhamami, Jabbar Y. AL-Mayah, Najah R. AL-Mousawi, Hayder I. J. AL-Mousawi. Pleiotropic Effects of Antihypertensive Drugs. The Islamic University Journal (Series of Natural Studies and Engineering) 2007; 15(1): 1-12.
4. Kapanadze S, Dolidze N, Bakhutashvili Z, Chapidze L, Shengelia E. Pleiotropic effects of cardilopin (secondary coronary prevention). Georgian Med News. 2005; Feb; (119): 46-8.
5. Weintraub HS.; Basile J. The pleiotropic effects of antihypertensive agents: do they account for additional cardiovascular benefit beyond BP reduction? Southern Medical Journal 2008; 101(8): 818-823.
6. Burges RA, Dodd MG. Amlodipine. Cardiovasc Drug Rev 1990; 8: 25-44.
7. Neyler WG. Amlodipine. Springer, Berlin-Heidelberg-New York, 1993, 292 pp.
8. Steffen H.-M. Amlodipine - a third generation dihydropyridine calcium antagonist. J Clin Basic Cardiol 1999; 2: 45-52.
9. Flynn JT. Efficacy and safety of prolonged amlodipine treatment in hypertensive children. Pediatric Nephrol 2005; 20(5): 631-635.
10. Fleckenstein-Grűn G, Frey M, Thimm F, Fleckenstein A. Protective effects of various calcium antagonists against experimental atherosclerosis. J Hum Hypertens 1992; 6 (Suppl 1): S13-S18.
11. Mason RP, Marche P, Hintze TH. Novel vascular biology of third-generation L-type calcium channel antagonists. Ancillary actions of amlodipine. Arteriosclerosis, Thrombosis, and Vascular Biology (ATVB) 2003; 23: 2155-2163.
12. Mason RP, Trumbore MW, Mason PE. Interactions biophysiques membranaires de l'amlodipine et proprietes antioxydantes [Membrane biophysical interaction of amlodipine and antioxidant properties]. Drugs 2000; 59: 9-16.
13. Sathish V, Ebenezar KK, Devaki T. Synergistic effect of Nicorandil and Amlodipine on tissue defense system during experimental myocardial infarction in rats. Mol Cell Biochem 2003; 243: 133-8.
14. Jukema JW, van der Hoorn JW. Amlodipine and atorvastatin in atherosclerosis: a review of the potential of combination therapy. Expert Opinion on Pharmacotherapy 2004; 5/2: 459-468.

15. Berkels R., Taubert D., Bartels H., Breitenbach Th., Klaus W., Roesen R. Amlodipine increases endothelial nitric oxide by dual mechanisms. Pharmacol 2004; 70: 39-45.
16. Nayler WG. Review of preclinical data of calcium channel blockers and atherosclerosis. J Cardiovasc Pharmacol 1999; 33: S7-S11.
17. Yan AT, Yan RT, Liu PP. Narrative review: pharmacotherapy for chronic heart failure: evidence from recent clinical trials. Ann Intern Med 2005; 142: 132-145.
18. Vergouwen MD, Vermeulen M, de Haan RJ, Levi M, Roos YB. Dihydropyridine calcium antagonists increase fibrinolytic activity: a systematic review. J Cerebral Blood Flow & Metabolism 2007; 27: 1293-1308.
19. Mayer B. Translocation of endothelial nitric oxide synthase: another feat of amlodipine, a cardiovascular jack-of-all-trades. Cardiovasc Res 2006; 71: 411-413.