

In-vivo & In-vitro antioxidant Studies of Telmisortan Used in the Management of Hypertension and Diabetes Mellitus

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Abstract

Telmisartan is an angiotensin II type 1 receptor blocker and partial agonist of peroxisome proliferator-activated receptor gamma. Here, we investigated the protective capacity of telmisartan 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 telmisartan was determined by 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay and protein expression. Telmisartan exhibited antioxidant activity in the ABTS assay with the IC₅₀ value of 37.5 µM. Additionally, the cell viability, GSH level, SOD and CAT activity were notably elevated by telmisartan. These findings suggest that telmisartan has protective effects on Management of Hypertension and Diabetes Mellitus.

Keywords: Antioxidant; DPPH; SOD; LPO; Telmisartan.

Introduction Majority of The diseases/disorders are mainly linked to oxidative stress due to free radicals [1]. Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism [2, 3]. The most common reactive oxygen species (ROS) include super oxide anion (O₂·), hydroxyl radical (OH·), hydrogen peroxide (H₂O₂) peroxy radical (ROO·). The nitrogen derived free radicals are nitric oxide (NO·) and peroxynitrite anion (ONOO·)[4]. ROS have been implicated in over a hundreds of diseases states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and cardio vascular malfunction [5, 6]. Reactive oxygen species (ROS), such as superoxide anion, hydroxyl radicals, and hydrogen peroxide are generated in living organisms through numerous metabolic pathways. The oxidative damage caused by ROS may generate various diseases in the human body, such as aging, arthritis, cancer, inflammation, and heart diseases [7, 8, 9]. An increase in the incidence of macrovascular diseases in type 1 diabetes has long been recognized (1). The acceleration of macrovascular disease is partly due to the increased incidence of classical risk factors such as hypertension and dyslipidemia (2). However, recent evidence suggests that hyperglycemia also plays a significant role (3). The endothelium is a major organ involved in the development of cardiovascular disease (CVD), even in diabetes, and the presence of endothelial dysfunction has often been reported in diabetes and been found to be an independent predictor of future CVD (4). Several studies have shown that hyperglycemia induces endothelial dysfunction in both diabetic and non diabetic subjects (5–7); however, in type 1 diabetic patients, endothelial dysfunction has been reported to be present, even when normoglycemia is achieved (8, 9). Evidence indicates that hyperglycemia induces endothelial dysfunction through the generation of oxidative stress (10), which has been suggested to be the key player in the generation of both micro- and macrovascular diabetes complications (11). We have recently demonstrated that a near normalization of endothelial dysfunction can be achieved in type 1 diabetic patients by combining an optimal control of glycemia with the infusion of the antioxidant vitamin C (12). Several compounds already in clinical practice have the property of reducing the generation of oxidative stress (10). In particular, AT-1 receptor blockers have been shown to be effective (13, 14). Telmisartan is a selective angiotensin II type 1 receptor antagonist (angiotensin receptor blocker [ARB]) which does not affect the other receptor systems

involved in cardiovascular regulation. It is a more lipophilic compound than other ARBs, which facilitates oral absorption and benefits tissue and cell penetration.³ The drug is used for the management of hypertension, either as monotherapy or as combination therapy with other antihypertensive agents. It is considered as a first-line drug in mild-to-moderate hypertension with an excellent safety profile, and is used for the treatment of hypertension-related cardiovascular end-organ damage. A number of studies have shown that telmisartan has partial PPAR γ -agonist activity, activating 25–30% of the receptor compared to the full PPAR γ agonists (6–8).

Material & Methods Collection of drug

Telmisartan was collected from Crinova Healthcare Pvt Ltd. All the chemicals were collected from the School of Pharmacy, Dr. A.P.J. 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 Talmisotan in water for injection at a dose of 150-mg/kg bodyweight given by intraperitoneal 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. Radical scavenging activity of extracts was measured by slightly modified method of Brand-Williams *et al.* 1995

^[20]. 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 μ l extract solution in 1 cm path length disposable microcuvettes (final mass ratio of extracts with DPPH was approximately 3:1, 1.5:1, 0.75:1). Radical scavenging activity was calculated as follows: **Scavenging rate = $[(A_s - A_i)/A_s] \times 100$**

Where A_s is the absorbance of pure DPPH, A_i 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, 20 μ g riboflavin, 12 mM EDTA, 0.1 mg/3 ml NBT, added in that sequence. The reaction was started by illumination the reaction mixture with different concentrations (5-100 μ g/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 IC₅₀ were calculated.

ABTS radical scavenging assay

The ability to scavenge the ABTS radical cation (ABTS⁺) was determined according to Re *et al.*, 1999 and Cai *et al.*, 2004 ^[21]. The ABTS⁺ solution was prepared by the reaction of 7mM ABTS (5 mL) and 2.45mM (88 μ L) 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 \pm 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. The reducing power can be determined by the method of Athukorala et al. 2006. 1.0 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 FeCl₃ (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 (Green et al., 1982). 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% H₃PO₃) is added and absorbance of the chromophore formed is measured at 540 nm. Percent inhibition of the nitrite oxide generated is measured by comparing the absorbance values of control and test preparations. Curcumin, caffeic acid, sodium nitrite (Sreejayan, 1997), BHA, ascorbic acid, rutin (Jayaprakasha et al., 2004), BHT or tocopherol (Garrat, 1964) can be used as a positive control.

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 (Halliwell and Gutteridge, 1981). The model used is ascorbic acid-iron- EDTA model of HO[·] generating system. This is a totally aqueous system in which ascorbic acid, iron and EDTA conspire with each other to generate hydroxyl radicals. The scavenging ability for hydroxyl radicals is measured by the method of Kunchandy and Rao (1990). The reaction mixture (1.0 ml) consist of 100 µl of 2-deoxy-D-ribose (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 FeCl₃ (1:1 v/v), 100 µl of H₂O₂ (1.0 mM) and 100 µl ascorbic acid (1.0 mM) which is incubated at 37°C for 1 h. 1.0 ml of thiobarbituric acid (1 %) and 1.0 ml of trichloroacetic acid (2.8 %) are added and incubated at 100°C 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). The absorbance decrease is proportional to the antioxidant content (Benzie and Strain, 1996). 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 FeSO₄ equivalents per gram of sample. BHT, BHA, ascorbic acid, quercetin, catechin or trolox can be used as a positive control.

Oxygen radical absorbance capacity (ORAC) assay

The capacity of a compound to scavenge peroxy radicals, generated by spontaneous decomposition of 2, 2'-azobis, 2-amidinopropane dihydrochloride (AAPH), was estimated in terms of standard equivalents, using the ORAC assay (Prior et al., 2005). The method of Ou et al. (2002a, 2002b) is used for the estimation. 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 described by Kooy et al., 1994 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 authentic 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 µg) 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 °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 α-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. Fogliano et al. (1999) obtained 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 °C). The reaction takes place when 50 IL of the sample (a dilution of 1:10 in water) is added to 950 IL 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 FeCl₂.6H₂O. 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 °C) FRAP reagent 1 mL (10 mM) of 2,4,6 tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, 1 mL 20 mM FeCl₂.6H₂O, 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 °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 (Sapakal et al., 2008) 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 IL) 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 tubes are 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 (Paglia and Valentin, 1967; Yang et al., 1984). According to Wood (1970), 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 IL of 60 mM/L glutathione reductase solution (30 U/mL), 50 IL of 0.12 M/L Na₂S₂O₃, 0.10 of 0.15 mM/L Na₂EDTA, 100 IL of 3.0 mM/L NADPH, and 100 IL 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 IL 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 method can be used as described by Jocelyn (1972). The reaction mixture (1 mL) consisted of 0.1 N potassium phosphate (pH 6.5), 1 nM/L GSt, 1 M/L 1-chloro-2, 4-dinitrobenzene as substrate and a suitable amount of cytosol (6 mg protein/mL). The reaction mixture is incubated at 37 °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

This method is well described by Mccord and Fridovich (1969) and can be applied for determination of antioxidant activity of a sample. It is estimated in the erythrocyte lysate prepared from the 5% RBC suspension. To 50 IL 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 H₂O₂. 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, MgCl₂ and g- Glutamyl-p-nitroanilide in 0.05 M tris (free base), pH 8.2. The mixture is incubated at 37 °C for 1 min and the absorbance read at 405 nm at 1 min interval for 5 min. 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. The method illustrated by Kakkar et al. (1984) is as follows: 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 °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 IM 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. This method described by Okhawa (1979) is as follows: 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 °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 $1.56 \cdot 10^5 \text{ ML cm}^{-1}$.

LDL assay

The isolated LDL is washed and dialyzed against 150 mmol/L NaCl and 1 mmol/L Na₂EDTA (pH 7.4) at 4 °C. The LDL is then sterilized by filtration (0.45 μm), kept under nitrogen in the dark at 4 °C. LDL (100 μg of protein/mL) is incubated for 10 min at room temperature with samples. Then, 5 μmol/L of CuSO₄ is added, and the tubes are incubated for 2 h at 37 °C. Cu²⁺-induced oxidation is terminated by the addition of butylated hydroxytoluene (BHT, 10 μM). 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 as described by Buege and Aust, 1978; El-Saadani et al., 1989.

Result & Discussion In-Vitro Antioxidant activity of Telmisortan**Table 1(A): Antioxidant activity (%) of the Telmisortan**

Con. C ($\mu\text{g/ml}$)	DPPH		Nitric Oxide		Super Oxide		ABTS		ORAC		FRAP	
	Control	Test Drug	Control	Test Drug	Control	Test Drug						
5	9.6 \pm 1.0	45.6 \pm 0.3	7.65 \pm 1.5	14.1 \pm 0.5	22.8 \pm 0.5	24.1 \pm 0.1	21.2 \pm 0.3	6.25 \pm 0.2	20.8 \pm 0.5	22.1 \pm 0.2	23.2 \pm 0.4	7.25 \pm 0.2
10	17.9 \pm 0.2	77.9 \pm 0.7	10.4 \pm 0.8	57.6 \pm 0.6	22.9 \pm 0.4	35.4 \pm 0.1	32.4 \pm 0.5	34.81 \pm 0.1	21.9 \pm 0.4	33.4 \pm 0.4	33.4 \pm 0.5	33.81 \pm 0.1
50	34.4 \pm 0.1	94.9 \pm 0.2	42.4 \pm 0.1	99 \pm 0.1	29.6 \pm 0.7	65.1 \pm 0.2	43.9 \pm 0.9	55.47 \pm 0.3	23.6 \pm 0.7	61.1 \pm 0.4	42.9 \pm 0.8	54.37 \pm 0.4
100	62.5 \pm 0.6	96.7 \pm 0.2	71.3 \pm 0.3	99.2 \pm 0.1	33.7 \pm 0.4	82.8 \pm 0.1	56.4 \pm 1.0	96.09 \pm 0.2	31.7 \pm 0.4	81.8 \pm 0.2	55.4 \pm 1.0	95.09 \pm 0.2
500	86.1 \pm 0.5	96.9 \pm 0.1	98.7 \pm 0.1	99.6 \pm 0.1	73.6 \pm 0.2	95.1 \pm 0.3	92.9 \pm 1.0	98.69 \pm 0.2	72.6 \pm 0.2	92.1 \pm 0.4	93.8 \pm 1.0	99.59 \pm 0.2

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

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

Con. C ($\mu\text{g/ml}$)	Hydroxyl radical		Reducing power		Phospho Molybdenum		Peroxynitrite radical		DMPD	
	Control	Test Drug	Control	Test Drug	Control	Test Drug	Control	Test Drug	Control	Test Drug
5	9.9 \pm 1.0	44.6 \pm 0.3	8.65 \pm 1.2	15.1 \pm 0.5	21.8 \pm 0.6	23.2 \pm 0.1	20.2 \pm 0.3	7.25 \pm 0.2	21.8 \pm 0.5	23.2 \pm 0.1
10	18.8 \pm 0.2	77.9 \pm 0.7	11.4 \pm 0.5	53.6 \pm 0.6	23.8 \pm 0.6	36.5 \pm 0.1	33.3 \pm 0.5	35.81 \pm 0.1	23.9 \pm 0.4	34.3 \pm 0.1
50	36.6 \pm 0.1	93.5 \pm 0.2	44.3 \pm 0.1	99.4 \pm 0.1	28.7 \pm 0.8	66.3 \pm 0.2	44.7 \pm 0.9	56.47 \pm 0.3	28.4 \pm 0.7	66.2 \pm 0.2
100	64.5 \pm 0.6	95.4 \pm 0.2	73.2 \pm 0.4	99.5 \pm 0.1	34.7 \pm 0.3	83.9 \pm 0.1	57.3 \pm 1.0	95.09 \pm 0.2	34.6 \pm 0.4	83.8 \pm 0.1

500	88.3±0.5	97.8±0.1	99.1±0.2	99.7±0.1	75.9±0.1	96.3±0.3	94.8±1.0	99.69±0.2	74.7±0.2	96.2±0.3
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All values are expressed as mean \pm S.E.M (n=6)

In-Vivo Antioxidant activity of Telmisortan

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

Con. C (μ g/ml)	Ferric reducing		GSH		GSHPx		GSt		SOD	
	Control	Test Drug	Control	Test Drug	Control	Test Drug	Control	Test Drug	Control	Test Drug
5	16.80±10.84	45.4±0.3	0.65±1.5	0.70±0.5	0.8±0.5	0.1±0.1	21.2±0.3	6.25±0.2	17.1±0.6	20.5±0.1
10	27.4±10.2	67.5±0.7	0.41±0.8	0.76±0.6	0.9±0.2	0.4±0.1	33.2±0.5	31.81±0.1	20.2±0.1	31.3±0.3
50	34.5±11.1	74.2±0.2	0.48±0.1	0.81±0.3	0.12±0.3	0.1±0.2	41.3±0.9	43.27±0.3	22.3±0.2	64.2±0.4
100	42.1±13.6	86.5±0.2	0.56±0.3	0.89±0.4	0.17±0.5	0.8±0.1	53.5±1.0	51.19±0.2	35.7±0.3	82.8±0.2
500	56.3±14.5	91.3±0.1	0.70±0.1	0.96±0.6	0.2±0.1	0.9±0.3	91.1±1.0	78.59±0.2	715±0.2	96.2±0.5

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

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

Con. C (μ g/ml)	Catalase		GGT		Glutathione reductase		LPO		LDL	
	Control	Test Drug	Control	Test Drug	Control	Test Drug	Control	Test Drug	Control	Test Drug
5	0.6±1.0	0.7±0.3	6.6±1.5	12.1±0.5	12.8±0.3	21.2±0.3	20.2±0.3	5.25±0.2	18.2±0.5	20.2±0.2

10	0.9±0.2	0.9±0.7	8.4±0.8	47.6±0.6	21.9±0.2	34.4±0.5	31.4±0.5	31.61±0.1	20.3±0.4	37.4±0.3
50	0.4±0.1	0.9±0.2	32.2±0.1	69.3±0.3	28.6±0.4	55.1±0.2	40.2±0.9	45.37±0.3	21.6±0.7	53.1±0.5
100	0.5±0.6	1.0±0.2	41.2±0.4	79.2±0.4	32.2±0.4	62.2±0.2	51.2±2.0	64.19±0.4	22.7±0.4	70.8±0.3
500	0.1±0.5	1.2±0.1	78.6±0.2	83.6±0.2	53.1±0.1	75.3±0.6	89.3±4.0	72.49±0.2	42.6±0.2	83.1±0.5

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

This study found that telmisartan can exert a protective effect against ROS-mediated oxidative damage and apoptosis induced by high glucose in neuronal PC12 cells. Glucose induction of ROS is critical to the pathogenesis of diabetic neuropathy [20]. In addition, Hydroxyl radical is one of the highly ROS that causes the oxidative damage associated with diabetes [16]. Our results revealed that glucose enhanced ROS concentration in PC12 cells while simultaneously treating with telmisartan and glucose effectively attenuated ROS generation.

This finding corroborates with previous studies which have shown that telmisartan acted as efficient scavenger of ROS particularly hydroxyl radicals [3]. To evaluate the antioxidant capacity of telmisartan, the ABTS cation radical-scavenging assay, was employed [6]. The results clearly showed that telmisartan displays antioxidant properties, which is consistent with previous studies that have demonstrated excellent antioxidant activity for telmisartan [3]. Moreover, it has been suggested that telmisartan, an AT1receptor antagonist, inhibits intracellular oxidative stress, at least in part, in areceptor-independent manner, possibly owing to its lipophilic and antioxidant structure [24]. The chemical structure of telmisartan contains the benzimidazolic and benzoic groups that probably confer selective scavenging properties for hydroxyl radicals [3]. On the other hand, some studies have previously shown that PC12 cells derived from rat pheochromocytoma predominantly express AT2receptor [14,19]. Based on these observations, it is very likely that in PC12 cells, the neuroprotective effects of telmisartan are independent of AT1receptor inhibition. In the present study, we also found that NADPH-oxidase activity and protein expression of p47phox was significantly higher in HG-treated than control PC12 cells. p47phox is a cytosolic subunit of NOX and several reports support its importance in diabetes. For instance, it has been shown that deletion of p47 (phox) attenuates diabetes-induced glomerular injury and beta cell dysfunction [17]. Furthermore, superoxide production and neuronal death were also blocked in studies using mice or cultured neurons deficient in the p47phox subunit of NADPH oxidase [28]. Our results demonstrated that telmisartan pre- and co-treatment suppressed the upregulated p47phox, and NADPH oxidase activity. In agreement with our present findings, previous observations have also shown that ARBs decrease NADPH oxidase activation associated with oxidative stress and neuronal apoptosis [23]. ROS also is a well-known initiator of apoptosis in many cell types [20]. In this study DNA fragmentation assay, a hallmark of apoptosis, Bax/Bcl-2 index and cleaved caspase-3 expression were used for measurements of apoptotic cell death. Telmisartan prevented HG-induced increase in caspase-3 expression, DNA fragmentation in PC12 cells and decreased the ratio of Bax/Bcl-2. This ratio is supposed to dictate the relative sensitivity or resistance of cells to a wide variety of apoptotic stimuli [26]. Interestingly, telmisartan has the unique character of having both ARB and PPAR- α agonistic effect [10], and Fuenzalida et al. reported that PPAR- α up-regulates Bcl-2 in neurons [5]. Also, Tamami Haraguchi et al. suggested that telmisartan reduces neuronal apoptosis via a PPAR-dependent caspase-3 inhibiting mechanism [10]. We used the PPAR-antagonist GW9662 to examine the possible involvement of PPAR-activation in the protective effect of telmisartan. Results demonstrated that the beneficial effects of telmisartan were weakened by co-treatment with GW9662. Previous reports are in line with our current data [5, 10], which indicate that telmisartan diminishes HG-elicited apoptosis in neuronal PC12 cells, possibly, via down-regulation of Bax/Bcl-2 and caspase-3 expression. To mitigate cumulative burden of oxidative stress, cells generally utilize antioxidant defense systems and scavenge ROS. SOD and CAT are two important antioxidant enzymes [20]. In this regard, our present study indicated that PC12 cells treated with high glucose showed a marked rise in oxidative stress as evidenced by excessive ROS and MDA production, together with depletion of "endogenous antioxidant reserve," including GSH contents, SOD and CAT activity level. However, co-treatment with telmisartan significantly attenuated oxidative damage to PC12 under high glucose condition, as reflected by the augmentation of antioxidant defense system (CAT, SOD and GSH) with accompanying decrease in MDA and ROS levels. These results confirm the in vitro antioxidant activity of telmisartan. In summary, our results indicate that telmisartan plays a protective role against HG-induced cell death in PC12 cells in a dose dependent manner. This is possibly accomplished through diminution of NADPH oxidase activation and ROS formation in parallel

with the increases in the GSH level, SOD and CAT activity. Furthermore, the results show that PPAR- α activation is involved in the neuro protective effects of telmisartan. **References**

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