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Efficiency of mitochondrially targeted gallic acid in reducing brain mitochondrial oxidative damage

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Abstract

Oxidative stress is associated with mitochondrial impairments. Supplying mitochondria with potent antioxidants can reduce oxidative stress-induced mitochondrial impairment. Gallic acid can be used to reduce oxidative burden in mitochondria. In order to increase the bioavailability of gallic acid inside the mitochondria we synthesized mitochondrially targeted gallic acid and explored its preventive effects against sodium nitroprusside induced oxidative stress in isolated mitochondria. Our observations revealed an increase in oxidative stress,decrease in reduced glutathione in mitochondria and increase in the mitochondrial permeability pore transition due to sodium nitroprusside treatment. Pre-treatment of gallic acid and mitochondrially targeted gallic acid to sodium nitroprusside treated mitochondria not only significantly reduced the oxidative stress but also prevented mitochondrial permeability pore transition to a significant difference. Mitochondrially targeted gallic acid was found more effective in reducing oxidative stress and mitochondrial permeability pore transition than gallic acid. We conclude that mitochondrially targeted gallic acid can be used for preventing mitochondrial impairment caused by oxidative stress.

Key words: Mitochondrial impairment, Nitric oxide, Peroxynitrite, Gallic acid, Mitochondrially targeted gallic acid, Oxidative stress.

Introduction

Mitochondria play key roles in cellular bioenergetics and are termed as cell's biochemical powerhouse. Apart from this important function, mitochondria carry out several other crucial metabolic processes (1) including amino acid biosynthesis, fatty acid oxidation, urea cycle, lipid metabolism, homeostasis of cellular calcium ions and apoptosis (2). Therefore, it is unsurprising that mitochondrial dysfunction leads to variety of metabolic impairments such as reduced ATP production, impaired calcium buffering, accumulation of metabolic intermediates and increased generation of reactive oxygen species (ROS) (3) and reactive nitrogen species (RNS). Increased production of ROS and RNS can initiate a number of damaging effects in the organelle including lipid peroxidation (LPO) and protein oxidation of mitochondrial membranes. During many neurodegenerative conditions oxidative damage in the mitochondria can be referred as an etiological factor of brain degeneration (4).

The brain is highly susceptible to oxidative damage because of high metabolic rate, high oxygen consumption rate, and comparative scarcity of antioxidant enzymes (5). One of the most evident ways to reduce the oxidative damage in neurodegenerative disease is through the use of effective antioxidants. Although several conventional antioxidants such as vitamin E and vitamin C (6) and natural plant extracts such as Ginkgo biloba extract (7, 8) have been found to reduce mitochondrial oxidative damage in neurodegenerative diseases, but their efficiency is restricted because they do not accumulate within mitochondria (9). Thus, mitochondrially targeted antioxidants need to be developed which can easily pass through mitochondrial membrane (10) and preserve mitochondrial functions. Since mitochondria are the cytoplasmic organelles that are highly negatively charged so that the antioxidant compounds conjugated with mitochondrially targeted positively charged cations can be used to target mitochondria (11). In various studies including our present study lipophilic triphenylphosphonium ion (TPP) is used as a positively charged cation to target mitochondria (12, 13). TPP functions as an excellent vehicle to deliver antioxidants to mitochondria because of its property to rapidly permeate mitochondrial membrane and to accumulate inside mitochondria (14). This accumulation of TPP conjugated antioxidants inside mitochondria enhances the efficiency of antioxidants against oxidative stress (15).

Sodium nitroprusside (SNP) is a potent nitric oxide (NO) donor (16, 17) that reacts with ROS and produce cytotoxic RNS. A biochemical and pharmacological study have shown that plant derived polyphenols play important roles in improvement of mitochondrial functioning by neutralizing ROS (18, 19). Gallic acid (GA; 3,4,5-trihydroxybenzoic acid) is a naturally occurring polyphenol widely distributed throughout the plant kingdom (20, 21). A large family of plants such as strawberries, pineapples, bananas, lemons, red and white wines, gallnuts, sumac, witch hazel, tea leaves, oak bark, and apple peels contain GA (22, 23). GA is known to exert antioxidant, anti-bacterial, anti-viral, anti-inflammatory, anti-proliferative, antitumorigenic, antimutagenic and antimelanogenic activities via inhibition of tyrosine kinase activity (23 – 27). The antioxidant property of GA can prevent mitochondrial membrane permeability transition (MPT) and exert cyto-

protective role in brain cells (28). However, natural GA derived from plants has less significance to the multicellular systems because it lacks hydrophobicity which enables the antioxidant to reach the mitochondrial sites. Therefore, in the present study GA was chemically modified to specifically target the mitochondria. The results of the present study show that mitochondrially targeted GA (mt-GA) reduces brain mitochondrial nitric oxide and oxidative stress induced by SNP and thus protect the MPT.

Materials and methods

Chemicals

TPP, HCl, ethyl acetate, ethanol, sodium hydroxide (NaOH) were purchased from HiMedia Laboratories (Mumbai, India). Potassium dihydrogen orthophosphate (KH_2PO_4), dipotassium hydrogen orthophosphate (K_2HPO_4), metaphosphoric acid, sodium chloride and ethylene diamine tetra acetate (EDTA) were obtained from Merck, India. Streptomycin sulphate, GA, trichloroacetic acid (TCA), 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) and di-nitrophenyl-hydrazine (DNPH) were obtained from Sigma Chemical Company Inc. (St. Louis, MO, USA).

Experimental Animal

Adult Swiss albino mice (*Mus musculus albinus*) weighing 25-30 g were obtained from College of Veterinary Science and Animal Husbandry,Mhow, India. All studies using these mice were approved by the research committee of Vikram University in accordance with the International guidelines for the care and use of laboratory animals. All animals were housed at 25 ± 1°C with 12 hr light / dark cycle.

Mitochondrial preparation

The animals were euthanized by decapitation. Brains were rapidly excised and placed into ice cold isolation medium (0.25 M sucrose). Tissues were weighed, finely minced and homogenized in isolation medium (10% w/v) using glass homogenizer. Mitochondria were isolated from normal mice brain by conventional differential centrifugation method as described (29, 30).The pellet, i.e., the mitochondrial fraction, was re-suspended in 1 ml isolation buffer to obtain approximately 4 mg mitochondrial protein/ml. The experiments were performed immediately after purification of the mitochondria.

Synthesis of targeted antioxidant

Mitochondrially targeted GA was synthesized by covalent linkage of GA with a lipophilic cation, TPP as described (31, 32). TPP was reacted with a brominated precursor to obtain the lipophilicity. To synthesize targeted derivative of GA, a solution of lipophilic cation and the GA was refluxed and evaporated to obtained mt-GA.

Mitochondrial treatments

Mitochondria were incubated in a reaction mixture containing 120 mMKCl, 2mM phosphoric acid and 15 mM Tris. The experiment was performed in four groups. The first group served as control. In the second group, mitochondria were incubated with SNP (0.4 µM) for 30 minutes. In the third group, mitochondria were pre treated with five different concentrations of GA (i.e. 100 µmol to 500 µmol) for 15 minutes prior to incubating with SNP for 30 minutes. The incubation mixture of the mitochondria for the fourth group was the same except mt-GA was used in place of GA. Mitochondria were energized by pyruvate/malate (10/ 5 mM) before incubation with SNP, GA and mt-GA.

Sample preparation for assays

After incubation, different groups of mitochondria were assayed by using suitable buffers. The 0.05 M phosphate buffer was used for LPO assay. The phosphate buffer (100 mM; pH 7.4) containing 0.1% digitonin was used for protein carbonyl assay, and 0.1 M cold metaphosphoric acid containing 0.02% EDTA and 0.1 mM DTNB for reduced glutathione (GSH) assay.

Quantitation of NO

Nitric oxide was determined spectrophotometrically by measuring the accumulation of its stable degradation products, nitrite and nitrate. Nitrate was reduced to nitrite by incubating mitochondria in the presence of 0.1 unit/ml nitrate reductase, 50 mM NADPH, and 5 mM FAD for 15 min at 37 °C. NADPH is oxidized to avoid interference with the nitrite determination on completion of nitrate reduction and for this purpose samples were incubated with lactate dehydrogenase (10 units/ml) and sodium pyruvate (10 mM) for 5 min at 37 °C. All incubations were performed in red polypropylene tubes to protect the light-sensitive nitrate reductase. Total nitrite was then determined spectrophotometrically by using the Griess reaction as we have described (33).

Immunoblotting

Samples were immunoblotted against monoclonal anti-nitrotyrosine antibody (Alexis, San Diego, CA, USA) for detection of protein tyrosine nitration as we have described (30, 34).

Lipid peroxidation (LPO)

LPO was determined by measuring thiobarbituric acid reactive substance (TBARS) in terms of malonaldehyde equivalent (MDA) using the molar extinction coefficient of 1.56×10^5 $min^{-1}.cm^{-1}$ as described(35). Briefly, 0.1 ml of mitochondrial sample was mixed with 0.2 ml of 8.1% SDS, 1.5 ml 20% glacial acetic acid, and 1.5 ml of 0.8% thiobarbituric acid (TBA). Following these additions, tubes were mixed and heated at 95 °C for 60 min in a water bath, and cooled under tap water before mixing with 1 ml distilled water and 5 ml mixture of n-butanol and pyridine (15: 1). The mixture was centrifuged at 2,200 x g for 10 min. The TBARS value was determined by measuring the absorbance of upper organic layer at 532 nm. The results were expressed as nmol TBARS/mg protein.

Protein Carbonyl Content

Protein carbonyl content was assessed according to Levine *et al.* (36) with some modifications. Briefly, in a 0.5 ml of mitochondrial sample, streptomycin sulphate solution (10% w/v) is added to a final concentration of 1% to precipitate DNA.The solution is mixed and left to stand for 15 min at room temperature, and then it is

centrifuged at 2,800 x g for 10 min at room temperature. The supernatant is removed and 0.8 ml is divided equally between two test tubes. Now DNPH (1.6 ml, 10 mM in 2 M HCl) is added to 1 tube and 1.6 ml of 2M HCl to the other tube. The tubes are then incubated for 1 hr. at room temperature and then the protein is precipitated by adding an equal volume of 20% trichloroacetic acid (TCA) and leaving them for 15 min. The protein is centrifuged at 3,400 x g for 10 min at room temperature and the pellet is washed with 1.5 ml of an ethyl acetate: ethanol mixture (1:1, v/v) to remove excess DNPH. The final protein pellet is dissolved in 1.25 ml of 6 M guanidine hydrochloride and the absorbance of both DNPH and HCl solution were measured at 370 nm in a Perkin-Elmer UV spectrophotometer. A standard curve was obtained by using bovine serum albumin (BSA) and included in each assay to determine linearity and the extent of derivatization. The results of carbonyl contents were expressed in terms of nmol carbonyl group/mg protein.

Reduced glutathione (GSH)

The GSH content of tissue homogenates was quantitated as described (37), involving the spectrophotometric assessment of the formation of 5-thio-2-nitrobenzoate from DTNB in the presence of NADPH and glutathione reductase. Briefly, 0.5 ml mitochondrial samples were mixed with1.5 ml of 0.1 M ice cold metaphosphoric acid and centrifuged at 16,000 x g for 15 min at 4 ºC. The supernatant (0.5 ml) was equilibrated with 4 ml of ice cold 0.1 mM solution of DTNB in 0.1 M phosphate buffer pH 8.0 and the optical density was obtained at 412 nm in a Perkin-Elmer UV-Spectrophotometer. A calibration curve was prepared by using GSH as standard.

Determination of membrane permeability transition (MPT; mitochondrial swelling)

Isolated mitochondria (0.5 mg protein) were re-suspended in 2 ml Hank's balanced salt solution (HBSS; 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, 132 mMNaCl, 10 mM HEPES, pH 7.3, and pyruvic acid (10 mM) / mallate (5 mM). MPT was induced in the mitochondria by adding 3µM $CaCl_2$.Experiments were performed on isolated mitochondria in different test tubes at room temperature with SNP (1.6 µmol), SNP + GA (100 µmol to 500 µmol) and SNP + mt-GA (100 µmol to 500 µmol). Mitochondrial swelling was estimated from the changes of light scattering at 540 nm measured in mitochondrial suspensions (0.5 mg of protein in 2 ml) as described (38).

Assay of Total Protein

Total protein content of mitochondrial fractions was measured by Folin-phenol reaction as described by Lowry *et al.* (39). A standard curve of BSA was included in each assay to determine linearity and to measure the extent of derivatization.

Statistical Analysis

All data are expressed as mean ± S.E. Statistical comparisons were made relative to the appropriate control group using one way analyses of variance (ANOVA). The 0.05 level was selected as point of minimal statistical significance.

Results

Oxidative stress in isolated mitochondria was induced by treating mitochondria with an NO donor, SNP. Fig. 1A, shows an increase in NO in the mitochondria treated with SNP. Prior incubation of mitochondria with different concentrations of GA and mt-GA resulted in a significant reduction of NO level in comparison to SNP treated mitochondria.

To elucidate the effects of increased NO on mitochondrial peroxynitrite formation and oxidative stress, we performed western blot of nitrated proteins. As shown in Fig. 1B, mitochondria incubated with SNP, express detectable levels of protein tyrosine nitration. After pre-incubation of mitochondria with GA, a decrease in mitochondrial protein tyrosine nitration was obtained. Pre-incubation of mitochondria with mt-GA resulted in further decline in mitochondrially nitrated proteins.

Increased oxidative stress in the mitochondria causes peroxidation of membrane lipids. So next we tested the effect of SNP on mitochondrial LPO. As shown in Fig. 2A, the LPO was significantly increased by incubating mitochondria with SNP. The level of LPO increased from 0.18 ± 0.01 nmol TBARS/mg proteins in control to 0.96 ± 0.04 nmol TBARS/mg proteins in SNP-treated mitochondria. Pre-treatment of brain mitochondria with different concentration of GA and mt-GA deliberated the significant reduction of LPO as compared to group that were treated with SNP alone. These results suggest that mt-GA is more effective than GA in reducing SNP-induced LPO.

As mitochondrial proteins are considered to be one of the targets of oxidative damage, so testing the effect of SNP on protein carbonylation was mandatory, and our results revealed an increase in protein carbonyl con-

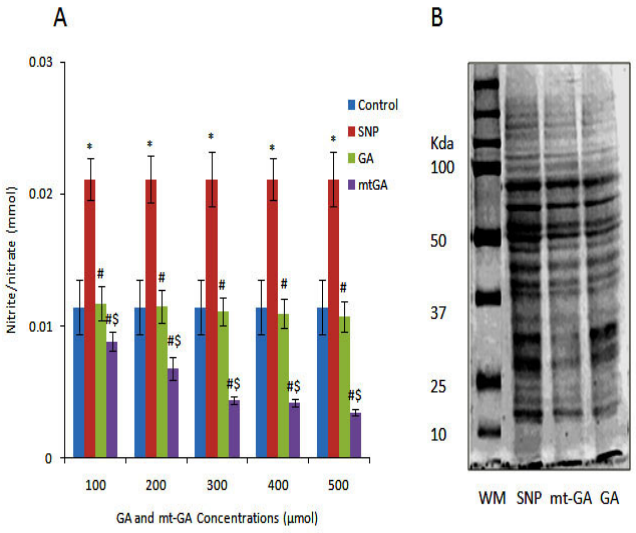

Figure 1. A: Showing nitric oxide levels (nitrite/nitrate; mmol) in mice brain mitochondria treated with SNP (0.4 µmol), SNP (0.4 µmol) + GA (100 µmol to 500 µmol), SNP (0.4 µmol) + mt-GA (100 µmol to 500 µmol). *Significantly different from control ($p<0.05$; n=5), #significant difference from SNP treated mitochondria ($p<0.05$; n=5). $Significant difference from GA ($p<0.05$; n = 5). **B:** Tyrosine nitration of mitochondrial proteins in mice brain mitochondria treated with SNP (0.4 µmol), SNP (0.4 µmol) + GA, SNP (0.4 µmol) + mt-GA.

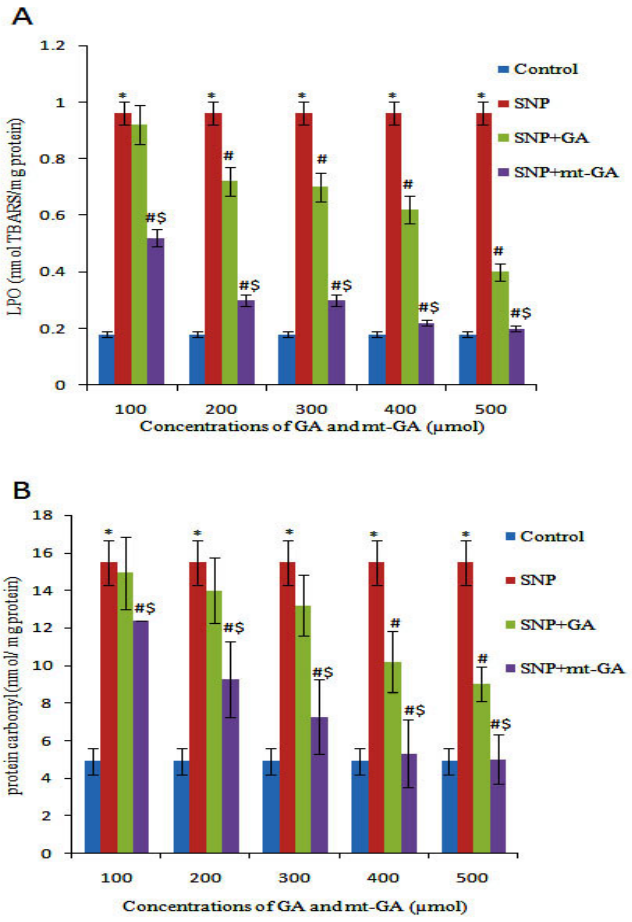

Figure 2. A: Showing lipid peroxidation (LPO; nmol TBARS/mg protein) in mice brain mitochondria (0.1 ml) treated with SNP (0.4 µmol), SNP (0.4 µmol) + GA (100 µmol to 500 µmol), SNP (0.4 µmol) + mt-GA (100 µmol to 500 µmol). *Significantly different from control ($p<0.05$; n=5), #significant difference from SNP treated mitochondria ($p<0.05$; n=5). $Significant difference from GA ($p<0.05$; n = 5). **B:** Showing protein carbonyl (nmol /mg protein) in mice brain mitochondria (0.5 ml) treated with SNP (0.4 µmol), SNP (0.4 µmol) + GA (100 µmol to 500 µmol), SNP (0.4 µmol) + mt-GA (100 µmol to 500 µmol). *Significantly different from control ($p<0.05$; n=5), #significant difference from SNP treated mitochondria ($p<0.05$; n=5). $Significant difference from GA ($p<0.05$; n = 5).

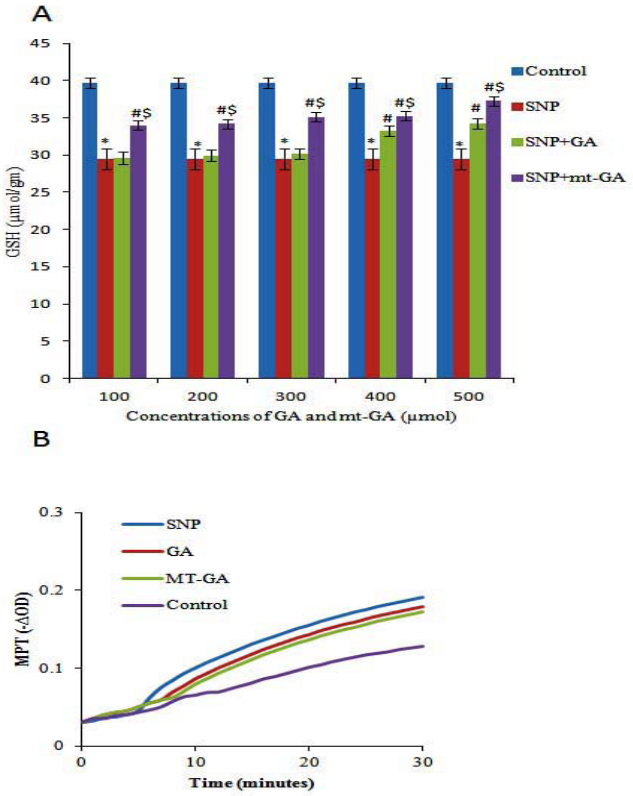

Figure 3. A: Showing reduced glutathione (GSH; µmol/g) in mice brain mitochondria (0.5 ml) treated with SNP (0.4 µmol), SNP (0.4 µmol) + GA (100 µmol to 500 µmol), SNP (0.4 µmol) + mt-GA (100 µmol to 500 µmol). *Significantly different from control ($p<0.05$; n=5), #significant difference from SNP treated mitochondria ($p<0.05$; n=5). $Significant difference from GA ($p<0.05$; n = 5). **B:** Showing mitochondrial permeability transition (MPT; -ΔOD) in mice brain mitochondria (0.5 mg of protein) treated with SNP (0.4 µmol), SNP (0.4 µmol) + GA (500 µmol), SNP (0.4 µmol) + mt-GA (500 µmol).

tent in SNP treated mitochondria (15.49 ± 1.20 nmol/ mg protein) compared to control (4.92 ± 0.70 nmol/mg protein). The mitochondria pre-incubated with GA and mt-GA showed lower protein carbonyl content compared to mitochondria that were only treated with SNP. As shown in Fig. 2B, the mt-GA decreases protein carbonylation more competently than GA.

Mitochondrial GSH is the most abundant thiol that serves as major reducing agent against oxidative damage. As shown in Fig. 3A, SNP treatment caused a significant ($p < 0.05$) decrease in GSH levels (29.53 ± 1.4 µmol/g) in the mitochondria as compared to control (39.72 ± 0.72 µmol/g). Pre-incubating mitochondria with GA and mt-GA increased the level of GSH.However, as shown in the Fig. 3A, mt-GA was most efficacious than GA in preserving the GSH content in SNP treated mitochondria.

MPT is a non-selective mitochondrial inner membrane permeabilization and, a catastrophic event preceded by oxidative stress. Increased MPT is regarded as the primary mediator of cell death. Therefore, we measured the MPT in mitochondria treated with SNP. Our results illustrate that treatment with SNP increases the MPT (Fig. 3B). Pre-incubation of mitochondria with GA and mt-GA prevents the MPT induced by SNP. As shown in Fig. 3B, mt-GA was more effective in prevention of MPT than GA.

Discussion

Mitochondria are the major source of ROS and, thus may become easy target of damage caused by various oxidants and oxidative metabolism. Mitochondrial ROS production appears to be an essential mediator of many age related diseases. One of the most prominent mechanisms to counter the oxidative stress and mitochondrial impairment is through the use of effective antioxidants. Supplying mitochondria with effective lower or higher concentration of antioxidants may have beneficial effects under mild or extreme oxidative stress conditions. GA, a plant polyphenol derived from various fruits and vegetables that exhibits antioxidant activity *in vivo* and *in vitro* (25), may be a reliable antioxidant to neutralize ROS in the mitochondria. To enhance the efficiency of GA against ROS in mitochondria, in the present study we synthesized mt-GA that can rapidly enter and accumulate inside the mitochondria and reduce oxidative stress and mitochondrial impairment. In order to induce oxidative stress under laboratory conditions the mito-

chondria were incubated with SNP, a potent NO donor. NO donated by SNP, combines with superoxide anion and form peroxynitrite that eventually contributes to oxidative stress (40) in the mitochondria. In addition to increasing oxidative stress the SNP are also used as potent vasodilators in arterioles and venuols. Our study showed an increase in NO level in the isolated brain mitochondria incubated with SNP alone, which consequently increased the oxidative stress. The increased mitochondrial oxidative stress finally increased MPT. Prior incubation of mitochondria with GA and mt-GA ameliorated the toxic effects of SNP, represented by decline in LPO and protein carbonyl content. mt-GA was more efficacious in reducing oxidative stress than GA.

SNP is a NO donor therefore first we tested effect of SNP on mitochondrial NO. Our results show that SNP increases the level of NO in brain mitochondria that was significantly decreased by GA and mt-GA. The mt-GA was more effective in ameliorating the levels of NO than GA, suggesting that mt-GA availability exceeds the level of GA in the mitochondria.

To test whether elevated NO levels in brain mitochondria cause oxidative stress, we determined mitochondrial protein tyrosine nitration. As shown in the Fig.1B, incubation of mitochondria with SNP increases mitochondrial protein tyrosine nitration. Nitration of tyrosine affects various mitochondrial protein functions. Accumulation of nitrotyrosine indicates the imbalance between formation of RNS and antioxidant defense mechanisms (41) in mitochondria. Prior incubation of mitochondria with GA and mt-GA decreases protein tyrosine nitration that suggests modulating effect of GA and mt-GA on nitrated proteins.

Mitochondrial membrane lipids are very susceptible to oxidation caused by ROS and RNS (42). LPO has potential applications as biomarker of oxidative stress caused by peroxidation of lipids (43). In our study mitochondria incubated with SNP alone showed increased levels of LPO, incubation of mitochondria with GA and mt-GA prior to SNP treatment showed a significant decrease in the level of LPO. The mt-GA was more effective than GA in preventing SNP-induced increase of LPO. Our observations are clearly consistent with previous studies on various cell types showing the effectiveness of GA and mt-GA against various drug toxicities. It has been shown that GA reduces streptozotocin induced LPO in hepatic cells (44) and brain cells (45). In addition, GA has been found to reduce LPO in oxytetracycline induced nephrotoxicity (46) and in left ventricular tissue of rat heart infused with Advanced Glycation End Products (47).

Protein carbonylation is the oxidation of proteins promoted by oxidative stress. Increased levels of protein carbonyls are widely used as markers of oxidative damage caused by peroxynitrite species that intervene with the redox signaling processes of mitochondria (48, 49). In the present study the protein carbonyl levels were significantlyincreased in brain mitochondria treated with SNP alone. Incubation of mitochondria with GA and mt-GA prior to the SNP treatment significantly reduced the protein carbonyl level. The mt-GA proved more effective in reducing the level of protein carbonyl than GA. Similar to our study the preventive effects of GA against protein carbonyl were also reported in left ventricular tissue of rat heart that was infused with Advanced Glycation End Products (47).

Oxidative stress induced MPT is an important biomarker of mitochondrial impairment. MPT occurs due to the opening of a mitochondria permeability transition pore in the inner mitochondrial membrane. In the present study, increased MPT was observed when isolated mitochondria were incubated with SNP. This suggests occurrence of mitochondrial functional impairment due to the SNP treatment. Antioxidant can prevent MPT and polyphenols like GA are considered as effective antioxidants for the prevention of oxidative stress. GA and mt-GA served as effective antioxidants against SNP-induced MPT. The mt-GA was found more effective in preventing MPT than GA.

GSH is the most abundant mitochondrial antioxidant and critical for regulating mitochondrial redox environment. Increased level of oxidized disulphide glutathione GSSG reflects oxidized environment (50). Depletion of GSH can disturb redox homeostasis and impair mitochondrial functions. The present study shows that SNP significantly obstruct this antioxidant defense machinery of mitochondria. GA and mt-GA significantly increased the level of GSH. According to Mitic *et al.* this effect of GA is due to its hydrogen donating nature (51) that retains GSH in its reduced form.

We conclude that SNP induces oxidative stress and mitochondrial impairment and that GA and mt-GA served as effective antioxidants in lowering SNP-induced mitochondrial oxidative stress and mitochondrial impairment. Although the effectiveness of mt-GA was not tested *in vivo* in the present study but in isolated brain mitochondria the mt-GA was found more effective in preventing oxidative burden and mitochondrial impairment than GA. Thus, targeting mitochondria with mitochondrially targeted antioxidants can be the preferred strategy to reduce oxidative burden in these cellular power houses.

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