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Nephroprotective effect of arjunolic acid on cytosolic lipids and mitochondrial enzymes against cyclosporine induced renal complications in rats

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Abstract

Cyclosporine (CsA) has considerably modified the graft survival in solid organ and bone marrow transplantations. Renal toxicity is the major adverse effect of chronic CsA administration. Deterioration of renal function and renal histopathology are the basic elements of the diagnosis.

The present study aimed at investigating the protective effects of Arjunolic acid (10mg/kg body weight) against Cyclosporine (25mg/kg body weight) on the activities of TCA cycle enzymes - SDH (succinate dehydrogenase), MDH (malate dehydrogenase) Aconitase, Citrate synthase and examined the activities of respiratory chain complexes I, II, III, and IV. Mitochondrial enzymes of NADH DH, ICDH and αKG DH activities in cytosol, Lysosomal enzymes of β-Cathepsin, β-Glucuronidase, β-Galactosidase, β-D-N-Acetyl Glucosaminidase in cytosol, lipids of Cholesterol, Triglycerides (TG), Free fatty acids (FFA), and phospholipids were estimated. Urea, Uric acid, and Creatinine were also estimated in 24hrs urinary sample. Results showed that CsA caused a marked decrease in the level of SDH, MDH, Aconitase, Citrate synthase, Respiratory chain complexes of I, II, III, IV, NADH DH, ICDH, αKG DH, in the kidney mitochondrial tissue homogenate and also urea, uric acid and creatinine in urine sample. The lysosomal enzymes of β-Cathepsin, β-Glucuronidase, β-Galactosidase, β-D-N-Acetyl Glucosaminidase in cytosol, lipids of Cholesterol, Triglycerides (TG), Free fatty acids (FFA), and phospholipids were found to be increased in the cytosol of kidney tissue homogenates. But AA successfully prevented the alterations of these effects in the experimental animals. Our study demonstrated that AA could protect the mitochondrial kidney tissues against CsA induced oxidative stress probably by increasing antioxidative defense activities.

Keywords: Cyclosporine, Nephrotoxicity, Mitochondria, Cytosol, Arjunolic acid.

1. Introduction

Cyclosporine exposure on renal dysfunction after cardiac transplantation, two large studies demonstrated that no relationship between cyclosporine concentration and the decline in renal function measured as the slope of serum creatinine as a function of time or by serial GFR measurements [1,2], and a case-control study [3] showed that cyclosporine doses and trough levels in the 24 heart transplant patients who developed end-stage renal disease were not different from those of patients who maintained stable renal function.

CsA binds an immunophilin molecule named cyclophilin-D, a peptidylpropyl cis-trans isomerase endowed in cell cytoplasm and in mitochondria [4-6]. CsA protects respiratory chain integrity preventing mitochondrial swelling and limiting apoptotic stress. An elegant model of '*in vivo*' CsA protection from mitochondrial damage was developed by Mott *et al*. [7]. Markers of tubular dysfunction like urinary N-acetyl β-D-glucosaminidase, which is a lysosomal enzyme that originates from proximal tubular microsomes, have also been associated with reduced GFR induced by cyclosporine A [8, 9].

Cells continuously receive survival or death signals from the local microenvironment [10]. In the kidney, death through apoptosis is a physiological process in nephrogenesis as well as in maintenance of tissue homeostasis. During drug exposure, apoptosis may become a double-edged weapon, causes tissue loss and organ dysfunction, also contributes to clear off intoxicated cells and to control compensatory proliferative responses. A large number of drugs are known to induce renal cell apoptosis in cell Culture or in vivo, and this is associated with renal dysfunction. In general, apoptosis occurs at low levels of drug exposure, whereas necrosis requires higher doses [11-13].

Mitochondria is the garden of cell death and plays crucial role in regulating cell death pathways [14, 15]. ROS are predominantly produced in mitochondria and plays important role in apoptosis. Mitochondria is the most

important cell organelle in research because of its crucial role as a regulator of energy balance [16]. Various NAD/NADP-linked enzymes are intricately involved in the maintenance of the reduced redox state in mitochondria in order to provide the reducing power to generate ATP *via* oxidative phosphorylation [17, 18]. There is a huge understanding between cytosolic and mitochondrial enzymes to maintain the favorable conditions to regulate various biological functions.

Mitochondrial antioxidant defense mechanisms counteract these reactive species, but are exhausted when there is inordinate production of free radicals leading to disruption of mitochondrial membrane [19]. Mitochondria involves diverse processes that modulate cell operation like cell cycle regulation and apoptosis. Inclusion bodies are sometimes noted in the tubular cytoplasm in association with cyclosporine use. Ultrastructurally, these inclusion bodies represent giant mitochondria and autolysomes [20, 21]. However, giant mitochondria are nonspecific and occur in a variety of conditions, including ischemic injury, and are often found in preimplantation donor biopsies, which limit their diagnostic value [22]. It is currently not known what triggers the formation of these giant mitochondria, but it is clear that cyclosporine has important effects on mitochondrial functioning [23, 24].

Despite the fact that the major respiratory pathways were elucidated decades ago, relatively little is known about their regulation and control. Respiration can be divided into three main pathways: glycolysis, the mitochondrial tricarboxylic acid (TCA) cycle and mitochondrial electron transport.

Glucose metabolism, the citric acid cycle, and oxidative phosphorylation are central biochemical pathways in cellular energy metabolism. Hyperglycemia glycates ICDH which results in reduced activity [25]. *α*-KGDH could be a crucial target of reactive oxygen species (ROS) and being an important regulatory site in the mitochondrial metabolism could play a key role in the bioenergetic deficit evolving oxidative stress. One of the mitochondrial proteins selectively targeted by 4-Hydroxynonenal (HNE) is the FAD-containing subunit of SDH and results in decreased oxygen consumption in the presence of succinate and in complex II activity as reported by Lashin *et al*. [26].

Mitochondria produce the majority of cellular ATP through oxidative phosphorylation and carry out several other crucial metabolic processes [27]. The oxidative phosphorylation is the major source of ATP in mammalian cells relying on aerobic energy metabolism. The electron transport chain (ETC) consists of: a) three major protein assemblies: mitochondrial respiratory complex I (NADH: ubiquinone oxidoreductase), complex III (ubiquinol: ferricytochrome *c* oxidoreductase) and complex IV (cytochrome *c* oxidase), which build up transmembrane electrochemical potential (y) by coupling their electron transfer activities to H+ translocation from the matrix (negative) to the outer (positive) side of the inner mitochondrial membrane, and b) two mobile carrier molecules, ubiquinone (Coenzyme Q) and cytochrome *c*. The electrochemical gradient is then utilized for ATP synthesis by complex V (ATP synthase). Succinate-Q oxidoreductase, which is part of the tricarboxylic acid cycle, is also assigned to the respiratory chain as complex II. All the respiratory chain complexes are made up of numerous polypeptides and contain a series of different protein-bound redox coenzymes, including flavins (FMN or FAD in complexes I and II), iron-sulfur clusters (in I, II, and III), and hemes (in II, III and IV) reviewed in [28].

The use of medicinal plant either as a single drug or in combination is increasing in the health care of human being. Medicinal plants can be important source of previously unknown chemical substances with potential therapeutic effect. *Terminalia Arjuna* (TA) bark is commonly known as Arjuna bark or Arjuna and abundantly available throughout India. TA contains 15 % tannin, tritepenoid saponins, flavonoids, calcium, aluminium and magnesium salts along with colouring matter and sugars are the other constituents of arjun. TA possess wide-ranging therapeutic properties and has the potential to treat numerous medical conditions, especially those pertaining to the heart and circulation system. TA considered to be an excellent hypolipidemic, anticoagulant, hypocholesteremic, antihypertensive, antiviral, antithrombotic, antifungal and antibacterial properties. The therapeutic properties of the tree regarding cardiovascular health are basically attributed to triterpenoids enclosed by the arjuna. Similarly, flavonoids and tannins naturally present in the herb possess anticancer properties.

The effect of a compound formulation (Abana) containing *Terminalia arjuna* 30 mg per tablet was studied in isoproterenol-induced myocardial necrosis in rats. Increase in serum CPK, SGOT, SGPT following myocardial necrosis were significantly reversed by Abana. The drug also showed 90% protection against reduction in glycogen levels in ischemic rats. The beneficial effect of Abana was further evident by reduction in mitochondrial enzymes such as α-kG and succinate dehydrogenase (SDH) by 44% and 48%, respectively [29]. Effect of arjunolic acid derived from *Terminalia arjuna* (15 mg/kg body weight) on antiplatelet activity, electrocardiographic changes, serum marker enzymes, antioxidant status, lipid peroxide and myeloperoxidase (MPO) were measured and compared with the acetyl salicylic acid (ASA) in rats subjected to isoproterenol challenge. The drug was given intraperitoneally before and after isoproterenol administration. Arjunolic acid treatment prevented the decrease in the levels of SOD, CAT, glutathione peroxidase, ceruloplasmin, tocopherol, reduced glutathione (GSH), ascorbic acid, lipid peroxide and MPO. Cardioprotection conferred by arjunolic acid could possibly be due to the protective effect against the damage caused by myocardial necrosis [30].

The purpose of this study was to gain the protective effect of Arjunolic acid in the specific targets of endogenously generated mitochondrial oxidative stress in the kidney of cyclosporine induced nephrotoxicity.

2. Materials and Methods

2.1 Extraction of Arjunolic Acid

The extraction of Arjunolic acid was carried out by following the methods of King *et al* [31] and Bag *et al*., [32] Briefly, after collection the bark of *Terminalia Arjuna* was cut into small pieces, dried and ground into powder (1 kg) which was then extracted with petroleum ether (20 h) to remove greasy non polar material. After removing the petroleum ether, the crude material was subsequently extracted with diethyl ether (40 h). The crude material was filtered off and the mother liquor was concentrated under reduced pressure. Next the compound was subjected to separation on silica gel column. Elution with chloroform: methanol and the arjunolic acid were obtained in the fractions were pooled & tested the identity of the presence of AA by TLC and colour reaction with anisaldehyde. The extract was lyophilized. The purity of the compound [Arjunolic acid] has been checked by using standard tool like NMR and IR. The extract containing Arjunolic acid is indicated as Arjunolic acid (AA).

2.2 Animal Model

Male albino rats of wistar strain weighing (180±20g) were obtained from the laboratory animal maintenance unit, Saveetha University, Vellapanchavadi, Chennai. The animals were acclimatized to the laboratory conditions for a period of one week. They were maintained at an ambient temperature of 25 ± 2^0 and were given standard rat feed and water *ad libitum*. The experiments were conducted according to the ethical norms approved by ministry of social; justice and empowerment, Government of India and Institutional Animal Ethics Committee guidelines (IAEC No. Biochem BWC.006 2009).

2.3 Experimental Design

The following groups of animals will be used for the present study. The rats will be divided into four groups (n=6) in each group.

Group I: Control treated with vehicle alone.

Group II: Cyclosporine (CsA) induced orally (25 mg/kg body wt) for a period of 21 days.

Group III: Cyclosporine A induced (25mg/kg body wt) + ajrunolic acid (10mg/kg body Wt) orally for a period of 21 days.

Group IV: Arjunolic acid treated orally (10mg /kg body weight) for a period of 21 days.

2.4 Collection of samples for Biochemical Analysis

After the experimental period, the animals were anaesthetized by intraperitonial injection of ketamine (30mg/kg B.Wt.) and sacrificed by cervical decapitation. Blood was collected and the kidney tissue was washed in physiological saline to remove blood clot and other tissue materials. The 24hrs urinary samples were collected for the analysis.

2.5 Preparation of kidney homogenate

After sacrifice kidney, samples were blotted to dryness. From these, a piece weighing about 100mg was taken and homogenized at 4^0c in Tris HCL buffer(0.1M, pH7.4).The tissue homogenate was centrifuged at 2500rpm for 30 minutes. The resultant supernatant was kept under refrigeration until further biochemical analysis. All the assay procedures were carried out within 24hrs after sample collection.

2.6 Isolation of Mitochondria

Mitochondria were isolated by the method of Johnson and Lardy (1967) [33]. The kidney tissue was homogenized in ice-cold sucrose (0.25 M). This homogenate was centrifuged at 600 x g for 10 minutes. The supernatant fraction was decanted and saked. The pellet containing cell debris and tissue fragments were discarded. The supernatant was then centrifuged in a refrigerated centrifuge at 10,000 x g for 5minutes. The pellet was taken as mitochondria and the supernatant was discarded. The mitochondrial pellet was suspended in 0.25 M sucrose containing 10 mM Tris-HCl (pH 7.4) and 1mM EDTA to a known volume (2ml).

2.7 Isolation of Lysosomes

Lysosomal fractions were isolated by the method of Wattiaux *et al*., (1977) [34]. Kidney was homogenized in 0.25 M sucrose solution. The homogenate was filtered and centrifuged at 3000 x g for 10 minutes in a refrigerated centrifuge. The pellet was removed and rehomogenised and resuspended as before. The supernatants were combined and centrifuged again at 15,000 x g for 20 minutes. The lysosomal pellet was suspended in 1.15 % KCL homogenized and used for the estimation of enzymes and lipid peroxides.

2.8 Biochemical Analysis

Urea was determined by the method of Geyer and Dabich (1971) [35]. Creatinine was estimated by the method of Broad and Sirota, (1948) [36] using Jaffe's color reaction. Uric acid was estimated according to the method of Caraway (1963) [37]. The purity of the obtained mitochondrial fraction was evaluated by enzymatic assay according to the method

of Slater and Bonner (1952) [38]. The activity of MDH was assayed by the method of Mehler *et al*., (1948) [39]. Citrate synthase was measured by the method of Srere (1969) [40]. Aconitase was assayed by the method of Racker (1950) [41]. Complex I activity was measured following the decrease in absorbance due to the oxidation of NADH at 340 nm with reference set at 425 nm using the method of Birch-Machin *et al*., (1994) [42]. Complex II activity was measured by following the reduction of 2, 6-dicholorophenol indophenol (DCPIP) at 600 nm according to the method of Birch-Machin *et al.*, (1994) [42]. Activity of complex III was measured following the reduction of cytochrome c Fe^{3+} by decylubiquinol by the method of Krahenbuhl *et al*., (1994) [43]. Complex IV activity was measured following the oxidation of cytochrome c Fe^{2+} by the method of Smith (1955) [44]. The activity of NADH-Dehydrogenase was determined according to the method of Minakami *et al*., (1962) [45]. The activity of isocitrate dehydrogenase (ICDH) was assayed by the method of King (1965) [46]. The activity of α-KGDH was estimated according to the method of Reed and Mukerjee (1969) [47]. Cathepsin- D activity was measured by the method of Sapolsky *et al*., (1973) [48]. β-D-Glucuronidase activity was determined by the method of Kawai and Anno (1971) [49]. β-D-Galactosidase was assayed according to the method described by Kawai and Anno (1971)[50]. β-D-N-acetyl galactosaminidase activity was assayed according to the method of Moore and Moris (1982)[51]. The lipids were extracted by the method of Folch *et al*., (1957) [52]. Cholesterol was estimated by the method of Zak *et al*., (1954) [53]. Free fatty acids were measured by the method of Horn and Menahen (1981) [54].Triglycerides was estimated by the method of Rice (1970) [55]. Phospholipids were estimated as inorganic phosphorous by the method of Fiske and Subbarow (1925) [56] after Barlette (1959) [57] perchloric acid digestion.

2.9 Data Analysis

All the values are represented as mean ± S.D. (n=6). The statistical differences among different groups were analyzed by student's t-test. P-Values of 0.05 or less were considered significant.

3. Results

Table-I: Effects of CsA and AA on the assessment of renal function in urine

Groups	Urea	Creatinine	Uric acid
Control	0.78 ± 0.06	1.27 ± 0.29	0.90 ± 0.18
CsA	0.34 ± 0.04 [a]	0.55 ± 0.16 [a]	0.14 ± 0.07 [a]
CsA+AA	0.47 ± 0.05 [b]	0.98 ± 0.29 [b]	0.57 ± 0.08 [b]
AA	0.43 ± 0.02 [c]	0.67 ± 0.20 [c]	0.50 ± 0.07 [c]

Values are expressed as mean ± S.D for six animals in each group
Units: Urea, Creatinine, Uric acid – mg/ 24hrs urine sample
Comparisons: [a]Control Vs CsA, [b]CsA Vs CsA + AA, [c]Control Vs AA.

Table-II: Effects of CsA and AA on the activity of TCA cycle enzymes in mitochondrial kidney homogenate

Groups	SDH	MDH	Aconitase	Citrate synthase
Control	0.07 ± 0.01	0.86 ± 0.38	0.79 ± 0.14	0.79 ± 0.04
CsA	0.05 ± 0.009 [a]	0.28 ± 0.16 [a]	0.49 ± 0.06 [a]	0.61 ± 0.09 [a]
CsA+AA	0.08 ± 0.02 [b]	0.68 ± 0.21 [b]	1.01 ± 0.12 [b]	1.12 ± 0.10 [b]
AA	0.12 ± 0.09 [ns]	0.96 ± 0.21 [ns]	1.23 ± 0.04 [c]	0.88 ± 0.13 [ns]

Values are expressed as mean ± S.D for six animals in each group
Units: SDH: nmoles of succinate oxidized/min/mg protein
MDH: nmoles of NADH oxidized/min/mg protein
Aconitase: nmoles of cis-aconitate formed/min/mg protein
Citrate synthase: nmoles of DNBA formed/min/mg protein
Comparisons: [a]Control Vs CsA, [b]CsA Vs CsA + AA, [c]Control Vs AA.

Table-III: Effects of CsA and AA on the activity of complexes I, II, III, IV in mitochondrial kidney homogenate

Groups	Complex-I	Complex-II	Complex-III	Complex-IV
Control	0.97 ± 0.17	0.61 ± 0.03	0.21 ± 0.03	0.13 ± 0.03
CsA	0.24 ± 0.08 [a]	0.47 ± 0.04 [a]	0.05 ± 0.01 [a]	0.09 ± 0.01 [a]
CsA+AA	1.18 ± 0.04 [b]	0.71 ± 0.05 [b]	0.13 ± 0.006 [b]	0.12 ± 0.02 [b]
AA	1.40 ± 0.09 [c]	0.87 ± 0.03 [c]	0.17 ± 0.01 [c]	0.12 ± 0.01 [c]

Values are expressed as mean ± S.D for six animals in each group
Units: Complex-I – nmoles of NADH oxidized/min/mg protein
Complex-II – nmoles of dichlorophenol/indophenols oxidized/min/mg protein
Complex-III – nmoles of cytochrome c reduced/min/mg protein
Complex-IV – nmoles of cytochrome c oxidized/min/mg protein
Comparisons: [a]Control Vs CsA, [b]CsA Vs CsA + AA, [c]Control Vs AA

Table-IV: Effects of CsA and AA on the activity of mitochondrial enzymes in the cytosol of kidney homogenate

Groups	NADH DH	αKG DH	ICDH
Control	0.11 ± 0.02	0.27 ± 0.04	0.19 ± 0.04
CsA	0.08 ± 0.01 [a]	0.78 ± 0.03 [a]	0.11 ± 0.02 [a]
CsA+AA	0.10 ± 0.01 [b]	0.65 ± 0.03 [b]	0.22 ± 0.07 [b]
AA	0.12 ± 0.01 [c]	0.47 ± 0.02 [c]	0.27 ± 0.04 [c]

Values are expressed as mean ± S.D for six animals in each group
Units: NADH DH – mg of NADH DH liberated/mg protein
αKG DH – mg of αKG DH liberated/min/mg protein
ICDH – nmoles of αKG DH formed/hr/mg of protein
Comparisons: [a]Control Vs CsA, [b]CsA Vs CsA + AA, [c]Control Vs AA.

Table-V: Effects of CsA and AA on the activity of Lysosomal enzymes in the cytosol of kidney homogenate

Groups	Cathepsin-D	β–D Glucuronidase	β –D Galactosidase	β–D-N-acetyl glucosaminidase
Control	7.48 ± 0.14	2.48 ± 0.86	9.36 ± 1.75	5.81 ± 0.18
CsA	9.1 ± 0.21 [a]	6.26 ± 0.91 [a]	14.38 ± 0.97 [a]	7.26 ± 0.25 [a]
CsA+AA	8.76 ± 0.11 [b]	4.90 ± 0.28 [b]	9.28 ± 0.12 [b]	5.96 ± 0.56 [b]
AA	7.97 ± 0.10 [c]	4.32 ± 0.73 [c]	6.96 ± 1.36 [ns]	4.90 ± 0.80 [ns]

Values are expressed as mean ± S.D for six animals in each group
Units: Cathepsin-D - µmole of tyrosine released/hr/mg protein
β–D Glucuronidase: µmole of p-nitrophenol liberated/min/mg protein
β –D Galactosidase - µmole of p-nitrophenol liberated/min/mg protein
β –D-N-acetyl glucosaminidase - µmole of p-nitrophenol liberated/min/mg protein
Comparisons: [a]Control Vs CsA, [b]CsA Vs CsA + AA, [c]Control Vs AA.

Table-VI: Effects of CsA and AA on the level of cytosolic lipids in the mitochondrial kidney homogenate

Groups	Cholesterol	Triglycerides	Free fattyacid	Phospholipid
Control	0.51 ± 0.05	0.79 ± 0.09	0.18 ± 0.03	0.03 ± 0.003
CsA	0.81 ± 0.12 [a]	1.43 ± 0.11 [a]	0.47 ± 0.09 [a]	0.05 ± 0.003 [a]
CsA+AA	0.51 ± 0.05 [b]	1.04 ± 0.24 [b]	0.31 ± 0.06 [b]	0.04 ± 0.006 [b]
AA	0.38 ± 0.07 [c]	0.91 ± 0.14 [c]	0.27 ± 0.05 [c]	0.03 ± 0.001 [ns]

Values are expressed as mean ± S.D for six animals in each group
Units: Cholesterol, TG, FFA, Phospholipids – mg/g tissue
Comparisons: [a]Control Vs CsA, [b]CsA Vs CsA + AA, [c]Control Vs AA.

Table-VII: Effects of CsA and AA on the level of cytosolic lipids in the lysosomal kidney homogenate

Groups	Cholesterol	Triglycerides	Free fatty acid	Phospholipid
Control	0.76 ± 0.15	0.87 ± 0.05	0.25 ± 0.01	0.03 ± 0.001
CsA	1.31 ± 0.10 [a]	1.72 ± 0.09 [a]	0.35 ± 0.02 [a]	0.06 ± 0.006 [a]
CsA+AA	0.99 ± 0.14 [b]	1.32 ± 0.21 [b]	0.29 ± 0.04 [b]	0.04 ± 0.006 [b]
AA	0.93 ± 0.15 [ns]	0.92 ± 0.34 [c]	0.24 ± 0.07 [c]	0.03 ± 0.003 [ns]

Values are expressed as mean ± S.D for six animals in each group
Units: Cholesterol, TG, FFA, Phospholipids – mg/g tissue
Comparisons: [a]Control Vs CsA, [b]CsA Vs CsA + AA, [c]Control Vs AA.

3. Results

The levels of Urea, Uric acid and Creatinine (Table I) were assessed in the urine of experimental animals. A significant ($P<0.05$) decrease in their levels were observed in CsA treated animals which marks the deterioration of renal function. Concomitant treatment with AA significantly ($P<0.05$) altered the levels up to a certain extent compared to control.

Table II, shows the activities of the TCA cycle enzymes SDH, MDH, Aconitase, Citrate synthase in the kidney of control and experimental group pf animals. The activities of TCA cycle enzymes showed a significant decrease ($P<0.05$) in CsA treated rats. Treatment with AA altered the activities of these enzymes to near normal levels when compared with CsA induced animals.

Mitochondrial respiratory complex I (NADH:ubiquinone oxidoreductase), complex II (succinate ubiquinone oxidoreductase) complex III (ubiquinol:ferricytochrome *c* oxidoreductase) and complex IV (cytochrome *c* oxidase) in Table III were assessed in the kidney of control and CsA induced experimental group of rats. The Group II rats induced with CsA showed considerable impairment ($P<0.05$) in the activities of mitochondrial respiratory complexes when compared to Group I control rats. However, these alterations in the drug metabolizing enzymes were appreciably prevented ($P<0.05$) in Group III rats treated with AA, when compared to induced animals.

Table IV, represents the levels of mitochondrial enzymes NADH DH, αKG DH, ICDH in the kidney cytosol of control and experimental groups. The activities of mitochondrial enzymes were significantly ($P<0.05$) reduced in CsA induced rats (Group II) as compared to the control rats (Group I). AA administration (Group III) significantly ($P<0.05$) increased the mitochondrial enzymes activity as compared with CsA induced rats (Group II). No significant change was found between control (Group I) and AA alone treated group or rats (Group IV).

Table V, shows the activities of lysosomal enzymes in induced and experimental group of animals. There was a significant increase ($P<0.05$) in the activities of lysosomal enzymes such as Cathepsin-D**,** β–D Glucuronidase, β –D Galactosidase, β–D-N-acetyl glucosaminidase in CsA induced rats when compared with control rats. Group III AA administered rats showed significant ($P<0.05$) decrease in the activity of lysosomal enzymes when compared to CsA treated rats. Treatment with AA significantly ($P<0.05$) altered the levels up to a certain extent compared to control.

Table VI, shows the levels of cholesterol, triglycerides, free fatty acids, phospholipids in the cytosol (mitochondrial kidney homogenate) of control and CsA induced experimental group of animals. The levels of these parameters were increased significantly ($P<0.05$) in CsA induced group of animals compared to control rats. AA treatment significantly ($P<0.05$) ameliorated the lipid levels compared to CsA induced animals.

Table VI, shows the levels of cholesterol, triglycerides, free fatty acids, phospholipids in the cytosol (lysosomal kidney homogenate) of control and CsA induced experimental group of animals. The levels of these parameters were increased significantly ($P<0.05$) in CsA induced group of animals compared to control rats. Treatment with AA significantly altered the levels up to a certain extent compared to control.

4. Discussion

The primary effect of CsA is to inhibit the maturation of T and B cells [58] by inhibiting the activation cascade [59] and the synthesis of gamma-interferon, the cytokine that amplifies the signal, activating macrophages and mast cells [60]. Haynes *et al*. (1985) [61] suggested that both the immunosuppressor role and the toxicity of CsA were related to its hydrophobic property which facilitates its solubilization within the lipid bilayer, diminishing membrane fluidity. This leads to a reduction in LDL clearance in the hepatocyte, increasing the plasma LDL levels and predisposing the individual to the development of atherosclerosis [62, 63].

The TCA cycle and electron transport chain are important determinants of mitochondrial function. In mitochondrial membranes, unsaturated fatty acids, being components of phospholipids, are very susceptible to oxidation by the hydroxyl radical.

The mitochondrial TCA cycle enzymes aconitase, succinate dehydrogenase (SDH), and KGDHC (α-Keto glutarate dehydrogenase) are sensitive to oxidative inactivation both *in vitro* and *in vivo* [64-66].

Cytosolic and mitochondrial enzymes such as, G6PD, LDH, SDH, MDH and GDH plays a crucial role for the maintenance of favorable physiological conditions in the cell. Fluctuations in these enzyme activities during pathological conditions like diabetes leads to severe physiological malfunctions in the kidney [67]. SDH, a marker enzyme for mitochondria, is usually far greater in activity than the other enzymes in both developing and adult animals. Decreased SDH activity in diabetic condition affects succinate-fumarate conversion, which indicates depressed oxidative metabolism in mitochondria. It has been suggested that the diabetogenicity of STZ is dependent on the inhibition of activity of citric acid cycle enzymes like SDH [67, 68]. MDH plays an important role in the TCA cycle as SDH. Remarkable decrease in renal MDH activity in diabetic rats indicates irregularity in the TCA cycle and ultimately affects other mitochondrial enzymes. Decreased MDH activity in diabetic rats was also reported by Pannerselvam and Govindaswamy [69].

Data support our hypothesis about the possible involvement of the mtETC components like complex I (P-site) and complex III (S-site) in the mitochondrial membrane permeabilization mediated by the MPT pore [70-73]. The hypothesis suggested [70] that both respiratory complexes I and III might be involved in the mitochondrial membrane permeabilization promoted by Cd^{2+} and/or Ca^{2+} plus Pi. On the basis of findings [74-76] and data existing in the literature postulated a hypothetical model of regulated MPT pore. The model of conventional Ca^{2+} activated CsA-sensitive pore originally proposed by Halestrap [77, 78] in which ANT (adenine nucleotide translocase) was considered its crucial core element (in the light of the modern findings it may be also phosphate carrier, PiC) had been integrated with an idea of Fontaine and Bernardi [79] concerning mitochondrial respiratory chain complex I involvement in the MPT pore formation and/or regulation. Both complex I and III of the mtETC are places of localization of Ca^{2+} (Me^{2+})-binding site(s), critical for the MPT induction, and depending on conditions and cell type, either one or both complexes could be involved in triggering of the MPT pore assembly; besides, the complex I of the respiratory chain likely constitutes the P-site while complex III—the S-site of the MPT pore [70]. Later, the model was extended by additional suppositions [80]. In particular, the complex I (P-site) and complex III (S-site) may constitute not only critical Me^{2+} binding sites but also main loci for ROS generation that was instrumental in oxidation of critical thiol groups and the MPT pore opening [80]. Moreover, the

super complex I-III could be the key component of the regulated (i.e., Ca^{2+} dependent CsA-sensitive) MPT pore complex, while complex III is likely involved in the "unregulated" (i.e., Ca^{2+} and/or CsA-insensitive) MPT pore assembly and might concern to the external (inhibitory) Ca^{2+} binding site.

Prior studies have demonstrated defects in specific respiratory chain complexes and TCA cycle enzymes in response to exogenous chemical insults and mitochondrial enzymes are sensitive to endogenously produced superoxide in the absence of chemical inhibitors. We further investigated the consequences of endogenous mitochondrial oxidative stress by enzymatic assays and revealed the electron transport chain complexes I, II (as well as SDH), III, and IV, in addition to the previously reported aconitase defect [81], are sensitive to mitochondrial ROS.

The important data were obtained by several groups of investigators under study of mechanism of action of arachidonic acid [82], ceramides [83-86], and tumor necrosis factor-alpha [87, 88] during induction by MPT and apoptosis indicates the direct participation of complex I and/or complex III in the processes. In addition, data concerning the contribution of the respiratory chain components in ischemia/reperfusion (hypoxia/reoxygenation) injury [89-96] and the modern knowledge about respiratory super complexes [97, 98] including findings on the structure of mitochondrial super complex formed by complexes I and III [99-103] are also very impressive.

Our previous studies demonstrated a highly significant decrease in the catalytic activity of mitochondrial aconitase from cortex of sod2–/– mice, which is partially rescued with EUK-8 treatment [104]. In order to evaluate other mitochondrial enzymes for vulnerabilities to mitochondrial oxidative stress, we assessed enzyme activities of ETC complexes I, II, III, IV and the TCA cycle enzymes.

Ferns *et al*. (1990) [105] observed that CsA has a cytopathic effect on the cells of the macrovascular tissue. CsA appears to provoke changes in lipid metabolism [106-111].

MRS (Magnetic resonance spectroscopy) data indicate that cyclosporine inhibits the Krebs' cycle (decreased glutamate/glutamine concentrations) and mitochondrial oxidative phosphorylation (decreased concentrations of NAD^{+}), resulting in a significant cellular reduction in ATP and phosphocreatine with a concomitant increase of ADP concentration. The inhibition of mitochondrial energy production caused lactate accumulation followed by elevated fatty acid oxidation at the high cyclosporine doses. The increased lactate concentrations in the cyclosporine-treated rats can be explained by compensatory stimulation of anaerobic ATP synthesis via glycolysis [112]. The decrease of fatty acids at the highest treatment dose (25 mg/kg/day cyclosporine) can be attributed to the increased stimulation of fatty acid oxidation, which is another additional alternative pathway for energy production [112], when oxidative mitochondrial pathway remains largely inhibited by cyclosporine. This may explain the increased lipid peroxidation in the 25 mg/kg/day cyclosporine-treated group. Malondialdehyde, one of the main products of lipid peroxidation, indirectly measured in TBA test, is generated during hydrolysis by oxidation of PUFA [113, 114]. PUFA concentrations, as well as the total fatty acid pool, were decreased in 25 mg/kg/day treated rats and correlated with the increased LPO. The biochemical depression of mitochondrial glucose and high-energy phosphate metabolism found in our study are very similar to those induced by cyclosporine in the brain [115, 116]. The ability of cyclosporine to decrease the energy state by inhibiting mitochondrial metabolic pathways has also been reported for the kidney [117] and, more recently, it has been shown that cyclosporine produces hypoxia-like conditions in the normoxic liver [118]. It can be speculated that by inducing metabolic changes similar to hypoxia, cyclosporine also possesses ischemic preconditioning effects. Besides direct depression of mitochondrial respiration, another possible mechanism, such as induction of heat shock protein expression as shown for the kidney [119], may be involved in cyclosporine-induced pharmacological preconditioning. However, the potential contribution and the exact mechanism of cyclosporine-induced preconditioning will require further assessment.

5. Conclusion

From the above observations it can be concluded that, AA is able to favorably modulate the activities of the enzymes involved in carbohydrate metabolism. It was also able to restore the altered activities of the TCA cycle enzymes and normalize the alteration in energy production. Our work elucidates the mechanism of oxidative stress observed due to administration of Cyclosporine and proves beneficial effects of *arjunolic acid* in reducing mitochondrial renal damages.

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