Research Article

Beneficial effects of sulfated polysaccharides from *Sargassum wightii* against mitochondrial alterations induced by Cyclosporine A in rat kidney

Anthony Josephine[1], Ganapathy Amudha[1], Coothan Kandaswamy Veena[1], Sreenivasan P. Preetha[2], Arjunan Rajeswari[1] and Palaninathan Varalakshmi[1]

[1] Department of Medical Biochemistry, Dr. ALM. Post Graduate Institute of Basic Medical Sciences, University of Madras, Chennai, India
[2] Department of Zoology, Division of Biochemistry, University of Madras, Chennai, India

Sulfated polysaccharides from marine seaweeds are receiving continuous attention owing to their wide therapeutic applications and are known to inhibit free radical generation. It has been well known that mitochondria are the major sources as well as the target of free radicals. The renal tubules have high density of mitochondria and therefore show structural and functional defects in acute renal failure. Hence, the present study is designed to appraise the mitochondrial status during Cyclosporine A (CsA)-induced nephrotoxicity and the effect of sulfated polysaccharides over it. Sulfated polysaccharides (5 mg/kg body weight, subcutaneously) treatment significantly prevented the CsA-induced (25 mg/kg body weight, orally) mitochondrial damage. CsA-induced mitochondrial oxidative stress in rat kidney was evident from increased reactive oxygen species level, decreased antioxidant defense system, coupled with enhanced lipid peroxidation. Further, the activities of tricarboxylic acid cycle and electron transport chain enzymes were decreased in CsA-induced rats, along with a significant increase in the activities of urinary enzymes, thus indicating renal tubular injury. Ultrastructural changes were also in accord with the above aberrations. The above abnormalities were favorably modulated by sulfated polysaccharides supplementation, thus highlighting the significance of sulfated polysaccharides in preventing the renal mitochondrial dysfunction allied with CsA-provoked nephrotoxicity.

Keywords: Antioxidants / Cyclosporine A / Mitochondrial enzymes / Mitochondrial swelling / Sulphated polysaccharides

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1 Introduction

Cyclosporine A (CsA) is the most widely used immunosuppressive agent in the field of transplantation. However, CsA-induced nephrotoxicity greatly impedes its immunosuppressive potential [1]. It has been found that CsA-induced nephrotoxicity is not only due to vasoconstriction, which obviously decreases oxygen supply to the cells, it also promotes cellular hypoxia, which might be the consequence of Ca^{2+} accumulation inside the mitochondrial matrix. As a result, ATP synthesis is decreased, which is considered to be one of the determinants of chronic renal insufficiency. Further, there is evidence, suggesting that CsA induces oxidative damage to lipids and proteins in the kidney cortex mitochondria and microsomes under *in vitro* conditions [2]. It has been found to increase malondialdehyde (MDA) formation, stimulate hydrogen peroxide production and depress the activities of endogenous antioxidant enzymes (Manganese-superoxide dismutase (MnSOD) and glutathione peroxidase (GPx)), strongly reflecting the role of oxidative stress in the CsA toxicity on kidney mitochondria.

Mitochondria play a vital role in the body's metabolism and hence protecting mitochondria is essential for sustaining life. On the other hand, mitochondria are targets of their own oxidant by-products, with the steady-state oxidative damage

Correspondence: Professor Dr. Palaninathan Varalakshmi, Department of Medical Biochemistry, Dr. A.L.M. Postgraduate Institute of Basic Medical Sciences, University of Madras, Taramani, Chennai – 600 113, India
E-mail: drvlakshmi@yahoo.com
Fax: +91-44-2454-0709

Abbreviations: ALP, alkaline phosphatase; **CsA**, cyclosporine A; **GAG**, glycosaminoglycans; **GPx**, glutathione peroxidase; **LDH**, lactate dehydrogenase; **LPO**, lipid peroxidation; **MDA**, malondialdehyde; **MDH**, malate dehydrogenase; **ROS**, reactive-oxygen species

being high relative to other organelles. This leads to a viscous cycle of decreasing mitochondrial efficiency, increasing free radical production and thereby increased mitochondrial damage [3]. Moreover, mitochondrial electron transport is not completely efficient, as 1 – 3% of inspired oxygen being constitutively converted to damaging superoxide radicals. The renal tubules were found to have high density of mitochondria and so during renal failure, marked structural and functional defects were seen in the mitochondria [4]. Although an abundance of research suggests that CsA leads to renal damage, the exact significance and nature of CsA toxicity to renal mitochondria under *in vivo* condition requires further elucidation. Thus the proposed concept at this context is to obtain a cytoprotection by administration of a drug that can stabilize mitochondrial functions, ATP synthesis, reactive-oxygen species (ROS) generations and Ca^{2+} homeostasis inside the mitochondrial matrix, as mitochondrial decay may eventually lead to apoptosis.

The application of modern scientific methodologies to novel model organisms is opening new avenues of research in marine biology. In recent years, algal polysaccharides have been widely accepted to be one of the important candidates for the development of effective and non-toxic medicines with stronger free radical scavenging and antioxidant actions [5] and renoprotective effect [6]. Sulfated polysaccharides are a class of compounds with hemi-ester sulfate groups on a polysaccharide backbone, and they are widespread in nature occurring in various organisms. *Sargassum wightii* is one such brown algae with wide pharmacological actions. Recently, it has been explored that sulfated polymannuroguluronate exhibits potent effect on mitochondria by enhancing the antioxidant status and diminishing the ROS accumulation, improving the mitochondrial membrane potential, preventing the cytochrome c release and by improving the ATP-energetic status [7]. However, the role of sulfated polysaccharides from *Sargassum wightii* on mitochondria has not been explored yet, and hence the present study would be the new approach towards mitochondrial alterations during CsA-induced nephrotoxicity under *in vivo* conditions.

2 Materials and methods

2.1 Drugs and chemicals

CsA was purchased from Sandoz, Basel, Switzerland. BSA, heparin, fucoidan (*Fucus vesiculosus*, marine brown algae) and 2, 4, 6-tri [2-pyridyl-5-triazine] (TPTZ) were obtained from Sigma Chemicals, St. Louis, MO, USA. All other chemicals and solvents used were of analytical grade.

2.2 Seaweed collection and extraction

The marine brown algal species *Sargassum wightii* was collected from Mandapam, Gulf of Mannar region, Rameswaram, India. Sulfated polysaccharides were extracted from *Sargassum wightii* according to the method of Vieira *et al*. [8], as described in our previous report [9].

2.3 Analysis of components present in sulfated polysaccharides

Total sugar content was estimated by the method of Dubois *et al*. [10]. The method of Lowry *et al*. [11] was followed for the estimation of protein content. Sulfate content was determined by Barium chloride-gelatin method of Dodgson and Price [12]. Uronic acid estimation was carried out according to the method of Dische [13]. Sulfated glycosaminoglycans (GAG) were estimated by the method of Farndale *et al*. [14].

2.4 Potential antioxidant activity of sulfated polysaccharides by the ferric reducing/ antioxidant power assay

The ferric reducing/antioxidant power (FRAP) assay offers a putative index of antioxidant potential of the sample. The reducing power of polysaccharides fractions from *Sargassum wightii* was estimated by the method of Benzie and Strain [15], with slight modifications by Pulido *et al*. [16]. This method allows the determination of the ferric reducing ability [μmol Fe (III) converted into Fe (II)] of the samples in aqueous solutions, which serves as a measure of their antioxidant power. The FRAP reagent contained 2.5 mL of a 10 mM TPTZ solution in 40 mM HCl containing 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ and 25 mL of 0.3 M acetate buffer, pH 3.6. In brief, 900 μL of freshly prepared FRAP reagent was mixed with 90 μL of distilled water and 30 μL of test sample (or water for the reagent blank) at 37°C. Absorbance readings at 595 nm were taken for every 15 s from 0 to 30 min using a Shimadzu Spectrophotometer. Aqueous solutions of known Fe (II) concentrations (100 – 2000 μM $FeSO_4 \cdot 7H_2O$) were used for calibration purpose. Results were expressed as mmol Fe (II)/g sample dry weight.

2.5 Experimental protocol

Male albino rats of Wistar strain (180 ± 20 g) procured from Tamil Nadu University for Veterinary and Animal Sciences, Chennai, India, were used for the study. Animals were fed with commercially available standard rat pelleted feed (M/s Pranav Agro Industries, India) under the trade name Amrut rat/mice feed and water was given *ad libitum*. The rats were housed under conditions of controlled temperature (25 ± 2°C) and acclimatized to 12-h light:12-h dark cycle. Animal experiments were conducted according to the guidelines of institutional animal ethical committee (IAES No. 02/052a/06).

Rats were categorized into four groups, each consisting of six animals. Group I served as vehicle (olive oil)-treated con-

trol. Group II animals were administered CsA orally at a dose of 25 mg/kg body weight for 21 days. Group III animals received sulfated polysaccharides (5 mg/kg body weight, subcutaneously) for 21 days as drug control. In Group IV, animals were administered CsA as in Group II along with concomitant administration of sulfated polysaccharides (5 mg/kg body weight, subcutaneously) for 21 days.

The animals were placed in a separate metabolic cage for 24 h after the last dose for urine collection, which was collected in the ice-cold containers at 0°C. It was then centrifuged to remove the debris and used for further analysis. After the experimental period of 21 days, all the animals were anesthetized and decapitated. Kidney tissues were immediately excised and immersed in ice-cold physiological saline and processed for the isolation of mitochondria. A section of kidney tissue was set aside for electron microscopic studies.

2.6 Assay of urinary enzymes

The activities of certain enzymes were assayed in the urine. The activity of alkaline phosphatase (ALP) was determined by the method of King [17] using disodium phenyl phosphate as the substrate. N-acetyl-β-D-glucosaminidase (NAG) was estimated by the method of Maruhn [18] using p-Nitrophenyl β-D-glucosaminide as substrate. The procedure of King [19] was adopted for the assay of lactate dehydrogenase (LDH). Activity of γ-Glutamyl transferase (γ-GT) was determined by the method of Orlowski and Meister [20].

2.7 Isolation of renal mitochondria

Mitochondria were isolated from kidney by the method of Mingatto *et al*. [21]. The kidney tissue was rapidly removed and homogenized in a medium labeled I (250 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EDTA, 120 mM KCl and 50 mM Tris-HCl, pH 7.4). The homogenate obtained was centrifuged at $755 \times g$ for 5 min, and the resulting supernatant was again centrifuged at $13\,300 \times g$ for 15 min. The pellet was then suspended in a medium labeled II (250 mM mannitol, 70 mM sucrose, 10 mM HEPES and 50 mM Tris-HCl, pH 7.4) and washed twice with the same buffer by centrifugation at $13\,300 \times g$ for 15 min. The final mitochondrial pellet was then resuspended in the same medium and used for further analysis. Mitochondrial protein was estimated by the method of Lowry *et al*. [11].

2.8 Assessment of mitochondrial oxidative stress

ROS generation in renal mitochondria was assessed using the fluorescent probe, 2′,7′-dichlorodihydrofluorescein-diacetate (DCFH-DA) [22]. The enzyme, SOD was assayed according to the method of Marklund and Marklund [23]. The unit of enzyme activity is defined as the amount of enzyme required to give 50% inhibition of pyrogallol autooxidation. GPx was assayed according to the method of Rotruck *et al*. [24], where the color developed was read at 412 nm. GPx activity was assessed in terms of utilization of glutathione. Total reduced glutathione (GSH) was estimated by the method of Moron *et al*. [25]. This method was based on the reaction of GSH with 5,5′dithio-bis (2-nitrobenzoic acid) (DTNB) to give a compound that absorbs at 412 nm.

2.9 Assay of lipid peroxidation

The level of lipid peroxidation (LPO) in the renal mitochondria was assessed by the method of Hogberg *et al*. [26]. MDA, formed as an end product of the peroxidation of lipids, served as an index of the intensity of oxidative stress. MDA reacts with thiobarbituric acid to generate a colored product, which absorbs at 532 nm.

2.10 Determination of TCA cycle enzymes

The method of Bernt and Bergmeyer [27] was followed for the estimation of the activity of isocitrate dehydrogenase (ICDH). The amount of isocitrate oxidized was measured by the increase in extinction due to the formation of NADPH. The activity of succinate dehydrogenase (SDH) was estimated by the method of Slater and Borner [28], wherein the rate of reduction of potassium ferricyanide was measured by following the decrease in optical density at 400 nm, in the presence of sufficient potassium cyanide to inhibit cytochrome c oxidase. Malate dehydrogenase (MDH) was assayed by the method of Mehler *et al*. [29]. Oxaloacetate was used as the substrate and the enzyme activity was determined by measuring the rate of oxidation of NADH in the presence of enzyme and excess substrate.

2.11 Enzyme complexes of electron transport chain

The activity of NADH dehydrogenase or Complex I was determined by the method of Minakami *et al*. [30], in which the rate of NADH oxidation was measured at 340 nm. The activity of succinic-coenzyme Q or Complex II was estimated by the method of Ziegler and Doeg [31]. The reaction was initiated with Coenzyme Q and the rate of reduction of 2,6-dichlorophenolindophenol (DCIP) was read at 600 nm. The activity of Coenzyme Q-cytochrome reductase or Complex III was assayed by the method of Green and Burkhard [32] by measuring the rate of reduction of ferricytochrome by the reduced form of coenzyme Q at 550 nm. The activity of cytochrome c oxidase or Complex IV was estimated by the method of Wharton and Tzagoloff [33]. The rate of oxidation of ferrocytochrome was measured as the rate of decrease in absorbance at 550 nm.

2.12 Transmission electron microscopic studies

Kidney tissues were excised and fixed in 3% glutaraldehyde, 2% formaldehyde solution in 0.1 M phosphate buffer at pH 7.4, and then post fixed in 1% osmium tetroxide. After dehydration in graded ethanol, the material was embedded in an epon araldite. Ultra thin sections were counterstained with uranyl acetate and lead citrate. Sections were observed in transmission electron microscope (TEM) Philips 201 C, Netherlands, and photographed.

2.13 Statistical analysis

The results are expressed as mean ± SD for six animals in each group. Differences between groups were assessed by one-way analysis of variance (ANOVA) using the SPSS software package for windows. Post hoc testing was performed for inter-group comparisons using the least significance difference (L.S.D.) test; significance at P – values <0.001, <0.01 and <0.05 have been given respective symbols in the tables and figures.

3 Results

Figure 1 displays the percentage of various components present in the sulfated polysaccharides. It contained the following components: total sugar (69.26%), protein (3.01%), uronic acid (15.62%) and sulfate (12.09%). The percentage of sulfated GAG was presented in Fig. 2. In this assay, sulfated polysaccharides from *Sargassum wightii* were tested for its sulfated GAG content by comparing with heparin. Along with this, sulfated GAG content from fucoidan was also examined. Hence considering heparin as standard, fucoidan was found to contain 78.53% of sulfated GAG and sulfated polysaccharides from *Sargassum wightii* was found to contain 74.21% of sulfated GAG, comparatively.

The antioxidant activity of sulfated polysaccharides from *Sargassum wightii* was assessed under *in vitro* condition. Sulphated polysaccharides were able to reduce the TPTZ resulting in the formation of blue colored complex. The

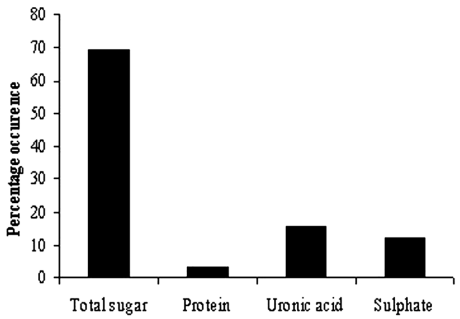

Figure 1. Levels of total sugar, protein, uronic acid and sulfate in the extract of *Sargassum wightii*. Values are expressed as mean of three experiments.

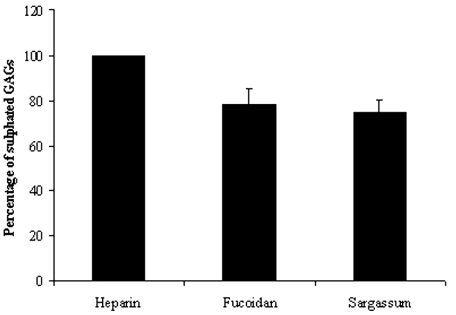

Figure 2. Levels of sulfated GAG in the extract of *Sargassum wightii*. Values are expressed as mean ± SD of three experiments.

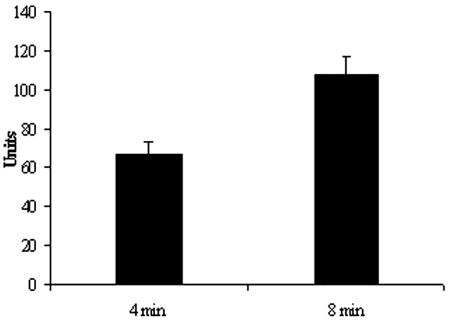

Figure 3. Ferric reducing ability/antioxidant power values of sulfated polysaccharides from *Sargassum wightii*. Values are expressed as mean ± SD of three experiments. Units: μmol Fe(II)/g of sulfated polysaccharides.

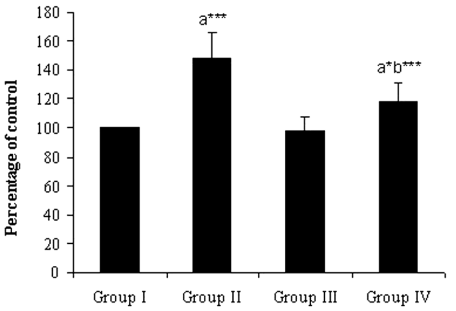

Figure 4. Effect of sulfated polysaccharides on CsA-induced mitochondrial ROS production. Values are expressed as mean ± SD for six animals in each group. Comparisons are made between a-Group I and Group II, III, IV; b-Group II and Group IV. The symbols (***) and (*) represent statistical significance at P <0.001 and P <0.05, respectively.

antioxidant activity was found to be 66.27 and 107.25 μmol Fe (II)/g at 4 and 8 min, respectively (Fig. 3). The above observation allows determination of the ferric reducing ability of sulfated polysaccharides, as more the reducing ability, the more the antioxidant power.

Administration of CsA resulted in adverse effects on renal mitochondria. Figure 4 represents the ROS level in

Table 1. Effect of sulfated polysaccharides on mitochondrial oxidative stress in CsA-challenged rats[a)]

Parameters[b)]	Group I	Group II	Group III	Group IV
SOD	25.16 ± 3.32	16.20 ± 2.64 a***[c)]	26.19 ± 3.73	23.78 ± 2.61 b***
GPx	16.05 ± 1.28	12.25 ± 1.61 a**	16.11 ± 1.95	15.49 ± 2.36 b**
GSH	3.27 ± 0.36	1.67 ± 0.25 a***	3.30 ± 0.43	2.71 ± 0.44 a*b***
LPO	1.78 ± 0.20	2.57 ± 0.43 a***	1.73 ± 0.15	1.92 ± 0.25 b***

a) Values are expressed as mean ± SD for six animals in each group.
b) Units: SOD: Units/mg protein, one unit is equal to the amount of enzyme that inhibits the autooxidation reaction by 50%; GPx: μg of reduced GSH consumed/min/mg protein; GSH: μg/mg protein; LPO: nmoles of MDA formed/min/mg protein.
c) Comparisons are made between a-Group I and Group II, III, IV; b-Group II and Group IV. The symbols (***), (**) and (*) represent statistical significance at $P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively.

Table 2. Altered TCA cycle enzyme activities during CsA administration and the effect of sulfated polysaccharides[a)]

Parameters[b)]	Group I	Group II	Group III	Group IV
ICDH	5.95 ± 0.66	3.15 ± 0.38 a***[c)]	5.93 ± 0.65	5.08 ± 0.83 a*b***
SDH	19.41 ± 1.43	13.75 ± 2.25 a***	19.24 ± 2.54	18.15 ± 2.19 b**
MDH	5.86 ± 0.77	2.69 ± 0.24 a***	5.90 ± 0.78	4.47 ± 0.68 a**b***

a) Values are expressed as mean ± SD for six animals in each group.
b) Units: ICDH: nmoles of NADPH formed/min/mg protein; SDH: nmoles of succinate oxidized/min/mg protein; MDH: nmoles of NADH oxidized/min/mg protein.
c) Comparisons are made between a-Group I and Group II, III, IV; b-Group II and Group IV. The symbols (***), (**) and (*) represent statistical significance at $P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively.

Table 3. Effect of sulfated polysaccharides on CsA-induced changes in electron transport chain enzymes[a)]

Parameters[b)]	Group I	Group II	Group III	Group IV
Complex-I (NADH dehydrogenase)	1.51 ± 0.20	1.01 ± 0.16 a***[c)]	1.55 ± 0.15	1.46 ± 0.27 b***
Complex-II (Succinic-coenzyme Q)	0.79 ± 0.13	0.58 ± 0.09 a**	0.80 ± 0.10	0.76 ± 0.11 b**
Complex-III (Coenzyme Q-cytochrome reductase)	1.85 ± 0.19	1.42 ± 0.23 a**	1.86 ± 0.15	1.70 ± 0.26 b*
Complex-IV (Cytochrome c oxidase)	2.31 ± 0.28	1.65 ± 0.22 a***	2.29 ± 0.30	2.13 ± 0.32 b**

a) Values are expressed as mean ± S.D. for six animals in each group.
b) Units: NADH dehydrogenase: μmoles of NADH oxidized/min/mg protein; Succinic-coenzyme Q: μmoles of DCIP reduced/min/mg protein; Coenzyme Q-cytochrome reductase: μmoles of cytochrome c reduced/min/mg protein; Cytochrome c oxidase: μmoles of cytochrome c oxidized/min/mg protein.
c) Comparisons are made between a-Group I and Group II, III, IV; b-Group II and Group IV. The symbols (***), (**) and (*) represent statistical significance at $P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively.

the control and experimental groups. Increase in mitochondrial oxidative stress on CsA exposure was apparent from increased ROS level (1.48-fold) in Group II animals. Co-administration of sulfated polysaccharides significantly (P <0.001) prevented the abnormal increase in ROS level, thus establishing its free radical scavenging activity. Table 1 portrays the alterations in mitochondrial antioxidant defense system and LPO induced by CsA and the protective effect of sulfated polysaccharides. A concomitant drop in the antioxidant status along with marked increase in LPO was noted in CsA given Group II animals. While simultaneously, considerable protection was seen upon sulfated polysaccharides treatment, thereby proving its antioxidant effect.

The effect of sulfated polysaccharides and CsA on TCA cycle enzymes was highlighted in Table 2. The activities of TCA cycle enzymes such as ICDH, SDH and MDH were decreased by 47.06, 29.16, and 54.10%, respectively, during CsA induction. The above alterations in the activities of TCA cycle enzymes were restored to near control values with sulfated polysaccharides administration. Table 3 shows the activities of electron transport chain enzymes. Group II animals revealed a significant decrease (P <0.001and P <0.01) in the enzyme activity, which was brought back to control value by simultaneous administration of sulfated polysaccharides.

The activities of urinary enzymes were displayed in Table 4. A significant increase (P <0.001and P <0.01) in the activities of ALP, LDH, NAG and γ-GT were observed in the urine of CsA-induced Group II animals. Sulfated polysaccharides treatment restored the activities of these urinary enzymes to near control value.

Table 4. Effect of sulfated polysaccharides on CsA-induced alterations in the activities of urinary enzymes[a)]

Urinary enzymes[b)]	Group I	Group II	Group III	Group IV
ALP	0.79 ± 0.10	1.01 ± 0.16 a**[c)]	0.76 ± 0.08	0.82 ± 0.07 b**
LDH	0.32 ± 0.04	0.49 ± 0.08 a***	0.29 ± 0.04	0.35 ± 0.05 b***
NAG	0.36 ± 0.05	0.69 ± 0.11 a***	0.35 ± 0.03	0.44 ± 0.08 b***
γ-GT	1.26 ± 0.15	2.18 ± 0.29 a***	1.25 ± 0.13	1.46 ± 0.18 b***

a) Values are expressed as mean ± S.D. for six animals in each group.
b) Units: ALP: μmoles of phenol liberated/mg creatinine/h; LDH: μmoles of pyruvate liberated/mg creatinine/h; NAG: nmoles of p-nitrophenol liberated/mg creatinine/h; γ-GT: μmoles of p-nitroaniline liberated/mg creatinine/h.
c) Comparisons are made between a-Group I and Group II, III, IV; b-Group II and Group IV. The symbols (***) and (**) represent statistical significance at $P < 0.001$ and $P < 0.01$, respectively.

Figure 5 shows the ultrastructural changes in the kidney tissue of control and experimental groups. Renal architecture with normal mitochondria was seen in the control group (Fig. 5a). CsA given animals (Group II) show morphological changes like clumping of nuclear chromatin, mitochondrial swelling, autophagy by lysosomes and dispersion of ribosomes (Fig. 5b). Sulphated polysaccharides alone treated group (Group III) reveal normal kidney structure as that of control (Fig. 5c). Sulphated polysaccharides treated Group IV shows considerable reduction in the pathological changes as that seen in Group II animals and minimized mitochondrial swelling (Fig. 5d), thereby strongly suggesting its protective role on kidney mitochondria.

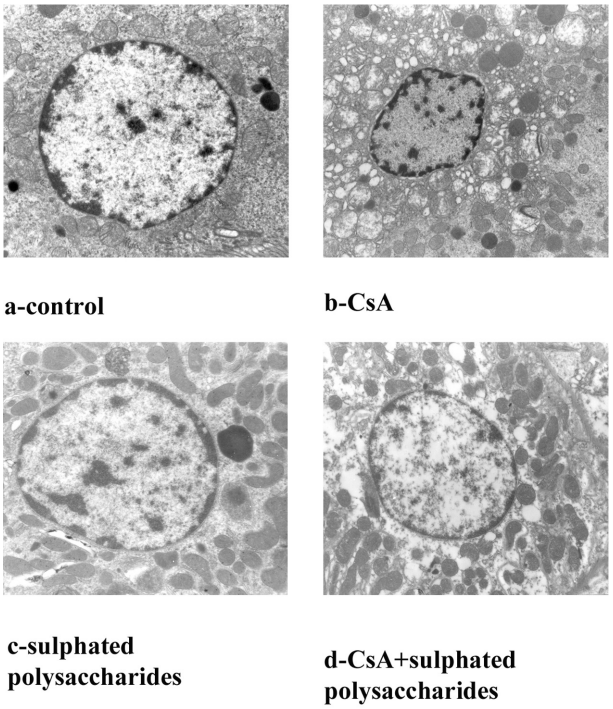

Figure 5. Transmission electron microscopic studies of renal cortex in the four experimental groups (15 000 ×) (a) control group shows normal architecture; (b) CsA induced group II animals show pathological changes like mitochondrial swelling, autophagy by lysosomes, along with clumping of nuclear chromatin and dispersion of ribosomes; (c) sulfated polysaccharides drug control group maintains the normal cellular architecture; (d) sulfated polysaccharides treatment in CsA induced group IV animals show considerable recovery from mitochondrial and other changes when compared to CsA induced group II animals.

4 Discussion

The present study is the first report exploring the status of mitochondria during CsA-induced nephrotoxicity under *in vivo* conditions, and the cytoprotective role of sulfated polysaccharides on such alterations. Sulfated polysaccharides are widely employed as a therapy for various diseases associated with impaired energy metabolism and that their dietary supplementation could reduce oxidative stress by imparting effective antioxidant defense [34].

Sulfated polysaccharides extracted from *Sargassum wightii* were found to be rich in polysaccharides with sulfate content in it. Fucoidan, a fucose-rich sulfated polysaccharide from marine brown algae (*Fucus vesiculosus*) which has also been found to exhibit renoprotective effect [6] was used for comparison in the present study. Moreover, previous studies show that sulfated polysaccharide extracts from *Sargassum* species and fucoidan exhibits similar activity to that of heparin [35], which is again a standard and potent nephroprotective agent among the sulfated GAG known [36]. Therefore, in the present study, sulfated polysaccharides from *Sargassum wightii* were tested for its sulfated GAG content by comparing with heparin. In this study, sulfated polysaccharides were found to contain significant amount of sulfated GAG, and hence the extract can be designated as sulfated polysaccharides. Moreover, it has been well established that polysaccharides possesses strong antioxidant activity [37, 38]. Additionally, it has been found that sulfation of the polysaccharides further increases its antioxidant capacity against various antioxidant systems i*n vitro* [39]. In the present study, sulfated polysaccharides were found to exhibit potent antioxidant effect. This is in consonance with the study of Ruperez *et al*. [40], which documents that fucoidan exhibits its antioxidant power at 4 and 30 min, respectively. Previous report shows that *Sargassum* species were found to exhibit the highest antioxidant activity [41]. This was also in agreement with Matsukawa *et al*. [42], who reported that the antioxidant activity of brown algae was superior to that of red or green groups. These reports support our present observation.

Recent interest has been focused on the role of altered mitochondrial bioenergetics in renal cell injury due to the following reasons: the central role of oxidative metabolism in renal tubular cell function, the susceptibility of the mitochondrial membrane to toxic injury, the high toxin levels which may be achieved within renal tubular cells and the relative ease with which mitochondria may be studied as a marker of cellular injury [43]. Copious reports hypothesize that oxidative stress is well accompanied with CsA-induced nephrotoxicity [44] and since, mitochondria are found to be the major source as well as the major target of ROS, a study to investigate the mitochondrial oxidative stress during renal injury is needed. In the present study, increased level of ROS in renal mitochondria indicates the onset of oxidative stress during CsA induction. Further, the involvement of ROS in CsA toxicity in the kidney mitochondria was studied in relation to the status of endogenous antioxidant defense system, wherein SOD and GPx activities were found to be decreased. SOD and GPx within mitochondria are supposed to eliminate ROS by reducing them to water while transferring their extra electrons to metals. In addition, a drastic decline in the GSH level was noted in the renal mitochondria of CsA given rats. GSH plays a critical role in cell viability through the regulation of mitochondrial inner membrane permeability by maintaining sulfydral groups in the reduced state [45], which suggests that oxidative stress and GSH depletion affect the functions of the mitochondria. Our observation corroborates with the report, which demonstrates that CsA decreases the ratio of GSH/GSSG within the renal cortex significantly [46], and hence the altered redox state by cellular GSH may in turn further aggravate the CsA toxicity.

Sulfated polysaccharides, through its antioxidant effect, remarkably restored the antioxidant enzymes back to its normal activity. Numerous evidences support the antioxidant activity of sulfated polysaccharides from brown algae [47], especially from *Sargassum* species [41] and have documented that sulfated polysaccharides possess higher free radicals (superoxide and hydroxyl radicals) scavenging activity. Furthermore, algal polysaccharides were found to increase the activities of SOD and GPx, via increasing their mRNA expressions [48, 49], which might lend support to the present study. Sulfated polysaccharides also found to exhibit metal chelating activity [47, 50], which might also contribute to its protective effect. The effect of sulfated polysaccharides in replenishing GSH activity was reported in our earlier study [9]. The above reports support our present observation, and that sulfated polysaccharides in the present study prevented mitochondrial oxidative stress by scavenging free radicals and boosting up the endogenous antioxidant enzymes and improving the GSH level to an appreciable extent.

Further, CsA-induced rats showed a sharp increase in LPO, suggesting damage to the mitochondrial membrane. Mitochondrial membrane is known to contain comparatively large amounts of polyunsaturated fatty acids in its phospholipids, which could probably be attributed to the increase in LPO level. Peroxidation of cellular membrane lipids is indeed associated with alteration of membrane function and inactivation of integral enzymes [51]. On the other hand, LPO was significantly inhibited by sulfated polysaccharides administration. It has been recently reported that sulfated polysaccharides like low molecular weight fucoidan acted as antioxidant and hepatoprotective agent by improving the activities of SOD and GPx and by significantly inhibiting LPO [52].

It has been well established that certain enzymes located in the mitochondria catalyze the oxidation of a number of substrates via, the citric acid cycle yielding reducing equivalents. These are then channeled through the respiratory chain eventually resulting in the synthesis of ATP by oxidative phosphorylation, and thereby afford the energy required for numerous cellular functions. Fall in the activities of TCA cycle enzymes in CsA-induced rats in the present study indicates the defect in aerobic oxidation of pyruvate, which might end up in the lesser production of ATP. CsA-induced increased LPO due to enhanced free radicals production, might contribute to the drop in the activities of TCA cycle enzymes, as these enzymes are located in the outer mitochondrial membrane. The inhibition of these enzymes in turn may affect the mitochondrial substrate oxidation and the electron transport chain, resulting in reduced oxidation of succinate and reduced rate of transfer of reducing equivalents to molecular oxygen [53].

Electron transport chains (also called electron transfer chains) are biochemical reactions that produce ATP (the energy currency of life). However, at the same time, the mitochondrial electron transport chain is an important source of ROS, which in turn coerces the mitochondria to be recurrently exposed to the accumulated ROS and hence mitochondria are said to be more vulnerable to oxidative damage than the rest of the cell [54]. In the present study, CsA stressed rats showed a significant decrease in the activities of electron transport chain enzymes. The possible reason for this can be ascribed to the shortage in the formation of reducing equivalents, which is due to the shut down of TCA cycle enzymes. Suppression of the mitochondrial electron transport chain may also contribute to the decline in GSH level [55]. Aupetit *et al*. [56] have suggested that although the mitochondrial damage induced by CsA appears to be limited at first glance, proposed that there exists a close contact between mitochondria and CsA-induced nephrotoxicity, with reference to its effect of inhibiting respiratory parameters and reducing mitochondrial protein levels. Moreover, Christians *et al*. [57] have demonstrated that CsA treatment due to its enhanced ROS production can lead to impaired energy yield through altered TCA cycle enzymes and oxidative phosphorylation. Recently, Indo *et al*. [58] have reported that increased ROS generations are found in mitochondria due to the impairment in

the electron transport chain. On one hand, enhanced ROS production occurs during electron transport chain reaction, while on the other hand, this augmented oxidative stress brings out enhanced LPO, which damages the mitochondrial membrane and thereby the leakage of these enzymes, which in turn eventually results in the production of more and more ROS again. Thus, mitochondria serve as a source as well as the target of ROS.

Numerous polysaccharides have been identified to protect cells from death, due to their ability to scavenge the excessive free radicals and ROS [59]. Miao *et al*. [7] have reported that sulfated polymannuroguluronate protected mitochondria from oxidative damage by targeting mitochondria and scavenging free radicals effectively, which emphasizes that it might block ROS generation from the start and thus prevent the accumulation of ROS in mitochondria. The above findings substantiate our observation, wherein sulfated polysaccharides were found to potentially improve the mitochondrial transport chain enzymes, through inhibiting the primary check, *i. e*. the ROS production by its natural free radical scavenging activity and by boosting the antioxidant defense system. Thus, through improving electron transport chain reaction, it might enhance ATP production and thereby the energy yield.

It has been widely suggested that renal cellular damage might result in the release of cellular enzymes into the urine. When numerous tubular cells disintegrate or when tubular permeability is disturbed, renal enzyme excretion is markedly increased. ALP is abundant in kidney and being membrane bound, serves as the marker for damage to the renal proximal tubules, and hence increased activity of ALP in the urine of CsA administered Group II animals indicates tubular damages. The kidney is known to be probably the major contributor of urinary LDH [60] and therefore its increase in the CsA-induced rats is highly suggestive of tubular lesions. NAG, a sensitive hydrolytic enzyme, is also said to be released in the urine after renal tubular damage. Elevation in the activities of these enzymes in the CsA administered animals could also be due to the lysosomal damage, leading to the leakage of these enzymes into the urine, and this is largely supported by Robinson *et al*. [61]. Augmented activity of γ-GT in the urine perhaps means injury to the proximal tubular epithelium and may reflect accelerated turnover of brush border membrane [62], thus indicating the ability of CsA to induce profound brush border membrane damage. The above observation is further strengthened by the report of Pons *et al*. [63], which strongly suggests that CsA-induced proximal tubular toxicity was characterized by an increased urinary excretion of NAG and γ-GT. However, simultaneous treatment with sulfated polysaccharides prevented the abnormal elevation in the activities of urinary enzymes, thus indicating its protective effect on tubular damage. The protective effect of sulfated polysaccharides on proteinuria and glycosaminoglycanuria due to its polyanionic nature [64], might contribute to the protection against abnormal leakage of urinary enzymes in the present study.

Ultrastructural changes also strongly confirm the above alterations. Animals administered with CsA reveals marked morphological changes like mitochondrial swelling and autophagy by lysosomes accompanied with clumping of nuclear chromatin and dispersion of ribosomes. Our results are in consonance with the findings of Jiang and Acosta [65], who documented that CsA-induces alteration in mitochondrial calcium homeostasis and a subsequent loss of energy supply, which might be the cause for mitochondrial swelling and subsequent apoptotic changes. In addition, Justo *et al*. [66] have reported that CsA leads to the loss of mitochondrial membrane potential, hallmark of mitochondrial injury, and thereby leads to cytochrome-c release, caspase activation and subsequent apoptosis. Further, it has been documented that CsA also leads to apoptosis in mesangial cells, by activating the pro-apoptotic factors [67]. Sulfated polysaccharides treatment ameliorated the mitochondrial swelling and other associated changes to a considerable extent, thereby proving its efficacy on mitochondria.

To sum up, our data offer a potential new therapeutical approach for prevention of CsA-induced renal damage. Sulfated polysaccharides effectively prevented the mitochondrial damage and tubular injuries associated with CsA-induced nephrotoxicity. Additional studies are needed to address these possibilities, which await future investigations.

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