ANTI-APOPTOTIC EFFECT OF SULPHATED POLYSACCHARIDES THROUGH DOWNREGULATING THE EXPRESSION OF CYTOCHROME C AND CASPASE 3 IN CYCLOSPORINE A-INDUCED APOPTOSIS

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Abstract

The adverse effect of CsA on the morphology and function of kidney makes nephrotoxicity its key limiting factor. However, the mechanisms of chronic CsA-provoked nephrotoxicity seem to be multifactorial. An *in vitro* study carried out with LLC-PK1 cell line has provided evidence that the major side effect of CsA might exhibit significant alterations on intracellular calcium pathway. Further, cytochrome C and caspase-3 expressions were also augmented during Cyclosporine A (CsA) exposure, which eventually led to apoptosis. Yet, the underlying molecular mechanism behind the effect of CsA on calcium signaling pathway is inadequate. The present study unravels the status of calcium signaling, cytochrome C and caspase-3 in CsA-induced apoptotic mechanism and the role of sulphated polysaccharides will be evaluated for its beneficial role in the above mechanism. Co-treatment of CsA-induced LLC-PK1 cells with sulphated polysaccharides (extracted from marine brown algae, "*Sargassum wightii"* prevented abnormal increase in intracellular calcium concentration due to CsA administration, as reflected by an intense fluorescence using Fura-2AM staining. Decrease in caspase activation and cytochrome C level on sulphated polysaccharides administration also shows the anti-apoptotic role of sulphated polysaccharides through its antioxidant nature.

Keywords: Cytochorme C, Caspase 3, Sulphated polysaccharides, Cyclosporine A, Apoptosis, Intracellular calcium.

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2. Introduction

In many models of apoptosis changes in the redox status of the cell is one of the hallmark features observed. This fact is further supported by the ability of various antioxidants such as N-acetylcysteine, in blocking apoptosis in a similar way as that of caspase inhibitors (McGowan *et al*., 1996). It has been strongly proposed that Cyclosporine A (CsA) increases reactive oxygen species production both *in vivo* and *in vitro*, and hence this might also be the major contributor of CsA-induced apoptosis. CsA induced changes such as structural injury and loss of cell viability are documented in LLC-PK1 cells (Polte*et al*., 2002), which were previously introduced as a model system to study the mechanisms of CsA-induced nephrotoxicity and its prevention. Alteration in calcium regulatory mechanism results in mitochondrial transmembrane potential damage, thus causing free radical production and apoptosis. Thus maintenance of intracellular calcium level is indispensable for the function and survival of the cell. Additionally, CsA is also found to affect the extracellular domain of calcium signaling pathway (Lo Russo *et al*., 1997). Previous studies demonstrate that the calcium channel inhibitors like verapamil and diltiazem exerted a positive influence on CsA-treated patients. The above reports strongly suggest that CsA-induced apoptosis is associated with increased intracellular calcium concentration (Cheng *et al*., 2002).

Sulphated polysaccharides from marine brown algae, *Sargassum wightii (S. wightii*), are known to reveal diverse pharmacological and biomedicinal effects like anti-coagulant, anti-tumor, hypoglycaemic, antioxidant, anti-lipemic, anti-ulcerogenic and anti-inflammatory actions (Berteau and Mulloy, 2003). The stability of seaweeds to oxidation upon storing also suggests that their cells have phenomenal defense mechanisms such as antioxidant effect. Free radicals, being the major root cause for apoptosis, antioxidant that could curtail the free radical production might be an ideal therapeutic strategy to prevent CsA-induced apoptosis (Josephine and Ashok Kumar, 2014). Sulphated polysaccharides, being the significant antioxidant was evaluated in the present study for its effect on intracellular calcium, cytochrome c and caspase3 activation, which is a mediator of apoptosis upon CsA administration.

3. Materials and Methods

2.1. Drugs and Chemicals

CsA was procured from Sandoz Ltd., Basel, Switzerland. *S. wightii* was collected from Mandapam, Gulf of Mannar region, Rameswaram, India. Extraction of sulphated polysaccharides from *S. wightii* was done according to the method of Vieira *et al*. (1991). LLC-PK1 cells (Porcine kidney epithelial cells) were purchased from National Centre for Cell Sciences (NCCS), Pune, India.

2.2. Assessment of intracellular calcium accumulation by Fura-2 AM staining

For the measurement of intracellular calcium concentrations, LLC-PK1 cells were treated with fura-2 AM (5 μM) at 37°C for 30 min. Cells after washing with PBS were visualized with a Nikon fluorescent microscope, using a 364-nm excitation and emission at 515–560 nm.

2.3. Estimation of cytosolic cytochrome c level and caspase-3

For analysis of cytochrome c, the protocol of Kim *et al*., (2005) was followed. The expression of caspase-3 was assessed by western blotting technique.

2.4. Statistical Analysis

The results are expressed as mean ± S.D. for three experiments. Differences between groups were assessed by ANOVA using the SPSS (Statistical Package for Social Sciences) software package for Windows. Post hoc testing was performed for inter-group comparisons using the Least Significance Difference (LSD) test; significance at P - values < 0.001, < 0.01 and < 0.05 has been given respective symbols in the figures.

4. Results and Discussion

Numerous evidences show that CsA-induces elevation in intracellular calcium concentration (Cheng *et al*., 2002), which then favors the release of pro-apoptotic proteins and finally leading to apoptosis. In the present study, CsA exposed LLC-PK1 cells exhibited a significant increase in the accumulation of intracellular calcium (Figure 1b), as reflected by intense fluorescence. Abnormal intracellular calcium loading was considerably restored by sulphated polysaccharides treatment (Figure 1d). It has also been found that closing of potassium channels depolarizes mitochondrial membrane leading to the opening of voltage-gated L-type calcium channels, as well as influx of calcium and vasoconstriction (Weir and Archer, 1995). On the other hand, co-treatment of CsA-induced LLC-PK1 cells with sulphated polysaccharides prevented the abnormal increase in intracellular calcium concentration (Figure 1d). Since, it is known that ROS induces an alteration in the intracellular calcium level and apoptosis, key antioxidants that are known to block free radical

productions, might prevent the influx of calcium in the cells.

The succeeding step after the release of cytochrome c is eventually the activation of caspases in the apoptotic machinery. Caspases also appear to be redox sensitive. Even mild oxidative stress has been shown to activate caspases, such as caspase-3 and caspase-8, although the exact mechanism is unclear. Multiple stimulators such as such as free radicals and calcium overload can activate caspases, the cell death executioners during the process of apoptosis (Green and Reed, 1998). Among the different types of caspases, caspase-3 plays a key role in the eventual development of apoptosis. This is evident from the results of the present study, wherein increased cytochrome C level (Figure 2) and caspase-3 activation was seen in CsA-exposed LLC-PK1 cells (Figure 3). The present observation is in consonance with various reports, showing that caspase-3 activity and caspase-3 mRNA expression were augmented during CsA exposure, which is associated with apoptosis (Shi *et al*., 2002). Thus increase in cytosolic cytochrome c level coupled with increased caspase-3 activation in CsA exposed LLC-PK1 cells in the present study is an evident of the apoptotic stimuli by CsA.

Decrease in caspase activation on sulphated polysaccharides treatment show the anti-apoptotic role of sulphated polysaccharides. Although multiple factors or stimuli are involved in apoptosis behind caspase-3 activation, free radicals were also found to play a major role in caspase-3 activation and hence supplementation of antioxidants would be a prophylactic strategy to inhibit the cell death by preventing the activation of apoptotic cascades. Similarly, in the present study, sulphated polysaccharides through its antioxidant effect prevented cytochrome c release and thereby effectively inhibited caspase-3 activation. The present observation is in corroboration with the report of Jhamandas*et al*. (2005), which showed that caspase activity was decreased on administration of a sulphated polysaccharides, fucoidan to neuronal cultures. Overall, the study is a pointer to prove the anti-apoptotic role of sulphated polysaccharides in CsA-induced apoptotic condition.

5. Conclusion

To sum up, the present study documents the anti-apoptotic role of sulphated polysaccharides through its antioxidant nature, by decreasing the intracellular calcium level, expressions of cytochrome C and caspase-3, which was significantly upregulated upon CsA administration.

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Conflict Of Interest

The Authors declare that there is no conflict of interest.

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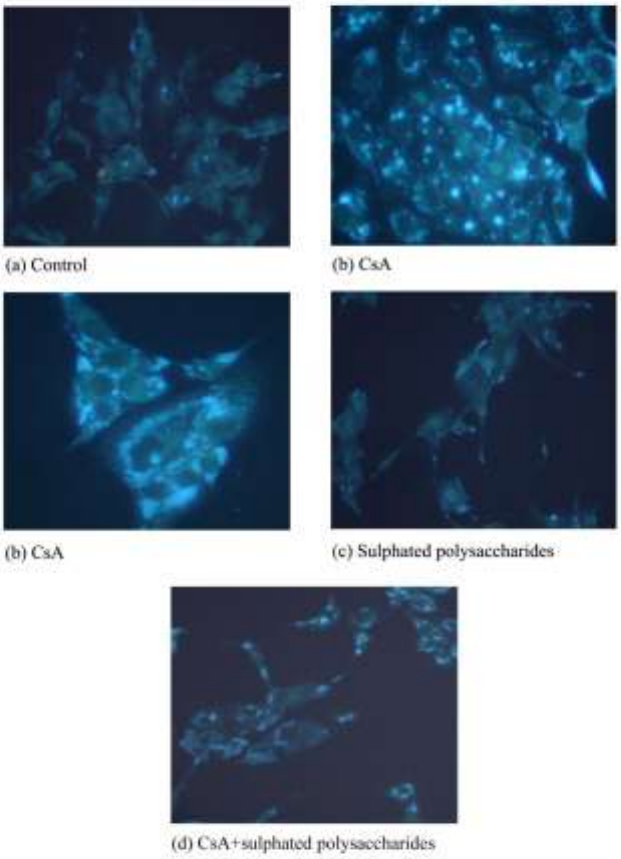
Figure 1. Effect of sulphated polysaccharides on CsA-induced intracellular calcium level

Figure 3.6. Effect of sulphated polysaccharides on CsA-induced intracellular calcium accumulation



Control and drug control cells show normal level of intracellular calcium, as evident by less fluorescence (a and c); CsA-induced LLC-PK1 cells show intense fluorescence, indicating significant increase in intracellular calcium accumulation (b); Sulphated polysaccharides treated CsA-induced cells prevented abnormal increase in intracellular calcium accumulation (d).

Figure 2. Effect of sulphated polysaccharides on cytochrome c levels in CsA-induced LLC-PK1 cells

Figure 3. Effect of CsA and sulphated polysaccharides on caspase-3 activation in LLC-PK1 cells