

c-myc Regulation and Apoptosis in Assessing the Beneficial Effect of Apigenin in Cyclosporine Induced Nephrotoxicity

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Abstract: Cyclosporine-A (CsA) is an immunosuppressant prescribed in organ transplants to prevent rejection. It is a calcineurin inhibitor produced by the fungi *Trichoderma polysporum* and *Cylindrocarpon lucidum*, its adverse effect of renal dysfunction has limited its use in a clinical setting. Apigenin (4', 5', 7'-Trihydroxyflavone), a herbal extract, with anti-inflammatory and anti-tumour properties has shown to reverse this adverse effect. This research was conducted to study the effects of apigenin on reversal of Cyclosporine-A induced damage and this was assessed by immunohistochemical estimation of expression of c-myc and estimation of apoptosis in histopathological sections. Rats were divided into groups and administered with CsA with Apigenin in different doses. The kidneys from the rats were harvested, weighed and observed for gross pathology changes. The renal tissue was processed and stained for haematoxylin and eosin staining, to assess the apoptotic index and stained by immunohistochemistry, for the analysis of the apoptosis regulatory gene c-myc. The apoptotic index was then compared with the c-myc intensity to observe for any correlation. It was found that there was a high apoptotic index and c-myc intensity in the Cyclosporine-A group. Apigenin managed to reduce the values of both parameters. The apoptotic index correlated with the c-myc intensity, especially in the glomeruli. The study proved that Cyclosporine-A enhanced the expression of c-myc in the rat kidney, which signifies accelerated apoptosis. Therefore, c-myc and apoptotic index may be used to assess apigenin and its effect on Cyclosporine-A induced renal damage.

Key words: Cyclosporine, nephrotoxicity, apigenin, 4', 5', 7'-Trihydroxyflavone, apoptosis, c-myc

INTRODUCTION

Cyclosporine-A or Cyclosporin is widely used as an immunosuppressing agent in organ transplantation. It was formally found in the fungi *Trichoderma polysporum* and *Cylindrocarpon lucidum* (Borel *et al.*, 1977). Besides being prescribed for organ transplant patients, it is also used in patients with rheumatoid arthritis and psoriasis (Webster, 2005). Cyclosporine-A acts by inhibiting Interleukin-2 and cytokine production. It is specific for T-lymphocytes and does not affect haematopoietic tissue. Cyclosporine-A successfully inhibited rejection in patients who received kidney transplants from mismatched donors but nephrotoxicity and hepatotoxicity were clearly visible side effects in patients. Other minor side effects of Cyclosporine-A have been identified as hirsutism, hyperglycemia, hypertension, hyperuricemia, hyperkalemia, hypertrichosis, tremors and gingival hyperplasia. The major advantage of Cyclosporine-A

compared to other immunosuppressant drugs is its lack of bone marrow toxicity. Cyclosporine-A acts on proliferating T-cells but not on mature T-cells. In addition, functions of mature B-cell and macrophages remain unaffected by Cyclosporine-A (Chong *et al.*, 2009).

Cyclosporine-A nephrotoxicity can be characterized by the presence of interstitial fibrosis, isometric tubular vacuolisation and the thickening of arteriolar walls (Shihab *et al.*, 1999). It may be difficult to distinguish acute Cyclosporine-A toxicity from acute organ transplant rejection. However, it was suggested that the former can be discriminated from each other by radiological methods.

Apigenin (4',5',7'-Trihydroxyflavone), a herbal extract was reported to have antiviral, anti-allergic, antiplatelet, anti-inflammatory, antitumour and antioxidant activities (Chong *et al.*, 2009). Apigenin has shown to arrest the proliferation of several cancer cell lines through several mechanisms such as by decreasing the expression

of bcl-2 and inducing the expression of p53 gene (Zheng *et al.*, 2005; Srikumar *et al.*, 2009). In addition, studies found that apigenin inhibits the proteasome activity that is required by cancer cells for survival (Chen *et al.*, 2005). Apigenin was also found to inhibit the motility and invasiveness of carcinoma cells *in vitro*. This was observed in HeLa wild-type cells and HeLa Cx43 transfectants, which were found to be highly invasive in the control group but were significantly reduced in by apigenin (Czyz *et al.*, 2005). Apigenin is a useful therapeutic management of inflammatory diseases. Its proposed mechanism is by inhibiting NO-mediated COX-2 expression and monocyte adherence (Lee *et al.*, 2007). Apigenin also has an antiallergic property. Hirano *et al.* (2004) discovered that the anti allergic property of Apigenin was due to the inhibition of IL-4 and 13 production by apigenin. IL-4 and 13 are cytokines produced by basophils that lead to an allergic reaction. Apigenin has shown to reverse the adverse effect of Cyclosporine-A on the kidney in preliminary studies. This research was conducted as a pioneer one to study the effects of apigenin on reversal of Cyclosporine-A induced damage and this was assessed by immunohistochemical estimation of expression of bcl-2 and estimation of apoptosis in histopathological sections.

c-myc is a nuclear transcription factor that functions as a master regulator of the cell cycle and thus regulates proliferation, differentiation, neoplasia and cell death. c-myc was identified as a proto-oncogene and immediate early response gene and thus, attention initially focused on its role in the cell cycle. However, enforced expression of c-myc augments the apoptotic program and thus, rapid cell death occurs when cells are deprived of survival factors. In addition, c-myc also plays an active role in cell death caused by environmental stresses including viral infection, T cell receptor activation, tumor necrosis factor and chemotherapeutic agents. Apoptosis induced by enforced c-myc expression requires a functional transactivation domain indicating that activation of gene targets is necessary (Cowley *et al.*, 1989).

In addition to other forms of renal damage, apoptosis is a critical early cellular event in the development of Polycystic Kidney Disease (PKD) in humans and mice. In the SBM transgenic model of PKD, both apoptosis and proliferation are c-myc driven and are independent of p53 and bcl-2 pathways (Couillard *et al.*, 2002). Although, enforced c-myc expression induces apoptosis after withdrawing survival factors, it is not clear if activation of the endogenous c-myc gene is an apoptotic signal after toxicant exposure. The renal tubular epithelium is a target for many toxicants. c-myc expression is activated by tubular damage (Zhan *et al.*, 1997).

Apoptosis is the physiological death of a cell and is tightly regulated intracellularly. Apoptosis does not induce an immune response compared to necrosis, as the cells are quickly cleared up by phagocytes.

The cell activates intracellular enzymes with function to digest or degrade the cell's own nuclear DNA and proteins of nuclear as well as cytoplasmic origin. An apoptotic cell is identified as a cell with membrane blebbing, condensed chromatin and cell shrinkage (Shihab *et al.*, 1996).

MATERIALS AND METHODS

Male sprague-dawley albino rats were used for the research, after due ethical committee approval. They were 6-8 weeks old, weighed between 200-250 g and were fed with standard rat chow and free access to water. The weight of the rats was recorded once every week.

Cyclosporine-A was acquired from Novartis, Switzerland and was used at dosage of 25 mg mL⁻¹. Apigenin was acquired in powdered form from Sigma Aldrich, with a purity of 98%. Apigenin was prepared at 3 different doses, 10, 15 and 20 mg mL⁻¹.

Groups and sample collection: There were a total of 6 experimental groups for this study (Table 1). Rats were dosed every 24 h for 21 days. After the 21st day, they were sacrificed and their kidneys harvested. The gross morphology was observed and the kidneys were then stored in 10% formalin. They were weighed (Mettler Toledo College B204-5) and measured using a caliper before they were sliced, processed and mounted on paraffin blocks.

Staining procedure: The paraffin blocks were sectioned and slides stained with haematoxylin and eosin. Mouse monoclonal (DAKO) antibody to c-myc diluted at a ratio of 1:100 (pH 7.6, 90 min, room temperature) was used for immunohistochemical staining of the paraffin blocks, after target retrieval at 95°C for 45 min. The slides were observed for the expression of c-myc in the glomeruli and tubules using a Nikon brightfield light microscope. Images were captured with a 5.1 megapixel evolution MP digital camera. Image proexpress software was used to process the images. The images were then analysed.

Apoptotic index in H and E: The apoptotic index was evaluated in the Haematoxylin and Eosin (H and E) stained slides. The number of apoptotic cells were counted in the glomeruli (500 cells) and tubules (500 cells) of the tissues. The apoptotic cells were identified as cells shrinkage, deeply eosinophilic cytoplasmic staining and dense nuclear staining compared to normal cells due to

Table 1: Groups used in this research and their respective administered drugs

Groups	Dosage
Control	Corn oil
CsA A	Cyclosporine-A at 25 mg kg ⁻¹ body weight
Apg 20	Apigenin at 20 mg kg ⁻¹ body weight
CsA + Apg (10)	Cyclosporine-A at 25 mg kg ⁻¹ body weight + Apigenin at 10 mg kg ⁻¹ body weight
CsA + Apg (15)	Cyclosporine-A at 25 mg kg ⁻¹ body weight + Apigenin at 15 mg kg ⁻¹ body weight
CsA + Apg (20)	Cyclosporine-A at 25 mg kg ⁻¹ body weight + Apigenin at 20 mg kg ⁻¹ body weight

Table 2: Grading of percentage of c-myc expression

Grades	Percentage of cells in tissue expressing c-myc
0	0% of the cells expressed c-myc
+	Less than 33% of the cells expressed c-myc
++	34%-66% of the cells expressed c-myc
+++	More than 67% of the cells expressed c-myc

the chromatin condensation. The cells were counted by selecting the first suitable field from the left side of the tissue and moving the stage towards the right side.

c-myc expression by immunohistochemistry: Cells that expressed c-myc positivity were characterized by a brownish bronze coloured pigmentation within the cytoplasm. Slides that exhibited positivity were categorised as (+), (++) or (+++) depending on the percentage of the staining. Slides that were negative for c-myc were labelled as 0 (Chong *et al.*, 2009) (Table 2).

Statistical analysis: In this study, 30 samples were studied and analysed. Statistical tests consisted of Kruskal-Wallis test for global comparison of groups; Non-parametric Mann-Whitney-U-test for comparison of apoptotic index in different groups and Spearman's rho test for correlation between apoptotic index and expression of c-myc. The statistical tests were employed on the data using SPSS software. For all individual tests, a (p<0.05) was taken and considered as significant.

RESULTS

Weight and volume of kidneys and apoptotic index: From the Table 3, it was observed that Group 1 has significant difference compared to Group 2 (p<0.01), Group 4 (p<0.01) and 5 (p<0.05). These 3 groups were administered with Cyclosporine-A. This shows that Cyclosporine-A has caused significant apoptosis in the kidney. Group 3 showed no significant change compared to Group 1 as Group 3 was administered with Apigenin only. This shows that Apigenin has no significant morphological effects on the kidney. Although, Group 6 was administered with Cyclosporine-A, there was no significant difference when compared to Group 1. This is probably due to the high Apigenin dosage administered

in this group. This shows that Apigenin at a dose of 20 mg kg⁻¹ body weight successfully protected the kidneys from the apoptotic damage caused by Cyclosporine-A.

Group 2 was also compared to Groups 3-6 and showed significant difference to the other groups. Group 2 was administered with Cyclosporine-A and no additional drugs. This proved that Cyclosporine-A has tremendous detrimental effects on the kidneys leading to severe apoptosis of cells in the kidneys. Apigenin, which was given in Groups 4, 5 and 6 along with Cyclosporine-A managed to protect the kidneys from the toxic effects of Cyclosporine-A, thus showing a significant statistical value when compared with Group 2.

Correlation of kidney weights, kidney volumes and apoptotic index: Non-parametric Spearman's rho test was utilised to determine if there was any correlation between the average kidney weight, average kidney volume and average apoptotic index in all groups and showed the kidney weight and volume were positively correlated to one another.

Immunohistochemistry for c-myc expression: Immunohistochemistry staining was performed to evaluate the expression of bcl-2 in the slides (Fig. 1). The grading was assessed on the percentage of cells that expressed bcl-2 (Srikumar *et al.*, 2009) (Table 4). Group 2 has the highest median value, while Group 1 and 6 have the lowest median value. In Group 3, the Apigenin 20 group showed a moderately high bcl-2 expression. From the Mann-Whitney U-test conducted, it was found that there was a significant difference in both the glomerular and tubular areas of the kidney in when group 1 was compared to Group 2 and when Group 2 was compared to Group 6. However, there was significant difference only in the glomerular area when Group 1 was compared to group 4. In the rest of the group comparisons, it was found that there was no significant difference between the groups.

Correlation of apoptotic index and expression of c-myc: The median of both Apoptotic index and c-myc expression was used to compare and to examine if any correlations existed between the two parameters. Non parametric Spearman's rho test was also used to find out if there was any correlation between the apoptotic index and c-myc expression. For each group, the median value was used. Glomerulus apoptotic index vs. glomerulus c-myc expression had a correlation coefficient of 0.820. Tubules apoptotic index vs. tubules c-myc expression had a correlation coefficient of 0.278. The values show that

Table 3: The average weight and average volume of kidneys in each group with the average Apoptotic Index and the p-values of the groups when compared with group 1 and group 2. Statistical method used was the Mann-Whitney U-test

Groups	Average weight	Average volume	Average apoptotic index	p-value of apoptotic index	
				Group 1	Group 2
1	2.513	1.163	22.0	-	0.008**
2	1.869	1.112	54.4	0.007**	-
3	2.436	1.232	18.8	0.053	0.008**
4	2.167	1.245	36.0	0.008**	0.024*
5	1.697	1.134	27.4	0.018*	0.008**
6	1.872	1.267	24.0	0.093	0.024*

*Significant difference at $p < 0.05$; **Significant difference at $p < 0.01$

Table 4: The median c-myc expression in the glomeruli and tubules as well as the results of the Mann-Whitney U-test conducted for comparison between 2 groups for c-myc expression

Groups	Glomeruli c-myc expression	p-value glomeruli		Tubules c-myc expression	p-value in the tubules	
		Group 1	Group 2		Group 1	Group 2
1	1+	-	0.005*	2+	-	0.013*
2	3+	0.007*	-	3+	0.014*	-
3	2+	0.089	0.051	3+	0.038	0.071
4	2+	0.014*	0.071	3+	0.048	0.081
5	2+	0.045	0.055	3+	0.048	0.044
6	1.5+	0.301	0.028*	1.5+	0.087	0.016*

*Significant difference at $p < 0.05$

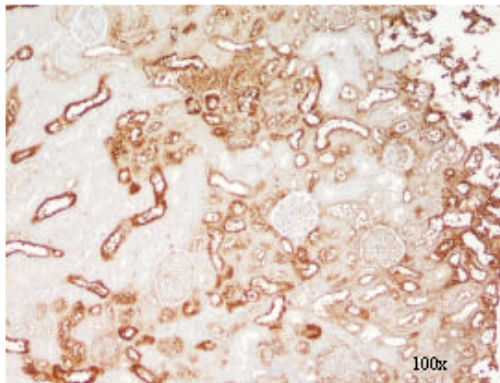


Fig. 1: Photomicrograph showing diffuse glomerular (+) and tubular cells (+++) positivity for c-myc (Immunohistochemistry, 100x magnification)

there was a positive correlation in both the comparisons. In the glomeruli, the apoptotic index (from H and E staining) has a strong correlation with the expression of c-myc (from IHC staining).

DISCUSSION

Apoptosis and Cyclosporine-A induced nephrotoxicity:

Numerous studies have been conducted to examine the clinical possibilities of Apigenin. However, its association with apoptosis and c-myc has not been studied yet. Apoptosis is a natural process of the body to eliminate unwanted or potentially harmful cell and cells that has outlasted its usefulness or importance. It occurs naturally in many situations (Shihab *et al.*, 1996). From the research conducted, the results showed that administration of

Cyclosporine-A increases the apoptotic index in rat kidneys (Table 3). Consequently, the administration of Apigenin successfully reduced the apoptotic index significantly. This shows that Apigenin may have a protective effect at a very high dose. In the study, the apoptotic index was calculated by an accurate method, which was counting the apoptotic cells in 400x and reconfirming by 1000x using oil immersion. This was done for every 1000 cells in the glomeruli and tubules in each case. The total apoptotic index was then calculated for the glomeruli and tubules and statistically evaluated. Although, the mechanisms of Cyclosporine-A nephrotoxicity are not fully known, few studies have shown that the toxicity produces glomerular and tubular damage (Thomas *et al.*, 1998).

One of these manifestations is by inducing apoptosis in cells, which resulted ultimately in the atrophy of the glomeruli and tubules, in addition to focal interstitial changes (Janku *et al.*, 2005). This feature was also confirmed by the study, where the apoptotic index was increased in rats treated with Cyclosporine-A, during the analysis of the histopathological sections. The cells included in the category of apoptotic cells in the study were cells with various stages of the process: cells displaying chromatin condensation; cells with densely eosinophilic cytoplasm and shrunk nuclei and cells with cytoplasmic blebs and nuclear fragments, forming apoptotic bodies.

The study has shown that Apigenin, when combined with Cyclosporine-A therapy was able to prevent the glomerular and tubular changes to a significant extent, which was reflected by the variation in the apoptotic indices in the various groups.

c-myc and its role in apoptosis: c-myc protein was stained in normal with greater intensity and focally in diseased renal tissue, showing an analogous expression. The antigen was expressed in a few parietal epithelial cells, in scattered proximal tubular epithelial cells and in the majority of distal and collecting tubular epithelial cells but not in the glomerular capillary tuft. The pattern of c-myc expression in normal and diseased glomeruli and tubules suggests and supports the reported notion that the mechanism of apoptosis may be increased in the injured glomerulus (Zhan *et al.*, 1997).

Based on the non-parametric Spearman's test conducted, there was a correlation between the Apoptotic Index and c-myc expression, especially in the glomeruli. This showed that the damage to the glomeruli and tubules in Cyclosporine-A mediated toxicity was facilitated through the expression of c-myc. This expression of c-myc in turn reflects the increased apoptotic death of the cells. These findings also correlated well with studies conducted by Cowley *et al.* (1989), Couillard *et al.* (2002) and Zhan *et al.* (1997).

There are a number of theories concerning how the c-myc gene family exert their pro-apoptotic effect. An important one states that this is similar to bcl-2 action, which is achieved by activation or inactivation of an inner mitochondrial permeability transition pore, which is involved in the regulation of matrix Ca^{2+} , pH and voltage.

It is also thought that some c-myc proteins act like bcl-2 family proteins which can induce (pro-apoptotic members) or inhibit (anti-apoptotic members), the release of cytochrome c into the cytosol, which once there activates caspase-9 and 3, leading to apoptosis (Kinnally and Antonsson, 2007).

Evaluation of the c-myc expression was done immunohistochemically to give additional evidence on the renal damage of Cyclosporine-A and also to observe the less extent of damage in the Cyclosporine-A with Apigenin group. Hence, it was used as a confirmatory parameter. Though statistical comparison was not necessary however, when it was done, it showed an acceptable correlation.

Moreover, from the observation of the median, there may be a change in the percentage of expression. Therefore, findings can be continued with a larger sample size. The weight and volume of the kidneys are directly proportional to each other. The apoptotic index and over expression of c-myc showed a significant correlation to both kidney weight and volume. Both these parameters adhered well with our hypothesis.

Although, all doses of Apigenin were beneficial to the morphology of the kidney, it was found that the ideal dosage of Apigenin in reversal of Cyclosporine-A

toxicity was 20 mg kg^{-1} body weight. Apigenin by itself at the dose of 20 mg kg^{-1} did show a high expression of c-myc. This may suggest that Apigenin can ideally be used in association with Cyclosporine-A rather than being given alone. This also gives scope for future studies into the effects of Apigenin when given alone.

Limitations to this study: This study focused more on the expression of c-myc, which was used as a parameter to assess the renal damage due to Cyclosporine-A. A comparative analysis with other apoptosis regulating markers like bcl-2 and TGF- β would help gain more insight into the mechanism. Further studies on the mitotic index may also help to analyse the comparison between mitosis and apoptosis and strengthen the evidence for our hypothesis.

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