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ORIGINAL ARTICLE

Expression of transforming growth factor-β and determination of apoptotic index in histopathological sections for assessment of the effects of Apigenin (4', 5', 7' - Trihydroxyflavone) on Cyclosporine A induced renal damage

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Abstract

Cyclosporine A (CsA), a calcineurin inhibitor produced by the fungi *Trichoderma polysporum* and *Cylindrocarpon lucidum,* is an immunosuppressant prescribed in organ transplants to prevent rejection. Its adverse effect on 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 been investigated for properties to reverse this adverse effect. This research was conducted to establish a standard protocol for immunohistochemical estimation of Transforming Growth Factor β (TGF-β) expression, as an indicator of Cyclosporine A induced damage, and to observe whether apoptotic index and TGF-β expression can be used to assess effects of Apigenin on CsA induced renal dysfunction. Six groups of 5 male Sprague-Dawley albino rats each were dosed once daily for 21 days, as follows: (1) negative control – oral corn oil, (2) positive control – Cyclosporine A (25 mg/kg), (3) Group 3 - Apigenin (20 mg/kg), (4) Group 4 - Cyclosporine A (25 mg/kg) + Apigenin (10 mg/kg), (5) Group 5 - Cyclosporine A (25 mg/kg) +Apigenin (15 mg/kg) and (6) Group 6 - Cyclosporine A (25 mg/kg) +Apigenin (20 mg/kg). Cyclosporine A was administered intra-peritoneally while Apigenin was given orally. The rat kidneys were harvested and examined microscopically to assess the apoptotic index, and stained by immunohistochemistry for multifunctioning polypeptide TGF-β expression. A high apoptotic index and TGF-β intensity was observed in the Cyclosporine A group. Apigenin significantly reduced the both apoptotic index and TGF-β intensity. The apoptotic index correlated with TGF-β intensity, especially in glomeruli. This study indicates that Cyclosporine A can enhance the TGF-β expression in rat kidney, signifying accelerated apoptosis. TGF-β and apoptotic index may be used to assess Apigenin and its effect on Cyclosporine A induced renal damage.

Keywords: Cyclosporine A, nephrotoxicity, Apigenin, Apoptotic Index, Transforming Growth Factor β (TGF- β)

INTRODUCTION

Cyclosporine A or Ciclosporin is widely used as an immunosuppressing agent in organ transplantation. It was formally found in the fungi *Trichoderma polysporum* and *Cylindrocarpon lucidum.*[1] Besides being prescribed for organ transplant patients, it is also used in patients with rheumatoid arthritis and psoriasis.[2] Cyclosporine A acts by inhibiting Interleukin-2 and cytokine production. It is specific for T-lymphocytes and does not affect haematopoietic tissue.[3] 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

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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.[4]

Cyclosporine A nephrotoxicity can be characterized by the presence of interstitial fibrosis, isometric tubular vacuolations and thickening of arteriolar walls.[5,6] It may be difficult to distinguish acute Cyclosporine A toxicity from acute organ transplant rejection. However, it was suggested that they can be discriminated from each other by radiological methods.[7]

Apigenin (4', 5, 7-Trihydroxyflavone), a herbal extract, has been reported to have antiviral, anti-allergic, antiplatelet, anti-inflammatory, antitumour and antioxidant activities.[8] Apigenin has been 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.[9] In addition, studies found that apigenin inhibits the proteosome activity that is required by cancer cells for survival.[10] 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 by apigenin.[11] Apigenin is also useful in the therapeutic management of inflammatory diseases. Its proposed mechanism is by inhibiting NO-mediated COX-2 expression and monocyte adherence.[12] Apigenin also has antiallergic property. Hirano *et al.* discovered that the anti-allergic property of Apigenin was due to the inhibition of IL-4 and IL-13 production. IL-4 and IL-13 are cytokines produced by basophils that can lead to allergic manifestations.[13]

Transforming growth factor β (TGF-β) is a pro-inflammatory cytokine which exists in 5 isoforms, namely TGF-β1, TGF-β2, TGF-β3, TGF-β4 and TGF-β5.[14] TGF-β is mainly produced and secreted by T-helper cells, B-cells, macrophages, and mast cells.[15] Some TGF-β variants inhibit the growth of endothelial cells[14] while others regulate the proliferation and differentiation of cells, embryonic development, wound healing and angiogenesis.[16] It has the property of inhibiting cellular immunity.[17]

TGF-β is found to be significantly elevated in Cyclosporine A treated rats. TGF-β is localized within the granular cells of the juxtaglomerular arterioles. On Northern Blot RNA analysis, there was increased staining, confirming the presence of increased transcription of TGF-β.[18] TGF-β causes renal disease by inducing fibrogenesis in the renal parenchyma. Rats given Cyclosporine A showed an increased expression of mRNA coding for TGF-β1 which occurs mostly in the tubulointerstitial and vascular compartment of the kidney. Salt depletion has been shown to accelerate the incidence of nephrotoxicity. Physiological changes that accompany the nephropathy are increased serum creatinine, reduced creatinine clearance, increased tubular enzymuria and the loss of medullary concentrating ability.[19]

Apoptosis is a form of physiological cell death and is tightly regulated intracellularly. Apoptosis does not induce an immune response compared to necrosis, as the cells are quickly cleared by phagocytes. The cell activates intracellular enzymes which 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.[20]

As mentioned, Cyclosporine enhances the expression of TGF-β in the juxtaglomerular cells of the rat kidney, while TGF-β in turn accelerates tubulointerstitial apoptosis. So expression of TGF-β and apoptosis may be suitable indicators to assess renal damage.

MATERIALS AND METHODS

Experimental rats

Thirty (30) male Sprague-Dawley albino rats comprising 6 groups of 5 rats each were used for this study (Table 1). They were acquired from the Institute for Medical Research (IMR), Malaysia. Dosing began when the rats were 6-8 weeks old and weighing between 200-250 grams. They were housed in groups of 5 rats in metal cages located within the Animal Housing Facility in the International Medical University. The rats were fed with standard rat chow and were given free access to water. The weight of the rats was recorded once every week.

Drug preparation

Cyclosporine A was acquired from *Novartis, Switzerland* at 100 mg/mL. To attain 25 mg/mL, 5 mL of Cyclosporine A was diluted into 15 mL of 0.9% saline using the syringe provided into a sterile measuring cylinder. The final solution was mixed thoroughly and kept in a conical tube.

TABLE 1: Grouping of experimental rats and their respective administered drugs

Group	Dosage
Group 1 – Control	Corn Oil
Group 2 – Cyclosporine A	Cyclosporine A at 25 mg/kg body weight
Group 3 - Apigenin 20	Apigenin at 20 mg/kg body weight
Group 4 - Cyclosporine A + Apigenin 10	Cyclosporine A at 25 mg/kg body weight + Apigenin at 10 mg/kg body weight
Group 5 - Cyclosporine A + Apigenin 15	Cyclosporine A at 25 mg/kg body weight + Apigenin at 15 mg/kg body weight
Group 6 - Cyclosporine A + Apigenin 20	Cyclosporine A at 25 mg/kg body weight + Apigenin at 20 mg/kg body weight

Apigenin was obtained in powdered form from Sigma Aldrich, with a purity of 98%. Apigenin was suspended in high grade corn oil. Apigenin was prepared at 3 different doses, i.e. 10 mg/mL, 15 mg/mL and 20 mg/mL. 200 mg of Apigenin was added into 20 mL of corn oil to acquire 10 mg/mL concentration whereas 300 mg of Apigenin was added into 20 mL of corn oil to acquire 15 mg/mL concentration. Finally, 400 mg of Apigenin was added into 20 mL of corn oil to attain 20 mg/mL concentration.

Groups and sample collection

There were 6 experimental groups for this study (Table 1). The negative control group was given corn oil orally whereas the positive control group was given Cyclosporine A intra-peritoneally to allow the rats to acquire maximum effect of the drug. There was one group given apigenin orally at 20 mL/kg body weight. The final 3 groups were administered with apigenin accompanied by Cyclosporine A. Apigenin was given orally while Cyclosporine A was administered intra-peritoneally. In these 3 groups, Apigenin was given at different doses, which were 10 mL/kg body weight, 15 mL/kg body weight and 20 mL/kg body weight. However, Cyclosporine A was administered at a constant dosage of 25 mL/kg body weight in the relevant groups. Rats were dosed once every 24 hours 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. For this study, only the right kidneys were analysed. They were weighed (*Mettler Toledo College B204-5*) and measured using a caliper before they were sliced, processed for analysis.

Staining procedure

Tris-buffered saline (*DAKO*) was prepared by dissolving one full sachet (16.15*g* $\pm$ 10%) into 1 litre of distilled water and mixed in a Schott & Duran bottle. 20 mL of TBS was pipette out into a coplin jar to ensure an ideal pH of 7.6 based on the literature provided by DAKO (*Mettler Toledo 320*).

Target retrieval solution (DAKO) was used on paraffin-embedded sections mounted on sialinised slides for heat-induced target retrieval before immunohistochemistry staining, at a dilution ratio of 1:10 with pH 9.0.

Mouse monoclonal [TB-21] (DAKO) antibody to transforming growth factor-β was diluted at a ratio of 3:1000 based on the literature provided by DAKO, with antibody diluent with background reducing components.

Substrate chromogen (DAKO) was used at a dilution of 1:50. For this experiment, 5*mL* of Dual Link System-HRP (DAB+) chromogen was diluted into 250 *mL* of DAB+ chromogen buffer.

The tissue was embedded in paraffin wax with ceresin using L-moulds. Sections of 4 µm were cut from the paraffin blocks. This was done using a rotary microtome knife (*Leica RM2135*). The cut sections were placed on the sialinised slide from DAKO and dried at 35 ˚C for 5 minutes before staining.

Hematoxylin and eosin staining

Before staining, the tissues were dewaxed using hot air oven at 60 ˚C for 45 minutes. Next, the slides were taken through xylene and decreasing strengths of alcohol till distilled water. The slides were then stained with and then differentiated in acid-alcohol, immersed in bicarbonate solution to 'blue' them and stained with Eosin Y. Slides were mounted with cover slips using DPX Vecta Mount Mounting Medium.

Immunohistochemistry Staining

Target retrieval was done using Target retrieval solution (TRS) at 95 ˚C for 45 minutes. The slides were brought to pH 7.6 by placing them in TBS for 10 minutes.

Next, Dual Endogenous Enzyme Block was applied to the tissue sections for 20 minutes, to prevent excess background staining by other tissue proteins. The primary antibody was then applied in amounts sufficient to cover the entire tissue section and left for 90 minutes at room temperature. Labeled Polymer HRP was then applied for 40 minutes. The slides were then rinsed in TBS and immersed for 10 minutes.

Substrate chromogen was applied on the tissue at room temperature for 20 minutes. The slides were then counter-stained with Meyer's Haematoxylin for 10 minutes, to allow for visualization of cell nuclei. The slides were then rinsed under tap water, blued, dried and coverslipped using DPX.

The slides were observed for the expression of TGF-β in the glomeruli and tubules using a Nikon brightfield light microscope. Images were captured with a 5.1 megapixel Evolution MP digital camera. Image Pro Express Software was used to process the images. The images were then analysed.

Analysis of sections

Apoptotic index

The apoptotic index was evaluated in H&E stained slides. The number of apoptotic cells in glomeruli (500 cells) and tubules (500 cells) were counted using a brightfield Nikon microscope at 400X magnification with built-in illumination. Apoptotic cells were identified as cells showing shrinkage, deeply eosinophilic cytoplasmic staining and dense nuclear staining compared to normal cells due to the chromatin condensation.

The cells were counted by selecting the first suitable field from the left side of the section and moving the stage towards the right side. The total number of cells counted both in the glomeruli and the tubules came up to 1000 cells. The tubular cells were the lining epithelial cells while the glomerular cells consisted predominantly of endothelial cells.

TGF-β expression by immunohistochemistry

A Nikon brightfield light microscope at a magnification power of 400x was used to grade the level of TGF-β expression. Cells that expressed TGF-β positivity were characterized by a brownish bronze-coloured pigmentation within the cytoplasm (Fig.1). Slides that exhibited positivity were categorised as (+), (++) or (+++) depending on the percentage of cells showing positive staining.[21] Slides that were negative for TGF-β were labelled as 0 (Table 2). The final score for each rat was based on a consensus of assessment of two viewers.

Statistical analysis

30 rat samples were studied and analysed (Table 2). Statistical tests used were:

(a) Kruskal-Wallis Test for global comparison of groups

TABLE 2: Grading of TGF-β expression in tissue secions

Grade	Percentage of cells in tissue expressing TGF-β
0	0% of cells expressed TGF-β
+	Less than 33% of cells expressed TGF-β
++	34%-66% of cells expressed TGF-β
+++	More than 67% of cells expressed TGF-β

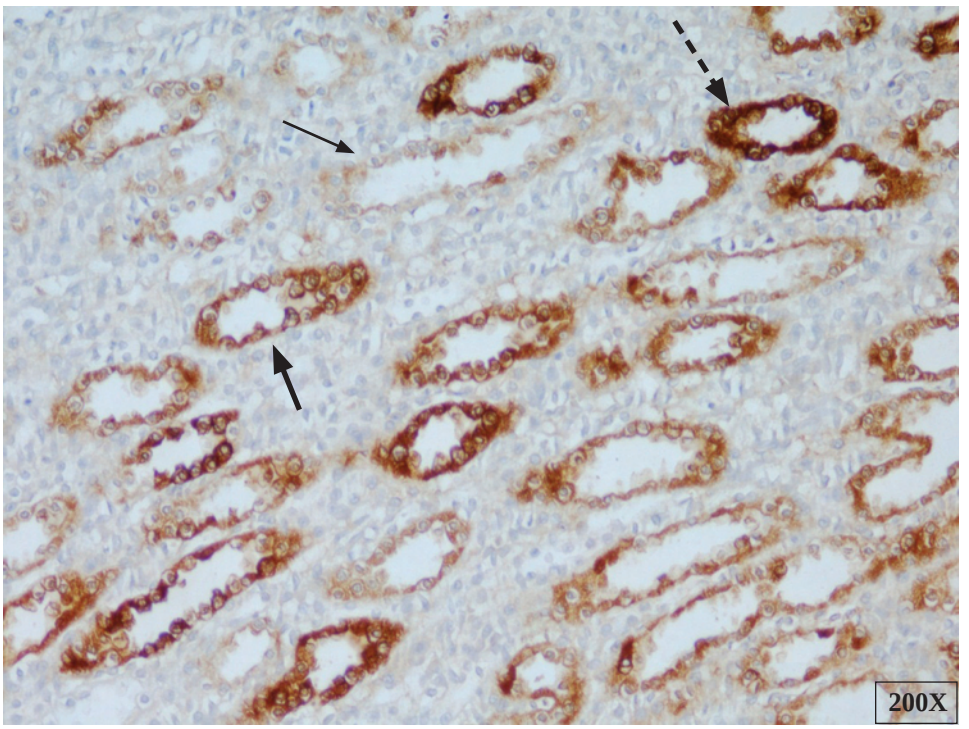

FIG. 1: Photomicrograph showing grades of TGF-β expressions: grade + (thin arrow), grade ++ (thick arrow) and grade +++ (dashed arrow). IHC x200

(b) Non-parametric Mann-Whitney-U Test for comparison of apoptotic index in different groups
(c) Spearman's rho Test for correlation between apoptotic index and expression of TGF-β

The statistical tests were employed on the data using SPSS software. For all individual tests, a *p-value* of less than 0.05 ($p<0.05$) was considered as significant.

RESULTS

Weight and volume of kidneys and apoptotic index

The average weight and volume of the kidneys as well as the average apoptotic index in each group are shown in Table 3. Group 1 showed significant difference in average apoptotic index compared to Group 2 ($p<0.01$), Group 4 ($p<0.01$) and Group 5 ($p<0.05$). Note that Groups 2, 3 and 4 had been administered Cyclosporine A. Group 3 showed no significant change in apoptotic index compared to Group 1. Although Group 6 was administered with Cyclosporine A, there was no significant difference in apoptotic index when compared to Group 1. This is probably due to the high Apigenin dosage administered in this group. This suggests that Apigenin at a dose of 20 mg/kg body weight successfully protected the kidneys from apoptotic damage caused by Cyclosporine A.

The apoptotic index of Group 2 was also significant difference from those of Groups 3, 4, 5 and 6. Group 2 had been administered Cyclosporine A and no additional drugs. This indicates that Cyclosporine A has 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 was able to protect the kidneys from the toxic effects of Cyclosporine A, thus showing a significant statistical difference 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 (Table 4). A value of +1 signifies that there is a very strong and established correlation between two variables and -1 signifies the correlation is inversely related. However, a correlation coefficient of 0 shows that the two variables have no correlation. Using the Spearman's Rho test,

TABLE 3. Average weight, volume of kidneys and apoptoxic index observed in each groups.

Group	Average Weight	Average Volume	Average apoptotic index	*p-value* of apoptotic index (vs. Group 1)	*p-value* of apoptotic index (vs. Group 2)
Group 1	2.516	1.168	21.0	-	0.009**
Group 2	1.998	1.072	59.4	0.009**	-
Group 3	2.578	1.218	19.8	0.530	0.009**
Group 4	2.126	1.238	34.0	0.009**	0.021*
Group 5	1.606	1.041	29.4	0.028*	0.009**
Group 6	1.873	1.276	22.0	0.623	0.014*

*p-values were calculated on the Mann-Whitney U Test. *Significant difference at $p < 0.05$*
*** Significant difference at $p < 0.01$*

the kidney weight and volume were positively correlated to one another. However, the kidney weight and volume were both inversely related to the apoptotic index. A reduction in the volume of the kidney was observed corresponding to the reduction in the weight of the kidneys. However, as the correlation was weakly associated, a greater sample population may clarify the situation.

Immunohistochemistry for TGF-β expression

Table 5 shows the median TGF-β expression grade score in glomeruli and tubules according to groups of rats. In the grading of TGF-*β* expression (Table 2), no half values were used. The half value of 1.5+ in Group 6 was acquired from statistical calculations. Group 2 has the highest median value while Group 1 and Group 6 have the lowest median value. Group 3, the Apigenin 20 group, showed a moderately high TGF-*β* expression. There was a significant difference in median values among the groups.

There was also a significant difference in TGF-*β* expression in both the glomerular and tubular areas in group 1 when compared to group 2, and between group 2 and group 6. However, there was significant difference only in glomerular TGF-*β* expression when group 1 was compared to group 4. In the rest of the group comparisons, no significant difference was shown.

Correlation of apoptotic index and expression of TGF-β

Tables 6 and 7 compares the average apoptotic index and median scores of TGF-β expression in the experimental groups. There was a positive correlation of both parameters in glomeruli and tubules. In the glomeruli, the apoptotic index showed a strong correlation with TGF-β expression.

TABLE 4 : Correlation coefficient between kidney weight, kidney volume and apoptotic index

Comparison	Correlation Coefficient
Kidney weight vs. kidney volume	0.257
Kidney weight vs. apoptotic index	-0.257
Kidney volume vs. apoptotic index	-0.543

Spearman's rho test was used to calculate the correlation coefficient.

TABLE 5: Median TGF-*β* expression grade scores in glomeruli and tubules of experimental groups of rats

Group	Glomeruli TGF-β expression	*p-value* in glomeruli (vs Group 1)	*p-value* glomeruli (vs Group 2)	Tubules TGF-β expression	*p-value* in the tubules (vs Group 1)	*p-value* in the tubules (vs Group 2)
Group 1	1+	-	0.007*	2+	-	0.014*
Group 2	3+	0.007*	-	3+	0.014*	-
Group 3	2+	0.189	0.054	3+	0.058	0.221
Group 4	2+	0.015*	0.072	3+	0.058	0.221
Group 5	2+	0.065	0.065	3+	0.058	0.221
Group 6	1.5+	0.371	0.018*	1.5+	0.777	0.018*

Mann-Whitney U Test was used for comparison between 2 groups for TGF-β expression.
** Significant difference at $p < 0.05$*

DISCUSSION

Apoptosis & Cyclosporine A induced nephrotoxicity

Numerous studies have been conducted to examine the clinical possibilities of Apigenin. However, its association with apoptosis and TGF-β has not well studied. Apoptosis is a natural process of the body to eliminate unwanted or potentially harmful cell and cells that have outlasted their usefulness or importance. It occurs naturally in many situations.[20]

Our study showed that administration of Cyclosporine A increases the apoptotic index in rat kidneys. Consequently, the administration of Apigenin significantly reduced the apoptotic index. At a dose of 10 mg/kg body weight (Group 4), the reduction in apoptotic index reached a *p-value* of 0.021 while a dose of 15 mg/kg body weight (Group 5) produced a *p-value* of 0.009. At 20 mg/kg body weight (Group 6), Apigenin showed significant *p-value* of 0.014 compared to the group administered with Cyclosporine A only. This suggests that Apigenin has a protective effect at a very high dose.

Although the mechanisms of Cyclosporine A nephrotoxicity are not fully known, some studies have shown that toxicity produces glomerular and tubular damage.[19] One of these manifestations is apoptosis, resulting ultimately in atrophy of glomeruli and tubules, in addition to focal interstitial changes.[22] This feature was also supported by our finding of an increased apoptotic index in rats treated with Cyclosporine A. Our study has shown that Apigenin, used together with Cyclosporine A, was able to reduce the glomerular and tubular changes to a significant extent, as reflected by the variation in the apoptotic indices in the various groups.

TABLE 6: A comparison of average apoptotic index and median TGF-β expression in glomeruli and tubules.

	Glomerular apoptotic index	Glomerular TGF-β expression	Tubular apoptotic index	Tubular TGF-β expression
Group 1	7	1+	12	2+
Group 2	29	3+	25	3+
Group 3	8	2+	11	3+
Group 4	16	2+	19	3+
Group 5	13	2+	16	3+
Group 6	9	1.5+	13	1.5+

TABLE 7: Correlation coefficient between apoptotic index and TGF-β expression in the glomeruli and tubules.

Comparison	Correlation Coefficient
Glomerular apoptotic index vs. glomerular TGF-β expression	0.820
Tubular apoptotic index vs. tubular TGF-β expression	0.278

Non-parametric Spearman's rho Test was used to calculate the correlation coefficient of median values.

Transforming growth factor β and its role in apoptosis

Transforming Growth Factor β (TGF-β) acts as a chemoattractive agent for monocytes and macrophages. However, it is also an anti-inflammatory agent, for instance, it is capable of inhibiting lymphocyte proliferation, both T-cell and B-cell, and NK cell activity.[16] The mechanism of TGF-β activation is not known.

Our study shows a correlation between the apoptotic index and TGF-β expression, especially in the glomeruli. This suggests that the damage to the glomeruli and tubules in Cyclosporine A mediated toxicity may be facilitated through the over-expression of TGF-β. This may in turn reflect the increased apoptotic death of the cells. These findings also correlated well with studies conducted previously.[18,19]

Our findings are based on observations of average and median scores, and have the limitation of small sample sizes. Extension of the study with a larger sample size may show a clearer picture. Although all doses of Apigenin appears to be beneficial to the kidney, it was found that the ideal dosage of Apigenin in reversal of Cyclosporine A toxicity was 20 mg/kg body weight. Apigenin by itself at the dose of 20 mg/kg showed a high expression of TGF-β. This also gives scope for future studies into the clinical utility of Apigenin.

The weight and volume of the kidneys have been shown to be directly proportional to each other. It is not surprising that apoptotic index and TGF-β expression showed an inverse relation to both kidney weight and volume, as these parameters would lead to cell loss, which in turn can be reflected by lowered weight and volume.

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