

Attenuation of Tacrolimus Induced Oxidative Stress, Apoptosis and Cell Cycle Arrest by Ferulic Acid in MDCK Cell Lines

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Abstract The current study aimed to understand the effect of Ferulic acid (FA), a phenolic compound with effective antioxidant properties on Tacrolimus (TAC) induced nephrotoxicity in MDCK cell lines. The distal tubular epithelial cell line, MDCK cells were tested for toxicity of TAC and FA using cell viability assay (MTT assay) and the apoptotic and necrotic morphology of cells were studied with AO/EB staining. The cell cycle arrest in TAC and FA co-treated cells was studied using flow cytometry. ROS level was measured using fluorogenic dye, 2,7-dichlorodihydrofluorescein diacetate (DCF-DA) and analysed by flow cytometry. The antioxidant analysis was performed by estimating GSH using 5,5-dithio-bis (2-nitrobenzoic acid) (Ellman's reagent, DTNB) and SOD was measured by the modified NBT method. TAC treated cells showed reduced cell viability, increased apoptosis with cell cycle arrest at S phase and sub G0/G1 phase cell population. But, FA co-treatment significantly ($p < 0.001$) increased cell viability with reduced apoptosis. Increased ROS generation, estimated by DCFH-DA in TAC treated cells, was comparatively reduced ($p < 0.0001$) in FA co-treated cells with concurrent increase in Glutathione (GSH) ($p < 0.001$) and Superoxide dismutase (SOD) ($p < 0.0001$) level in FA co-treated groups, which could be

attributed to its maintenance of intracellular redox balance. Thus, the maintenance of antioxidant defence status along with free radical scavenging property of FA may provide an adjuvant therapy to reduce the nephrotoxicity induced by TAC.

Keywords Ferulic Acid, Tacrolimus, Nephrotoxicity, Oxidative Stress, Cell Cycle, Apoptosis

1. Introduction

Ferulic acid (3-methoxy-4-hydroxycinnamic acid), a phenolic compound, is a derivative of hydroxycinnamic acid, with low toxicity [1] and an effective antioxidant activity against free radicals such as nitric oxide, superoxide and hydroxyl groups that damage DNA [2]. The phenolic nucleus and carboxylic side chain in FA is responsible for its strong antioxidant properties [3]. It is shown to increase the antioxidant enzymes levels with a significant reduction in free radical generating enzymes, thus protecting the membranes from lipoperoxides, neutralized alkoxyl and peroxy radicals [4], [5]. Its

bioavailability and distribution are comparatively more than other dietary flavonoids and phenolics so far studied [4]. Recently, FA has attracted researchers in terms of its wide range of potency including anti-inflammatory, anti-apoptotic, anti-aging, hepatoprotective, nephroprotective, neuroprotective, anti-atherogenic, hypotensive and vasodilation effects [2].

Drug induced nephrotoxicity is a major problem and studies have shown that drugs alter the levels of kidney markers, glutathione and other antioxidant enzymes, leading to oxidative stress [5]. Of late, there have been studies on protective effect of FA in kidney diseases, including drug induced nephrotoxicity via inhibition of free radicals' generation and lipid peroxidation (LPO) [2]. *In-vitro* and *in-vivo* studies showed its inhibitory effect against plasma angiotensin-converting enzyme activity, with a significant reduction in systolic blood pressure [3]. A study with methotrexate (MTX) and FA was shown to reverse MTX induced apoptosis via reactive oxygen species (ROS) reduction and enhancement of antioxidant defence mechanism [6]. Co-administration of gentamicin and FA, dose dependently increased the antioxidant markers such as SOD, glutathione peroxidase (GPx) and CAT, with a reduction in LPO marker (malondialdehyde) and tubular necrosis [2], [7]. FA co-treatment in cisplatin induced nephrotoxicity significantly prevented oxidative stress and apoptosis, increasing antioxidant status [8]. FA treatment significantly attenuated the glycerol-mediated increase in LPO levels and reversed the effect of glycerol by significantly increasing the activities of GSH, GPx, SOD, CAT and GST content in serum and kidney tissues [5].

Hence, the current study is focused to explore the protective effect of FA against nephrotoxicity induced by Tacrolimus (FK506), a Calcineurin inhibitor and primary immunosuppressant. TAC, an integral part of immunosuppressive regime [9] has been reported for renal failure during long term administration [10]. The global incidence of nephrotoxicity in renal and liver transplant patients has been reported as 17-44% and 18-42% respectively [11]. Prolonged intake of TAC showed various histopathological changes in kidney including hyalinosis in the arterial region of the kidney, atrophy in the tubular region, fibrosis in the Bowman's capsule and glomerular sclerosis. Despite the several mechanisms involved in TAC induced nephrotoxicity, reactive oxygen species (ROS) generation via NADPH oxidase activation pathway with simultaneous interruption in the antioxidant defense mechanism, was found to activate intrinsic apoptotic pathway with mitochondrial damage [12] due to disturbances in the renal antioxidant enzymes [13]. Therefore, in the present study, nephroprotective activity of FA has been exploited in terms of antioxidant activity against TAC induced nephrotoxicity in Madin-Darby Canine Kidney (MDCK) cells.

2. Materials and Methods

2.1. Chemicals and Reagents

Ferulic acid, Dulbecco's Modified Eagle's Medium (DMEM), foetal bovine serum (FBS), antibiotics, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), acridine orange, ethidium bromide, and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma Aldrich (Merck), India. Tacrolimus was purchased from Selleck Chem Pvt Ltd, USA.

2.2. Cell Culture

The distal tubular epithelial cell line, MDCK (RRID-CVCL_0422), was purchased from National Centre for Cell Sciences (NCCS), Pune, India. The cells were cultured and maintained in DMEM, supplemented with 10% FBS in a humidified 5% CO₂ incubator at 37°C.

2.3. Cell Viability

MTT assay [14] was carried out to determine the toxicity of TAC and FA on MDCK cells. Briefly, MDCK cells were cultured in 96-well plate at the density of 5×10^3 cells approximately and incubated in a 5% CO₂ incubator. The next day, cells were treated with TAC (50µM) with and without FA (20, 40, 60, 80 & 100µg/ml). Following 24h of incubation, 20µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added and incubated at 37°C for 2h. The formazan crystals formed were dissolved in DMSO and the absorbance was measured at 570nm in a spectrophotometer (UV-1800, Shimadzu UV-vis). Cell viability based on lysosomal uptake of neutral red (NR) assay was performed for quantitative estimation of viable cells after treatment [15]. NR was prepared in serum-free DMEM medium and incubated overnight at 37°C and centrifuged to remove fine precipitate of dye crystals. The cells were washed with PBS (pH 7.4) and incubated for 3h in 100µl of NR (0.25mg/ml). The cells were again washed with PBS and 200µl of elution medium (1% (v/v) acetic acid, 50% (v/v) ethanol, and 49% (v/v) distilled water) were added. After 15mins, absorbance was measured at 540nm (UV-1800, Shimadzu UV-vis).

2.4. Morphological Staining of Apoptotic & Necrotic Cells

2.4.1. Acridine Orange (AO) /Ethidium Bromide (EB) Staining

The apoptotic and necrotic morphology of cells was studied with AO/EB staining. After 24h post-treatment, cells were washed twice with PBS and double staining was performed with AO/EB staining (10µmol/ml). Cells were visualized immediately after staining, under Inverted fluorescent microscopy (Nikon) at 100x magnification [16].

2.5. Cell Cycle Analysis Using Flow Cytometry

The cell cycle arrest in TAC and FA co-treated cells was studied using flow cytometry. The control and treated cells were washed with ice cold PBS and disrupted in hypotonic solution (1% sodium citrate, 0.04% RNase, 0.05% PI, 3% Triton X-100). The cells were then scraped and centrifuged at 2000 rpm for 10mins. The cell pellet was mixed with 500 μ l of hypotonic solution and the distribution of cells in different phases of cell cycle was determined by C6 Accuri flow cytometer [17].

2.6. Qualitative analysis of ROS

2.6.1. Estimation of ROS

ROS level was measured with fluorogenic dye, 2,7-dichlorodihydrofluorescein diacetate (DCF-DA). After 24h post-treatment, cells were washed twice with PBS, trypsinized and incubated for 45mins with 10 μ mol/ml of DCFDA and flow cytometry analysis was performed with excitation at 475nm and emission at 525nm in BD C6 Accuri flow cytometer [18].

2.6.2. Estimation of Reduced Glutathione (GSH)

GSH was estimated using 5,5-dithio-bis (2-nitrobenzoic acid) (Ellman's reagent, DTNB) [19]. Briefly, the treated cells were washed twice with PBS and harvested with a cell scraper and lysed by sonicating thrice in 1ml 0.2% Triton X-100 and 20mM Tris-HCl buffer and centrifuged at 10,000g for 10mins at 4°C. To 160 μ l of cell lysate, 1.8ml of 0.2M Na₂HPO₄ buffer and 40 μ l of 10mM DTNB were added and incubated for 2mins. The absorbance was measured at 412nm using spectrophotometer.

2.6.3. Estimation of SOD

SOD was measured by the modified NBT method [20].

Briefly, to 1.4ml cell lysate, 1.2ml sodium pyrophosphate buffer (0.052 M, pH 8.3), 0.1ml PMS (186 μ M), 0.3ml NBT (300 μ M) were added and the reaction was initiated by adding 0.2ml NADH (780 μ M) and incubated at 30°C for 90sec. The reaction was stopped by adding 1ml glacial acetic acid and shaken vigorously after adding 4ml N-butanol. Following a 10mins incubation period, contents were centrifuged and the color intensity in the butanol layers was measured at 560nm in a spectrophotometer (Shimadzu UV-Vis 1800). The reaction mixture without cell lysate serves as a blank. 50% reduction in the color intensity of NBT in one minute measured at 560nm is defined as one unit of enzyme activity.

3. Results

3.1. Effect of TAC and FA Co-Treatment on Cell Viability

The effect of TAC (50 μ M) cell viability showed 72.37% cell death after 24h of incubation whereas, cells co-treated with FA (20-100 μ g/ml) showed concentration-dependent activity on cell death, with significant reduction at 40 μ g/ml (51.6%) and 80 μ g/ml (38.9%) (Fig 1). This effective increase in cell viability confirms the protective ability of FA and further studies were carried out using these two concentrations of FA. Further, cytoprotective effect of FA co-treatment on TAC induced toxicity was done based on the ability of viable cells to incorporate and bind the supravital neutral red dye in the lysosomes. We observed similar results, where TAC induced 73.60% cell death through lysosomal damage, but a good reduction in cell death was observed dose dependently in the presence of FA (Fig 2).

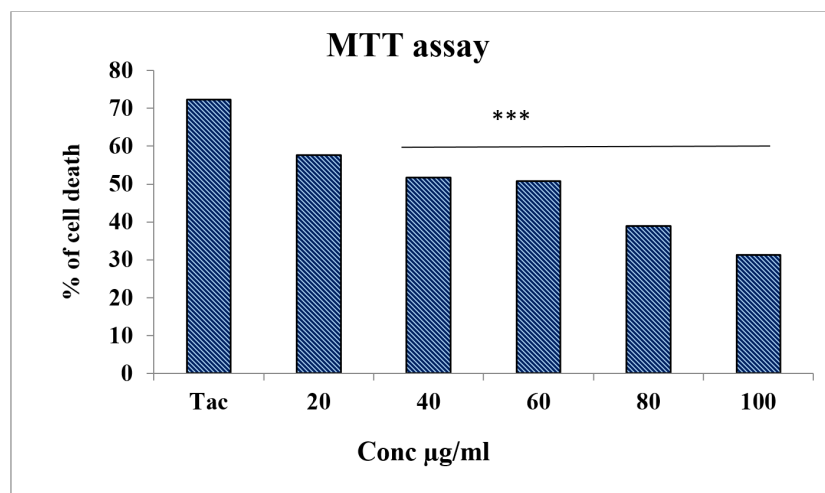


Figure 1. Effect of TAC and FA co-treatment on MDCK cells. The cell viability of TAC and FA co-treatment on MDCK cell was estimated by MTT assay. TAC (50 μ M) induced 72.36 % cell death, which was reduced significantly (***) p value <0.001 with increase in concentration of FA (dose dependent effect)

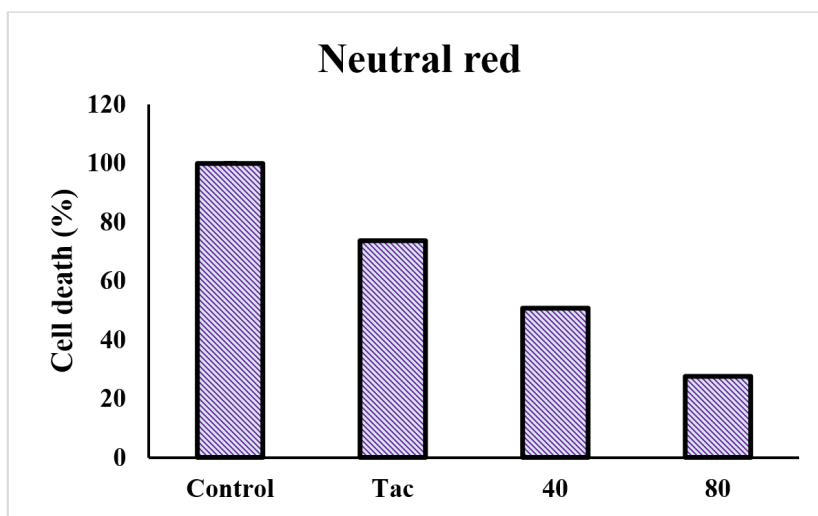


Figure 2. Cytoprotective effect of FA on TAC induced toxicity. Evaluation of protective effect of FA against TAC induced cellular damage by Neutral red assay. TAC (50 μ M) induced cellular damage was comparatively reduced with FA co-treatment (40 & 80 μ g/ml) (p value ***<0.001)

3.2. Morphological Evaluation of TAC and FA Co-Treatment

The cell viability was well corroborated with morphological changes in TAC and FA co-treated MDCK cells through Acridine orange/ethidium bromide (AO/EB) double staining. The profound change in cell morphology such as cell shrinkage, a known characteristic of apoptosis was found in TAC (50 μ M) treated cells (Fig 3B) compared to control (Fig 3A), but FA co-treatment could protect the cells from toxicity induced shrinkage (Fig 3C & D). AO/EB staining is used to detect the mild injuries in DNA and help to distinguish early and late apoptotic as well as necrotic cells [21]. AO is a cell permeable dye, whereas EB is a membrane impermeable dye [22]. Live cells emit uniform green colour while dark green rods appear in the nuclei of early apoptotic cell with intact membrane due to chromatin condensation and nuclear fragmentation. Late apoptotic cells uptake both AO & EB and emit orange colour whereas necrotic cells exhibit uniform orange or red colour nuclei with condensed chromatin [23]. In the current study, green colour was observed in the control (viable, Fig 4A) live cells while more of red and orange colour was observed in TAC treated cells, which confirms necrosis due to condensed chromatin (yellow arrow in Fig 4B). Co-treatment with FA prospectively reduced the late apoptosis and necrosis dose-dependently, which is evident from the absence of red or orange nuclei (Fig 4C & D).

3.3. Cell Cycle Analysis

Concurrently the intensity of apoptosis and changes in cell cycle distribution was further studied using flow

cytometry (Table 1). Cells treated with TAC alone showed an increase in accumulation of cells in S phase with a concomitant increase of cells at sub G0/G1 phase compared to control group, which indicates the TAC induced cell death. However, FA co-treated groups showed a significant reduction in cell death, which is evident from the increase in percentage of cells in G0/G1 phase. Further, accumulation of cells in S phase and sub G0/G1 phase was comparatively reduced dose dependently in FA co-treated groups. Hence, this validates the effect of FA on TAC induced cell death and cell cycle arrest at S phase in MDCK cells.

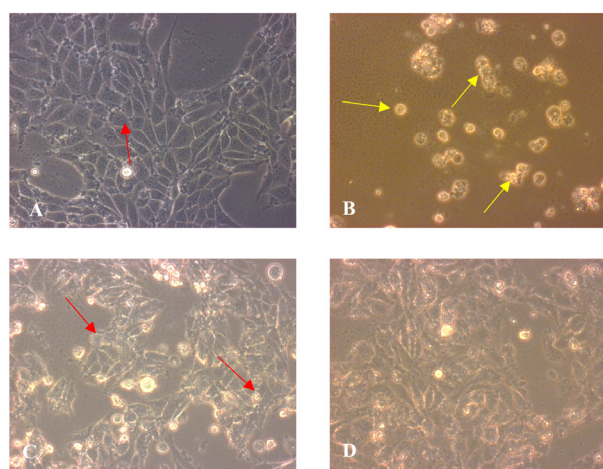


Figure 3. Morphological changes in TAC treated MDCK cells with and without FA. Cells were photographed using phase contrast microscopy. TAC treatment showed cell shrinkage whereas FA co-treatment reduced cell shrinkage with increase in cell density. A) Control B) TAC (50 μ M), C) TAC (50 μ M) & FA (40 μ g/ml) D) TAC (50 μ M) & FA (80 μ g/ml). Yellow arrow- dead cells, red arrow- normal cells. (magnification 200x)

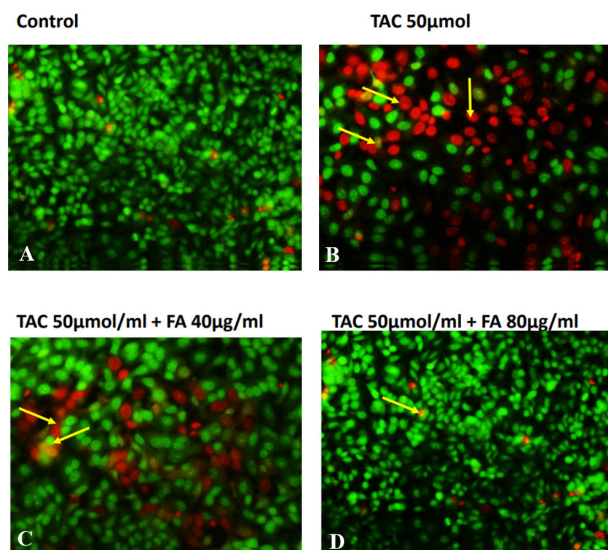


Figure 4. AO/EB Dual staining in TAC and FA co-treated cells. A) Control with only live cells (green colour) B) TAC (50 μ M) treated cells showed late apoptosis and necrosis (yellow arrows pointing out red & orange colour) C) Cells co-treated with TAC (50 μ M) and FA (40 μ g/ml) showed increase in cell viable and D) Cells co-treated with TAC (50 μ M) and FA (80 μ g/ml) showed increased cell density reduction in cell death (magnification 200x)

Table 1. Cell cycle analysis of TAC & FA co-treated cells

Groups	% of cells in each phase of the cell cycle			
	Sub G0/G1	G0/G1	S	G2/M
Control	5.08	47.76	15.45	31.81
TAC	12.39	40.42	26.54	20.65
TAC + FA (40 μ g/ml)	10.09	42.45	19.71	27.75
TAC + FA (80 μ g/ml)	6.74	45.27	17.64	30.35

TAC: Tacrolimus (μ M/L), FA: Ferullic Acid (μ g/ml)

3.4. Effect on ROS Production

In order to investigate the impact of toxicity induced by TAC exposure in terms of reactive oxygen species production, quantification of ROS was carried out by DCFH-DA probe. It is the most widely used probe for quantification of hydrogen peroxide and other oxidants generated intracellularly in response to redox signaling changes. It is a cell permeable probe which is deacetylated

by cellular esterases to 2',7'-dichlorodihydrofluorescein (H2-DCF) inside the cell, yet in the presence of ROS, H2-DCF is oxidized to 2',7'-dichlorofluorescein (DCF). Even though the exact mechanism of TAC induced nephrotoxicity is still not clear, studies have demonstrated ROS generation as one of the main contributing factors. Hence in the current study intracellular ROS levels were quantified with the DCFH-DA and found to be significantly higher in TAC treated cells (p value <0.0001) with respect to control cells. Nevertheless, FA treated groups showed a dose dependent reduction in ROS levels, which may be attributed to its significant anti-oxidant potential (Fig 5).

3.5. Effect on GSH & SOD

To authenticate the positive role of FA, the level of GSH was measured in the treatment groups. Glutathione, a tripeptide with thiol group, is a potent reducing agent which plays a significant role in the detoxification of electrophilic compounds and peroxides and cellular GSH levels are used as an indicator of cell toxicity and oxidative stress. Since, an increase in ROS disturbs the antioxidant status in the cells, it is imperative to measure GSH levels in TAC and FA co-treated cells. TAC treated cells showed a significant ($p < 0.01$) reduction in GSH content, which was concurrently increased in FA co-treatment, as FA is an effective ROS scavenger, probably by forming stable phenoxyl radicals (Fig 6). Hence the results confirm the protective effect of FA against GSH loss by eliminating oxidants and maintaining intracellular redox balance [19]. The benefit of FA was also proved in terms of increased SOD levels in the treated groups. The activity of SOD was assessed based on the superoxides generated in the cell lysate of TAC and FA co-treated cells. SOD levels were estimated with reduced NADH in the presence of PMS, a catalyst and NBT, an indicator. The SOD in the cell lysate competes with NBT to reduce superoxides, producing a blue color, where the color intensity is directly proportional to the SOD levels. TAC treated cells showed a significant reduction in SOD activity, which was dose dependently increased in FA co-treated cell. The antioxidant nurturing capacity of FA may be attributed for an effective rise in SOD during the co-treatment (Fig 7).

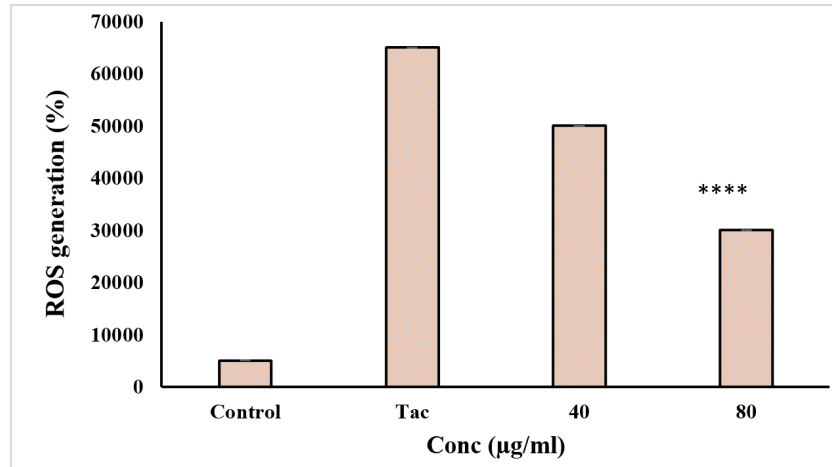


Figure 5. Fluorimetry analysis of DCFH-DA stained TAC and FA co-treated cells. High DCF fluorescence intensity observed in TAC treated cells, was reduced in FA co-treatment at 40 and 80 μg/ml, which may be attributed for its ROS scavenging activity (p value **** <0.0001)

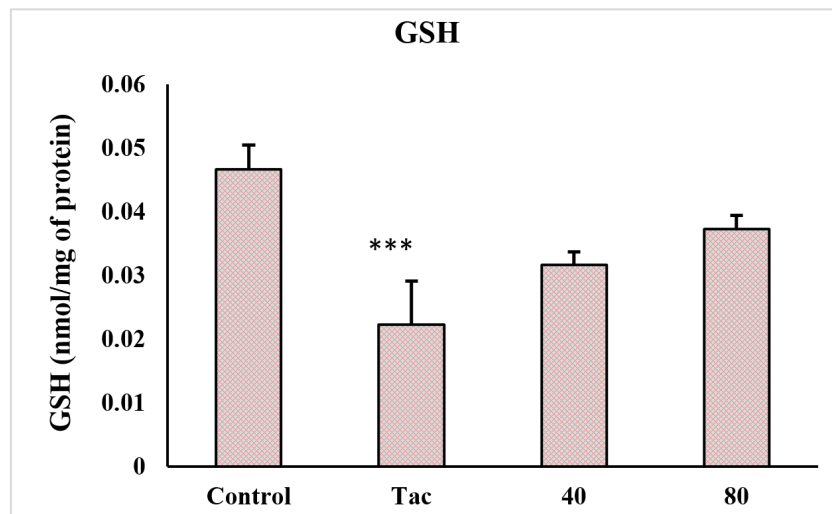


Figure 6. Effect of TAC and FA co-treatment on reduced glutathione (GSH) level. Significant depletion in GSH level compared to control was observed in TAC (50μM) treated cells (p value <0.001). FA Co-treatment from 40 and 80 μg/ml cells showed a remarkable replenish in GSH level in a concentration dependent manner

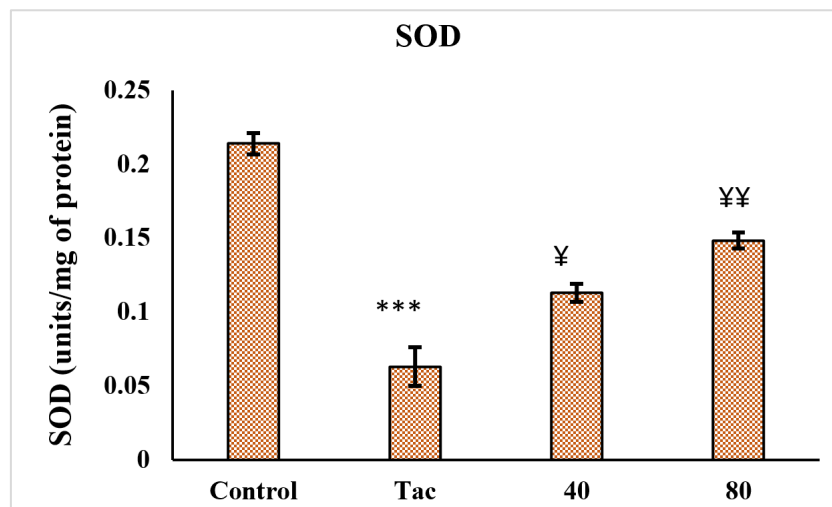


Figure 7. Evaluation of SOD in TAC and FA co-treated cells. Significant depletion of SOD level was observed in TAC (50μM) treated cells (**** <0.0001 compared with control). However, FA co-treatment (40 & 80 μg/ml) remarkably (p value ¥ <0.5 , ¥¥ <0.001 compared with tac treated group) amplified the SOD level

4. Discussion

There is an emerging interest in the naturally occurring antioxidant for therapeutic usage. FA, a superior antioxidant with good bioavailability compared to other phenolics [24], has been studied for its protective role against cisplatin and gentamicin induced nephrotoxicity through ROS generation, oxidative stress and inflammatory reactions. Research has shown the involvement of reactive oxygen species in the impairment of glomerular filtration rate [5]. Tacrolimus (FK506), a primary immunosuppressant in solid organ transplantation, was also found to cause nephrotoxicity, affecting the long-term graft and patient survival and thus, limiting its clinical usage. Although, the exact mechanism of TAC induced nephrotoxicity remains unclear, previous studies propose the ability of TAC to generate ROS via NADPH oxidase pathway, which results in disturbance in antioxidant defence process and renal toxicity. Hence, recent research is aimed to understand the protective effect of antioxidants against TAC induced toxicity, as an adjuvant therapy, without compromising the clinical application [25]. Therefore, the present study was performed to understand the protective role of FA against TAC induced nephrotoxicity.

Preliminary screening has shown that TAC induced cytotoxicity in MDCK cells, was reduced dose-dependently in FA co-treatment. Previous studies have reported that TAC induces ROS generation and oxidative stress, resulting in apoptosis and necrosis in the tubular kidney cells [12], [26]. In the current study, we have observed similar results that TAC induced apoptosis and necrosis have been significantly reduced in FA co-treatment, improving the survival of the cells. It has been reported that FA showed anti-apoptotic property against ethanol mediated hepatotoxicity. Previous studies by Zhou *et al* [26] demonstrated that tacrolimus increases the hypodiploid nucleus leading to accumulation of cells in subG0 phase. In the current study, TAC treatment was shown to disturb the cell cycle events with an increase in cell accumulation at S phase and sub G0/G1 phase. However, co-treatment with FA relatively reduced the cells accumulation at S and sub G0/G1 phase, with a subsequent increase in cell population at G0/G1 phase, which shows the protective role of FA against TAC induced apoptosis. ROS generation and oxidative stress are the major factors for TAC induced cell death. The generation of ROS occurs through a variety of processes, such as mitochondrial oxidation, the microsomal cytochrome P450 system, and plasma membrane NADPH oxidases [27], which finally leads to apoptosis in the kidney cells. In a normal cell, where the ROS generation is reduced by antioxidant enzymes, TAC was shown to affect this antioxidant defense mechanism, increasing the ROS generation and resulting in intrinsic apoptosis [28]. In the present study, GSH, an important non-antioxidant enzyme, and SOD, an anti-oxidant enzyme was reduced in the TAC treated cells.

However, FA co-treatment, noticeably replenished GSH & SOD levels, attributed to its antioxidant defense mechanism, thus, protecting against the membrane damage and apoptosis.

FA, is a phenolic compound with three peculiar structural motifs, responsible for its antioxidant mechanism through the formation of stable phenoxyl radicals on reaction with free radicals, affecting the complex reaction cascade of ROS generation [3], [4]. This compound also acts as hydrogen donor, which helps in protection of cell membrane lipid, against undesired auto-oxidation reactions. As a secondary antioxidant, FA and its related compounds are also shown to bind to transition metals such as iron and copper, preventing the toxic hydroxyl radical's formation and cell membrane peroxidation [24]. Therefore, the present study also showed that FA co-treatment has a significant role in protection against TAC induced cytotoxicity in MDCK cells through reduction in ROS production and apoptosis as well as increase in GSH & SOD.

5. Conclusions

FA has a protective effect on TAC induced nephrotoxicity. From the study result it could conclude that the anti-oxidative and anti-apoptotic properties are associated with protective effect of FA in TAC induced nephrotoxicity. Our study provides the rationale for the clinical utility of FA to be used as an adjuvant therapy to prevent TAC induced nephrotoxicity without compromising targeted therapy.

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Conflict of Interest

The authors declare no conflict of interest.

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