Clinical and Experimental Health Sciences

In Vitro Assessment of Anti-inflammatory Effect of Apigenin on Renal Cell Inflammation

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ABSTRACT

Objective: This study aimed to evaluate in vitro effect of apigenin on anti – and pro-inflammatory cytokines including interleukin-6 (IL-6), IL-10, tumor necrosis factor-alpha (TNF-α), and transforming growth factor-beta (TGF-β) levels in an in vitro model of renal cell inflammation induced with lipopolysaccharide (LPS).

Methods: For the in vitro renal cell inflammation model, the African green monkey kidney cell line (Vero) was used. Four groups as NC (without any treatment), LPS (Vero cells treated with 10 µg/mL of LPS for 4 hours), API (Vero cells treated with 5 µg/mL of apigenin for 12 hours), and LPS+API (Vero cells treated with 5 µg/mL of apigenin for 12 hours + 10 µg/mL of LPS for 4 hours) was formed. The non-cytotoxic dose of apigenin in Vero cells was evaluated by a cell count test. IL-6, IL-10, TNF-α, and TGF-β concentrations in the cell culture medium were measured by enzyme-linked immunosorbent assay kits. All analyses were performed in four repetitions.

Results: IL-6, IL-10, TNF-α, and TGF-β concentrations of the LPS group increased compared to NC, API, and LPS+API groups ($p<0.05$). We found that treatment with apigenin led to significant attenuation in the LPS-induced secretion of IL-6, IL-10, TNF-α, and TGF-β in the Vero cell line.

Conclusion: Our findings showed that apigenin significantly reduced LPS-induced IL-6, IL-10, TNF-α, and TGF-β formations in Vero cells. Taken together, these results suggest that apigenin may be a therapeutic candidate for relieving inflammatory renal cell damage. These results need to be supported by in vivo trials and clinical applications.

Keywords: *Apigenin, in vitro, lipopolysaccharide, renal cell damage*

1. INTRODUCTION

Inflammation and immune system activation play role in the etiopathogenesis of acute and chronic kidney diseases (1). Inflammation stimulates cytokines secretion and increases the production and activity of adhesion molecules in renal tissue (2,3). Patients with renal injury have higher serum interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α) levels (4-8). IL-6 induces the progression of chronic kidney disease by initiating chronic vascular disease, endothelial injury, and adiponectin expression (9,10). TNF-α stimulates the release of the inflammatory mediator Interleukin 1β (IL-1β), monocyte chemoattractant protein (MCP)-1), and transforming growth factor-beta (TGF-β) (11). TGF-β induces conditions causing chronic progressive kidney disease through regulation of cell proliferation, hypertrophy, apoptosis, and fibrogenesis (12). IL-10 is produced as a growth factor by mesangial cells in the normal adult kidney and induces pathological processes that lead to the progression of renal failure by inducing the synthesis and activity of Cystatin C and TGF-β. Increased TGF-β levels promote fibrosis and glomerulosclerosis with IL-10 (13).

In recent years, scientific studies on the treatment of inflammatory diseases have focused on the use of anti-inflammatory flavonoids. Many flavonoids show medical efficacy as antibacterial, antioxidative activity, free radical scavenging capacity, anti-inflammatory, anticancer and antiviral agents (14). Apigenin, found naturally in fruits and vegetables, has anti-inflammatory effects in various cellular processes (15,16). The prophylactic use of apigenin suppresses cyclooxygenase-2 (COX-2) and nuclear factor **κ**B (NF-**κB**) pathways by decreasing IL-1β, IL-6 and TNF-α concentrations, leukocyte quantity, and neutrophil percentage in bronchoalveolar lavage fluid and thereby alleviates inflammation (16). Apigenin exhibits an anti-inflammatory effect by suppressing chemokine production associated with T helper cell-1 and T-helper cell-2 and modulating mitogen-activated protein kinase (MAPK) in human monocyte cells (17). Apigenin inhibits LPS-induced NF-κB activity in lung tissue, reduces infiltration of inflammatory cells, and accumulation of chemotactic factors (18). It is known that apigenin has a good anti-inflammatory effect but the possible protective effect of apigenin on renal cell inflammation is unknown. The purpose of the present study was to investigate the effect of apigenin on anti-inflammatory cytokines IL-6, IL-10, TNF-α, and TGF-β levels in *vitro* renal cell inflammation model.

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2. METHODS

2.1. Cell Culture and Treatment

The Vero cell line used in this study was obtained from the Department of Virology, Faculty of Veterinary Medicine, Ondokuz Mayis University. The 10% calf serum, 2 mM l-glutamine, 100 µg/mL streptomycin, 100 U/mL penicillin, and 1 mM pyruvic acid in Minimum Essential Eagle's Medium (MEM, Sigma, Austria) were applied to the Vero cell line. The cells were maintained at 37 °C, 5% CO_2, 95% air, and 100% humidity. Filter cap cell culture flasks were used to prevent possible contamination and the medium was changed every two days. Cell survival and morphological structure were evaluated by an inverted microscope. The inflammation was induced by treatment with LPS (E. coli O111: B4) in Vero cells. In order to induce inflammation, the cells were incubated with culture medium containing different LPS concentrations (0.1 µg/mL, 1 µg/mL, 2 µg/mL, 5 µg/mL and 10 µg/mL) for 2, 4, 8, 16 and 24 hours. The inflammation dose of LPS was selected based on previous scientific studies (19,20). A previous *in vitro* study (21) was taken into consideration to determine the non-cytotoxic dose for apigenin treatment, cells were cultured with different apigenin concentrations (0.1 µg/mL, 0.25 µg/mL, 0.5 µg/mL, 1 µg/mL, 5 µg/mL, and 10 µg/mL) for 12 hours and the non-cytotoxic dose was determined according to the cell count test result. All experiments were performed in four repetitions.

In the study, four groups NC (negative control group, any application was not done), LPS (treatment with 10 µg/mL of LPS during 4 hours), API (treatment with 5 µg/mL of apigenin during 12 hours), and LPS+API (treatment with 10 µg/mL of LPS during 4 hours+5 µg/mL of apigenin during 12 hours) were formed. After all treatments, cell culture media were centrifuged at 1.550xg for 10 minutes and ELISA analysis was performed in supernatants (22).

2.2. Determination of Non-Cytotoxic Dose of Apigenin

The non-cytotoxic dose of apigenin in Vero cells was assessed by a cell counting kit-8 (96992, Sigma-Aldrich, USA). The test was performed according to the method notified by the manufacturer. For this purpose, 100 µL cell suspension including 1×10^5 Vero cells per milliliter was prepared and then cell suspension was added to the 96-well plate and incubated in a 37 °C incubator. When the cells covered the plate surface at the end of 24 hours, the cell production medium in wells was evacuated and the plates were incubated in a 22 °C incubator by adding stock apigenin from 3 different concentrations, 1 µg/mL, 5 µg/mL, and 10 µg/mL prepared in cell production medium and 10 µg/mL and from each dilution to 2 wells. In addition, 10 µL of cell-producing media was added into the wells of the control cells instead of apigenin. Apigenin in the wells of the plates was taken 12 hours later from the incubator and was evacuated using a pipette and 10 µL of cell counting solution was added to all wells and incubated at 37 °C for 2 hours. At the end of the time, the optical density of the microplate was read in 450 nm wavelength at the microplate reader (Tecan Infinite F50). The test was repeated 4 times. The half-maximal inhibitory concentration (IC50) value for apigenin was calculated and the graph was plotted using the GraphPad Prism 5 software (San Diego, CA, USA).

2.3. Main Outcome Measurements

IL-6 concentration:The monkey-specific ELISA assay (LS-F4822, LifeSpan Biosciences Inc., Seattle Downtown, Washington, USA) was used to measure the concentration of IL-6 in cell culture supernatants. All samples were studied double. ELISA steps were carried out as recommended by the manufacturer. The results were presented as pg/mL.

IL-10 concentration: The concentration of IL-10 in cell culture supernatants was determined using the ELISA kit specific to monkey (LS-F25130, LifeSpan Biosciences Inc., Seattle Downtown, Washington, USA) by the ELISA method. The measuring range of the kit was 0-1000 pg/mL and all samples were studied double. ELISA steps were carried out as recommended by the manufacturer. The results were presented as pg/mL.

TNF-α concentration: The concentration of TNF-α in cell culture supernatants was determined using the ELISA kit specific to monkey (LS-F4818, LifeSpan Biosciences Inc., Seattle Downtown, Washington, USA) by the ELISA method. All samples were studied double. ELISA steps were carried out as recommended by the manufacturer. The results were presented as pg/mL.

TGF-β concentration: The concentration of TGF-β in cell culture supernatants was determined using the ELISA kit specific to monkey (MBS737903, MyBioSource, Inc., San Diego, CA, USA) by the ELISA method. The measuring range of the kit was 0-1000 pg/mL and all samples were studied double. ELISA steps were carried out as recommended by the manufacturer. The results were presented as pg/mL.

2.4. Statistical Analysis

SPSS 22.0 package program was used for statistical evaluation of the findings obtained from the study. Before significance tests, all the data were evaluated using the parametrical test with Shapiro Wilk for normality assumptions and with the Levene test for homogeneity of the variance. Duncan test was used for variables that provide parametric test assumptions as a post-hoc test in cases where the difference between groups is significant. A minimum of $p < 0.05$ value was considered statistically significant for all statistical evaluations.

3. RESULTS

3.1. Non-Cytotoxic Dose of Apigenin on Vero Cells

As determined by the cell viability assay, different concentrations of apigenin resulted in cytotoxicity effects on

the Vero cell line in a dose-dependent manner for 12 hours. The growth of the cell line was strikingly inhibited by different concentrations of apigenin (0.1 µg/mL, 0.25 µg/mL, 0.5 µg/mL, 1 µg/mL, 5 µg/mL, and 10 µg/mL). In different apigenin concentrations, cell viability was found as 98.0 ± 1.56 % in 0.1 µg/mL, 90.6 ± 4.63 % in 0.25 µg/mL, 83.2 ± 2.92 % in 0.5 µg/mL, 75.8 ± 4.33 % in 1 µg/mL, 50 ± 5.68 % in 5 µg/mL, and 9.88 ± 0.68 % in 10 µg/mL (p<0. 05). Viability assay from four repetitions showed that IC50 of apigenin was 5 µg/mL (Figure 1).

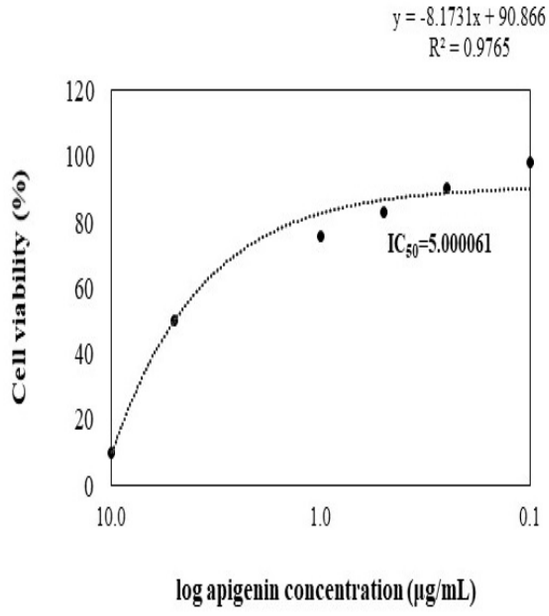


Fig 1. The dose-response curve for the half-maximal inhibitory concentration (IC50) for apigenin. Six different concentrations (ranging from 0.1 to 10 mg/mL) were tested. Data are represented as mean values of at least four independent experiments. The IC50 values were calculated using nonlinear regression analysis of GraphPad Prism software 5 by plotting log inhibitor versus normalized response.

3.2. Effect of Apigenin on IL-6 Concentration in LPS-Treated Vero Cells

The effect of apigenin administration of 5 µg/mL for 12 hours on IL-6 concentration in LPS-treated Vero cells and the differences between groups are presented in Figure 2. The results of the IL-6 levels of the groups were given in Table 1. The IL-6 concentrations were 159.3 ± 6.6 pg/mL in LPS group, 52.8 ± 3.9 pg/mL in NC group , 46.5 ± 1.3 pg/mL in API group and 86.5 ± 5.0 pg/mL in LPS+API group. Treatment of Vero cells with LPS alone notably increased the production of IL-6 compared with the negative control, API, and LPS+API groups. The LPS-treated group showed a 3.0-fold, 3.4-fold, and 1.8-fold increase in IL-6 levels when compared with the negative control group, API group, and LPS+API group, respectively (p<0.05). The secretion of IL-6 in the LPS+API group was significantly suppressed by apigenin compared with the level in the LPS-treated cells (p<0.05).

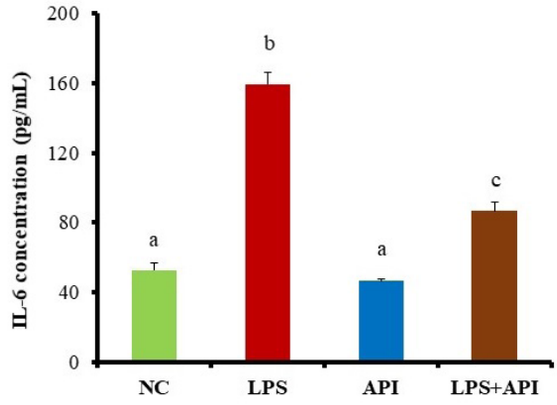


Fig 2. Effect of apigenin on IL-6 concentration in LPS-treated Vero cells. NC: Vero cells received no treatment; LPS: Vero cells treated with 10 µg/mL of LPS for 4 hours; API: Vero cells treated with 5 µg/mL of apigenin for 12 hours; LPS+API: Vero cells treated with 5 µg/mL of apigenin for 12 hours + 10 µg/mL of LPS for 4 hours. Data are mean ± SD for four independent experiments. a p<0.05: significantly different from LPS and LPS +API groups; b p<0.05: significantly different from NC, API and LPS+API groups; Duncan test.

Table 1. IL-6, 1L-10, TNF-α, and TGF-β levels of the groups

Cytokine	Groups			
	NC	API	LPS	LPS+API
IL-6 (pg/mL)	52.8 ± 3.9^a	46.5 ± 1.3^a	159.3 ± 6.6^b	86.5 ± 5.0^c
IL-10 (pg/mL)	62.3 ± 8.5^a	58.3 ± 6.4^a	359.0±16.9^b	155.0 ± 9.5^c
TNF-α (pg/mL)	67.3 ± 4.3^a	63.0 ± 4.5^a	292.3 ± 12.5^b	101.0 ± 3.2^c
TGF-β (pg/mL)	27.0 ± 4.2^a	32.3 ± 3.9^a	312.8 ± 10.2^b	150.0 ± 11.0^c

NC: Negative control (Vero cells received no treatment); API: Vero cells treated with 5 µg/mL of apigenin for 12 hours; LPS: Vero cells treated with 10 µg/mL of LPS for 4 hours; LPS+API: Vero cells treated with 5 µg/mL of apigenin for 12 hours + 10 µg/mL of LPS for 4 hours. a,b,c Different superscript letters indicate statistically significant differences in the same row (p<0.05; Duncan test)

3.3. Effect of Apigenin on IL-10 Concentration in LPS-Treated Vero Cells

The effect of apigenin administration of 5 µg/mL for 12 hours on IL-10 concentration in LPS-treated Vero cells and the differences between groups are presented in Figure 3. The results of the IL-10 levels of the groups were given in Table 1. The IL-10 concentrations were 359.0±16.9 pg/mL in the LPS group, 62.3 ± 8.5 pg/mL in the NC group, 58.3 ± 6.4 pg/mL in the API group, and 155.0 ± 9.5 pg/mL in the LPS+API

group. The LPS-treated group showed a 5.8-fold, 6.2-fold, and 2.3-fold increase in IL-10 levels when compared with the negative control group, API group, and LPS+API group, respectively ($p<0.05$). The secretion of IL-10 in the LPS+API group was significantly suppressed by apigenin compared with the level in the LPS-treated cells ($p<0.05$).

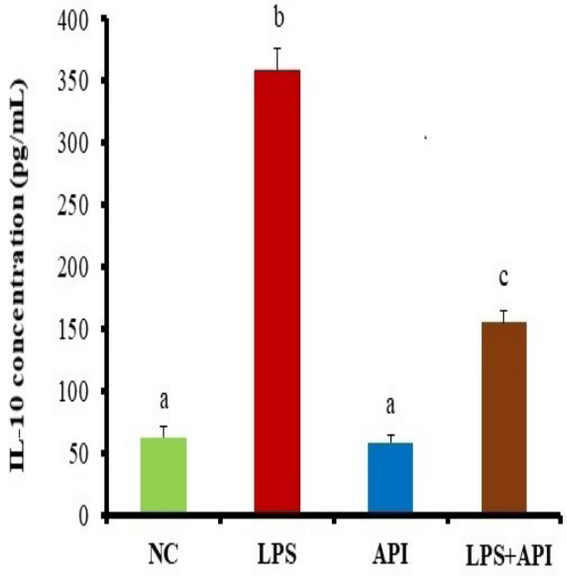
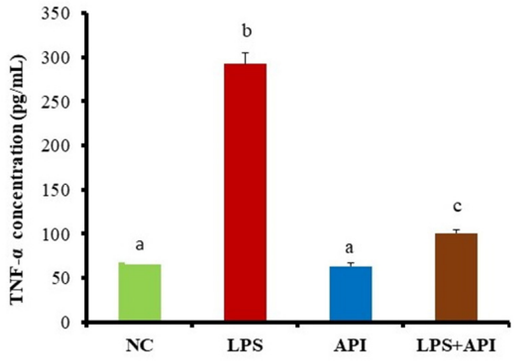


Fig 3. *Effect of apigenin on IL-10 concentration in LPS-treated Vero cells. NC: Vero cells received no treatment; LPS: Vero cells treated with 10 µg/mL of LPS for 4 hours; API: Vero cells treated with 5 µg/mL of apigenin for 12 hours; LPS+API: Vero cells treated with 5 µg/mL of apigenin for 12 hours + 10 µg/mL of LPS for 4 hours. Data are mean ± SD for four independent experiments. a p<0.05: significantly different from LPS and LPS+API groups; b p<0.05: significantly different from NC, API and LPS+API groups; Duncan test.*

3.4. Effect of Apigenin on TNF-α Concentration in LPS-Treated Vero Cells

The effect of apigenin administration of 5 µg/mL for 12 hours on TNF-α concentration in LPS-treated Vero cells and the differences between groups are presented in Figure 4. The results of the TNF-**α** levels of the groups were given in Table 1. The TNF-α concentrations were 292.3 ± 12.5 pg/mL in the LPS group, 67.3 ± 4.3 pg/mL in the NC group, 63.0 ± 4.5 pg/mL in the API group, and 101.0 ± 3.2 pg/mL in the LPS+API group. The LPS-treated group showed a 4.3-fold, 4.6-fold, and 2.9-fold increase in TNF-α levels when compared with the negative control group, API group, and LPS+API group, respectively ($p<0.05$). The secretion of TNF-α in the LPS+API group was significantly suppressed by apigenin compared with the level in the LPS-treated cells ($p<0.05$).



Fige 4. *Effect of apigenin on TNF-α concentration in LPS-treated Vero cells. NC: Vero cells received no treatment; LPS: Vero cells treated with 10 µg/mL of LPS for 4 hours; API: Vero cells treated with 5 µg/mL of apigenin for 12 hours; LPS+API: Vero cells treated with 5 µg/mL of apigenin for 12 hours + 10 µg/mL of LPS for 4 hours. Data are mean ± SD for four independent experiments a p<0.05: significantly different from LPS and LPS+API groups; b p<0.05: significantly different from NC, API and LPS+API groups; Duncan test.*

3.5. Effect of Apigenin on TGF-β Concentration in LPS-Treated Vero Cells

The effect of apigenin administration of 5 µg/mL for 12 hours on TGF-β concentration in LPS-treated Vero cells and the differences between groups are presented in Figure 5. The results of the TGF-β levels of the groups were given in Table 1. The TGF-β concentration were 312.8 ± 10.2 pg/mL in LPS group, 27.0 ± 4.2 pg/mL in NC group, 32.3 ± 3.9 pg/mL in API group and 150.0 ± 11.0 pg/mL in LPS+API group. The LPS-treated group showed an 11.6-fold, 9.7-fold, and 2.1-fold increase in TGF-β levels when compared with the negative control group, API group, and LPS+API group, respectively ($p<0.05$). The secretion of TGF-β in the LPS+API group was significantly suppressed by apigenin compared with the level in the LPS-treated cells ($p<0.05$).

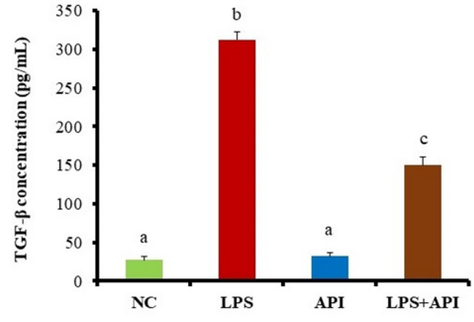


Fig 5. Effect of apigenin on TGF-β concentration in LPS-treated Vero cells. NC: Vero cells received no treatment; LPS: Vero cells treated with 10 µg/mL of LPS for 4 hours; API: Vero cells treated with 5 µg/mL of apigenin for 12 hours; LPS+API: Vero cells treated with 5 µg/mL of apigenin for 12 hours + 10 µg/mL of LPS for 4 hours. Data are mean ± SD for four independent experiments. a p<0.05: significantly different from LPS and LPS+API groups; b p<0.05: significantly different from NC, API and LPS+API groups; Duncan test.

4. DISCUSSION

Inflammation is known to play a role in the pathogenesis of kidney diseases by stimulating the release of cytokines and increasing the production and activity of adhesion molecules (3). Proinflammatory cytokines have a critical role in mediating irreversible tubular injury and nephron failure (23). Scientific reports have suggested that flavonoid supplements exhibit alleviate renal damage by suppressing inflammation (24,25). Apigenin has been shown to exhibit potent anti-inflammatory activity (15-18). Our present study focused on the anti-inflammatory activity of apigenin in renal cell inflammation. For this purpose, *in vitro* effect of apigenin on anti – and pro-inflammatory cytokines including IL-6, IL-10, TNF-α, and TGF-β in renal cell inflammation induced with LPS was evaluated by ELISA.

The prophylactic use of apigenin has been reported that alleviates inflammation by suppressing COX-2 and NF-κB pathways by decreasing IL-1β, IL-6, and TNF-α concentrations, leukocyte count, and neutrophil percentage in bronchoalveolar lavage fluid (16). In human monocyte cells, apigenin has been shown to exert an anti-inflammatory effect by suppressing T helper cell-1 and T helper cell-2-related chemokine production and modulating MAPK (17). It has also been stated that apigenin inhibits LPS-induced NF-κB activity in lung tissue, reduces the infiltration of inflammatory cells, and the accumulation of chemotactic factors (18). Hesperidin has been reported to alleviate the acetaminophen-induced inflammation in renal tubular cells (26). Consumption of isoflavone-rich soybean foods can alleviate systemic inflammation through inflammatory mediators in hemodialysis patients has been reported (27). Chrysin at different doses has been shown to alleviate kidney damage in adenine-induced experimental chronic kidney disease in rats (28). A previous study has reported that epicatechin supplementation with diet alleviated the renal cortex inflammation in high-fructose-diet-fed rats (29). Quercetin protects the kidney against lead-induced kidney injury by affecting the MAPK and NF-κB signaling pathways (30). Naringin treatment in cisplatin-induced nephrotoxicity has been reported to reduce TNF-α concentration in kidney tissue (31). Genistein has been shown to significantly inhibit urinary MCP-1 excretion and renal intercellular adhesion molecule – 1 expression in diabetic mice (32). Baicalin, which was used to ameliorate tubulointerstitial fibrosis in mice with unilateral ureteral obstruction, has been reported to inhibit the inflammatory process by inactivating NF-κB and MAPK signaling pathways (33). Malik, *et al.* reported that nobiletin alleviates acute kidney injury induced by cisplatin in a dose-dependent manner (34).

Markedly increased TNF-α concentration in HK-2 cells which were exposed to 10 μg/ml LPS for 3 hours has been reported (35). Similarly, in our study, the TNF-α level in the group treated with 10 μg/mL for 4 hours of LPS was significantly increased when compared to the negative control group, API group, and LPS+API group ($p<0.05$). TNF-α secretion in the LPS+API group was determined to be significantly inhibited by apigenin compared to the level in cells treated with LPS ($p<0.05$). In another study, it has been shown that apigenin inhibits the production of IL-1β, IL-8, and TNF-α, by suppressing NF-κB activity and modulating immune responses by suppressing inflammation in LPS-induced mouse macrophages (15). The researchers have stated that apigenin at the doses of 1 μg/mL and 10 μg/mL suppresses the LPS-induced IL-6 concentration, however, did not affect TNF-α concentration. Similar to these findings, we also determined that the IL-6 level was significantly increased when Vero cells were exposed to 10 mg/mL LPS for 4 hours compared to the negative control, API, and LPS+API groups. However, we determined that the increased IL-6 level due to LPS exposure was significantly suppressed by apigenin at a dose of 5 mg/ml administered for 12 hours. In addition, administration of LPS to Vero cells resulted in a significant increase in IL-10 levels. IL-10 level in the LPS+API group was determined to be significantly suppressed by apigenin compared to the level in cells treated with LPS ($p<0.05$). Apigenin inhibits LPS-induced IL-6 and/or TNF-α production in murine macrophage cells has been reported (21). It has also been reported that TNF-α-induced NF-kB transcriptional activation is significantly inhibited by apigenin (36). Apigenin administration significantly reduced the levels of TNF-α, IL-1β, and TGF-β in the kidneys in mice with cisplatin-induced kidney injury in a dose-dependent manner (37). Apigenin-rich diet has been reported to exert anti-inflammatory activity by reducing miR-155 and TNF-α expressions in the lungs of LPS-treated mice (38). In our study, the LPS-treated Vero cell line showed a significant increase in TGF-β levels when compared with the negative control group, API group, and LPS+API group ($p<0.05$). However, the elevated TGF-β level in LPS-treated cells was significantly suppressed by the administration of 5 mg/mL apigenin for 12 hours ($p<0.05$).

5. CONCLUSIONS

Our findings showed that apigenin significantly reduced LPS-induced IL-6, IL-10, TNF-α, and TGF-β formations in Vero cells. These findings indicate that apigenin has potent anti-inflammatory effects in LPS-induced *in vitro* renal cell damage by modulating pro-inflammatory and anti-inflammatory cytokine responses which are inflammatory mediators. These results need to be supported by *in vivo* trials and clinical applications.

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Conflict of Interest The authors declared that there is no conflict of interest.

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