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RESEARCH ARTICLE

Apigenin ameliorates streptozotocin-induced diabetic nephropathy in rats via MAPK-NF-κB-TNF-α and TGF-β1-MAPK-fibronectin pathways

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Malik S, Suchal K, Khan SI, Bhatia J, Kishore K, Dinda AK, Arya DS. Apigenin ameliorates streptozotocin-induced diabetic nephropathy in rats via MAPK-NF-κB-TNF-α and TGF-β1-MAPK-fibronectin pathways. *Am J Physiol Renal Physiol* 313: F414–F422, 2017. First published May 31, 2017; doi:10.1152/ajprenal.00393.2016.—Diabetic nephropathy (DN), a microvascular complication of diabetes, has emerged as an important health problem worldwide. There is strong evidence to suggest that oxidative stress, inflammation, and fibrosis play a pivotal role in the progression of DN. Apigenin has been shown to possess antioxidant, anti-inflammatory, antiapoptotic, antifibrotic, as well as antidiabetic properties. Hence, we evaluated whether apigenin halts the development and progression of DN in streptozotocin (STZ)-induced diabetic rats. Male albino Wistar rats were divided into control, diabetic control, and apigenin treatment groups (5–20 mg/kg po, respectively), apigenin per se (20 mg/kg po), and ramipril treatment group (2 mg/kg po). A single injection of STZ (55 mg/kg ip) was administered to all of the groups except control and per se groups to induce type 1 diabetes mellitus. Rats with fasting blood glucose >250 mg/dl were included in the study and randomized to different groups. Thereafter, the protocol was continued for 8 mo in all of the groups. Apigenin (20 mg/kg) treatment attenuated renal dysfunction, oxidative stress, and fibrosis (decreased transforming growth factor-β1, fibronectin, and type IV collagen) in the diabetic rats. It also significantly prevented MAPK activation, which inhibited inflammation (reduced TNF-α, IL-6, and NF-κB expression) and apoptosis (increased expression of Bcl-2 and decreased Bax and caspase-3). Furthermore, histopathological examination demonstrated reduced inflammation, collagen deposition, and glomerulosclerosis in the renal tissue. In addition, all of these changes were comparable with those produced by ramipril. Hence, apigenin ameliorated renal damage due to DN by suppressing oxidative stress and fibrosis and by inhibiting MAPK pathway.

diabetic nephropathy; oxidative stress; inflammation; fibrosis; apoptosis; apigenin

DIABETES MELLITUS is a complex metabolic disorder characterized by hyperglycemia resulting from defective insulin secretion and/or response (3). Diabetic nephropathy (DN) is one of the major microvascular complications of diabetes mellitus (19). Globally, it accounts for nearly half of all end-stage renal disease cases. Moreover, the high cost associated with dialysis and renal transplant also adds to the societal economic burden (12). Thus the high morbidity as well as management cost associated with DN makes it imperative to search for novel means to halt the development and progression of DN.

There is ample evidence that oxidative stress, inflammation, and fibrosis play a pivotal role in the progression of DN (29). Previous studies have shown that hyperglycemia-induced oxidative stress contributes significantly to kidney injury (22). This fact is given credence by the observation that attenuation of oxidative stress in streptozotocin (STZ)-induced diabetic rats prevents expansion of renal mesangial matrix (22). Oxidative stress leads to DNA damage, which, in turn, causes translocation of cytochrome *c* from the mitochondria, activation of various caspases, and induction of apoptosis in diabetic rats (6). Another key event in DN is the buildup of extracellular matrix (ECM) proteins collagen and fibronectin. Hyperglycemia stimulates overproduction of fibronectin and type IV collagen in the glomerular mesangial matrix (25). The accumulation of fibronectin and type IV collagen in the mesangium and renal tubulointerstitium ultimately leads to a form of epithelial-to-fibroblast transition resulting in fibrosis, which is a hallmark feature of DN (19). In vitro studies provide substantial evidence that transforming growth factor-β1 (TGF-β1) is a prominent profibrotic protein and its expression and secretion is increased in the diabetic kidney (14, 18).

Flavonoids are phenolic compounds that are ubiquitously present in fruits, vegetables, nuts, tea, soy, and red wine. Flavonoids have been reported to possess good antidiabetic properties. In fact, many flavonoids have been proposed as promising agents for management of diabetes and its complications (48). Apigenin belongs to the flavone subclass of flavonoids and is abundant in fruits and vegetables, such as onions, oranges, and parsley (42). Apigenin has been attributed with various biological activities, such as antioxidant (9), anti-inflammatory (4), antiapoptotic (52), antimutagenic (44), and antitumorigenic (49) in various cell types. The antidiabetic activity of apigenin has also been extensively studied. Apigenin administration to diabetic mice has been shown to reduce hyperglycemia (35). Furthermore, Suh et al. (46) demonstrated that apigenin mitigates damage in pancreatic β-cells by suppression of oxidative stress-related signaling. Recently, the use of apigenin has been reported to be beneficial in diabetic vascular complication as it ameliorated endothelial dysfunction in thoracic aorta of diabetic rats (41). However, there are scarce data on the usefulness of apigenin in diabetic nephropathy. The aim of this study was, therefore, to investigate the protective effect of apigenin in a rodent model of diabetic nephropathy

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with focus on delineating the underlying molecular pathway(s) involved in the renoprotection.

MATERIALS AND METHODS

Animals. Male albino Wistar rats (150–200 g) were used for the study. Animals were procured from Central Animal House Facility of All India Institute of Medical Sciences, New Delhi, India, and were housed under standard laboratory conditions in departmental animal house at an ambient temperature (25 ± 2°C) with a relative humidity (60 ± 5%) under a 12:12-h light-dark cycle. Animals were fed food pellets (Ashirwad Industries, Chandigarh, India) and tap water ad libitum. The study protocol was approved by Institutional Animal Ethics Committee (IAEC no. 671/12) and conforms to the Indian National Science Academy *Guidelines for Care and Use of Animals in Scientific Research*.

Chemicals. Streptozotocin (STZ; S0130), apigenin (A 2568), and ramipril (M03712) were obtained from Sigma-Aldrich, Otto-Chemie, and Ranbaxy, respectively. Isophane insulin injection vial (B-80024) was procured from Biocon. OneTouch Ultra 2 blood glucose meter was purchased from LifeScan. Blood urea nitrogen (BUN; 120214), serum creatinine (120246), and urinary albumin kits (120223) were obtained from Transasia Bio-Medicals. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay kit (ApoBrdU-IHC in situ DNA fragmentation assay kit, cat. no. K403-50) was procured from BioVision. Tumor necrosis factor-α (TNF-α; 865.000.096), interleukin-6 (IL-6; ELR IL-6), fibronectin (FN; EK0350), TGF-β1 (EK0514), and type IV collagen (E12410604) ELISA kits were obtained from Diaclone Tepnel, RayBiotech (Norcross, GA), Boster Biological Technology, and Sincere Biotech, respectively. Primary antibodies for extracellular signal-regulated kinase 1/2 (ERK1/2; no. 4695), phospho (p)-ERK1/2 (no. 4370), c-Jun NH_2-terminal kinase (JNK; no. 9252), phospho (p)-JNK (no. 9251), caspase-3 (no. 9662), and β-actin (no. 4967) were obtained from Cell Signaling Technology, whereas Bcl-2 (ab7973), Bax (ab32503), and p38 (ab7952) were purchased from Abcam. Primary antibodies against NF-κBp65 (sc-109) and phospho (p)-p38 (sc-7973) was procured from Santa Cruz Biotechnology. Secondary antibodies goat anti-rabbit (621140380011730) and goat anti-mouse (621140680011730) were procured from Merck Genei. All other chemicals used were of analytical grade and purchased from Sigma Chemical (St. Louis, MO). For administration, apigenin and ramipril were dissolved in 0.5% carboxymethyl cellulose (61799305001730).

Induction of diabetes. After acclimatization, in all animals except the control and per se group, type 1 diabetes mellitus was induced by a single intraperitoneal injection of STZ (55 mg/kg) freshly prepared in 0.1 M ice-chilled citrate buffer (pH 4.5) to overnight-fasted rats. After 3 days of STZ injection, blood was withdrawn from tail vein and diabetes was confirmed by estimation of blood glucose by using blood glucose meter. Rats with fasting blood glucose >250 mg/dl were considered diabetic and were used in the study.

Experimental design. Male albino Wistar rats were randomly divided into seven groups. The following treatment schedule was started and continued for 8 mo. *Group 1* (control; $n = 10$): rats were administered 0.5% carboxymethyl cellulose (1.5 ml/kg po). *Group 2* (diabetic control; $n = 12$): diabetic rats were administered 0.5% carboxymethyl cellulose (1.5 ml/kg po) once daily. *Groups 3–5* (diabetes + 5–20 mg/kg apigenin; $n = 12$ per group): diabetic rats were administered apigenin (5, 10, and 20 mg/kg po, respectively) once daily. *Group 6* (apigenin per se*;* $n = 10$): rats were administered apigenin (20 mg/kg po) once daily. *Group 7* (diabetes + ramipril; $n = 12$): diabetic rats were administered ramipril (2 mg/kg po) once daily. The doses of apigenin, i.e., 5, 10, and 20 mg/kg, were selected after a systematic literature search (2, 11, 35–37, 47). Isophane insulin (4 U) was administered once daily to diabetic rats to maintain blood glucose level between 300 and 400 mg/dl and to prevent ketoacidosis. The day before death, all of the experimental animals were individually transferred to metabolic cages for 24-h urine collection and stored at 4°C until analyzed. At the end of treatment, rats were anesthetized with injection of pentobarbitone sodium (60 mg/kg ip). Blood was withdrawn from the heart, collected in Eppendorf tubes, and centrifuged at 4,000 rpm to obtain the serum. The serum was used for the estimation of inflammatory cytokines (TNF-α and IL-6). The rats were then killed, and both kidneys were harvested, washed with normal saline, and processed for biochemical, histopathological, TUNEL assay, estimation of fibrotic markers (tissue FN, TGF-β1, and type IV collagen), immunohistochemical (IHC), and Western blot analysis.

Analysis of serum and urinary nephrotoxic markers. Serum creatinine and BUN levels were estimated using commercially available kits as per the manufacturer's instructions. Urinary albumin level was measured by bromocresol green (BCG) dye method using kit according to the manufacturer's instructions.

Biochemical estimation. For determination of biochemical parameters, the kidneys were removed from liquid nitrogen and thawed. The kidneys were weighed, and a 10% homogenate was prepared in ice-chilled phosphate buffer (0.1 M, pH 7.4). An aliquot of the homogenate was used for the estimation of malondialdehyde (MDA) and reduced glutathione (GSH; Refs. 33, 34). The remaining kidney homogenate was centrifuged at 5,000 rpm for 20 min at 4°C, and supernatant was used for the estimation of superoxide dismutase (SOD), catalase (CAT) enzyme activities, and protein content (1, 8, 28).

Tissue preparation and histological analysis. After the animals were killed, kidneys were immediately stored in 10% neutral buffered formalin and embedded in molten paraffin to make tissue blocks. Then, tissue sections of 5-μm thickness were cut using microtome (Leica RM2125). These sections were stained with hematoxylin-eosin (H&E) for any histopathological changes. Furthermore, tissue sections were subjected to Masson trichrome (MT) and periodic acid-Schiff (PAS) stains to demonstrate fibrosis and glomerulosclerosis in the renal tissues and visualized under light microscope (Dewinter Optical).

Assessment of proinflammatory and fibrotic markers. The levels of inflammatory cytokines, i.e., serum TNF-α and IL-6, and fibrotic markers, i.e., tissue FN, TGF-β1, and type IV collagen, were measured using commercially available ELISA kits as per manufacturer's instructions.

TUNEL assay. The TUNEL assay was performed by using ApoBrdU-IHC in situ DNA fragmentation assay kit. After death, the kidneys were removed and stored in 10% neutral buffered formalin. The fixed kidney tissue was then embedded in a paraffin block, and slices of 5-μm thickness were cut using a microtome. For deparaffinization and rehydration, sections were passed through xylene and graded series of ethanol and finally washed with distilled water. Sections were incubated with proteinase K for 20 min at room temperature followed by incubation with 30% H_2O_2 for 5 min to enhance tissue permeability and to reduce endogenous peroxidase activity, respectively. The sections were then incubated with complete labeling reaction mixture followed by addition of antibody solution. Antigen-antibody reaction was visualized using 3,3′-diaminobenzidine (DAB) as chromogen. At least five areas in each section were observed to check for TUNEL positivity.

IHC analysis for detection of apoptosis. IHC analysis was performed according to the method described in our previous paper (27).

The pathologist performing the histological, TUNEL, and IHC evaluation was blinded to the treatment assigned to different study groups.

Western blot analysis. Kidney tissue was removed from liquid nitrogen, weighed, and homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer (150 mM NaCl, 10% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 50 mM Tris base) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Homogenates were then centrifuged at 12,000 rpm for 20 min at 4°C, and total protein concentrations were measured using Bradford

method. Protein mixtures were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was blocked with 3% bovine serum albumin (BSA) and 0.5% Tween 20 in Tris-buffered saline (TBS). After washing, the membrane was incubated with primary antibodies, i.e., ERK1/2, p-ERK1/2, JNK, p-JNK, p38, p-p38, NF-κBp65, and β-actin (1:3,000). These primary antibodies were detected by adding horseradish peroxidase-conjugated secondary antibodies after incubating for 2 h at room temperature. Later, the bound antibodies were visualized with an enhanced chemiluminescence (ECL; Thermo Fisher Scientific) kit and quantified by densitometric analysis using ImageJ software.

Statistical analysis. All results are expressed as means ± SE. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc test using GraphPad InStat software. The value of $P < 0.05$ was considered to be statistically significant.

Table 2. *Effect of apigenin on kidney function tests in STZ-induced diabetic nephropathy in rats*

Group	BUN, mg/dl	Sr. Creatinine, mg/dl	Urinary Albumin, g/dl
Control	22.5 ± 0.76	0.7 ± 0.1	0.58 ± 0.052
Dia-control	49.33 ± 1.74[b]	1.46 ± 0.14[a]	1.08 ± 0.049[b]
Dia+APG 5	47.5 ± 1.76	1.22 ± 0.12	0.99 ± 0.064
Dia+APG 10	44 ± 1.34	1.07 ± 0.11	0.88 ± 0.065
Dia+APG 20	41.5 ± 1.47[d]	0.89 ± 0.08[c]	0.78 ± 0.069[c]
APG 20ps	24.33 ± 1.02	0.77 ± 0.11	0.60 ± 0.051
Dia+RAM	36.33 ± 1.58[e]	0.87 ± 0.14[c]	0.67 ± 0.063[e]

Data are presented as means ± SE; $n = 6$ in each group. BUN, blood urea nitrogen; Sr. Creatinine, serum creatinine; Dia-control, diabetic control; Dia+APG 5, diabetes + 5 mg/kg apigenin; Dia+APG 10, diabetes + 10 mg/kg apigenin; Dia+APG 20, diabetes + 20 mg/kg apigenin; APG 20ps, 20 mg/kg apigenin per se; Dia+RAM, diabetes + 2 mg/kg ramipril. [a]$P < 0.01$, [b]$P < 0.001$ vs. control rats, [c]$P < 0.05$, [d]$P < 0.01$, [e]$P < 0.001$ vs. diabetic control rats.

RESULTS

Effect on body weight. Body weight of all experimental groups was measured on *day 1* and after 2, 4, 6, and 8 mo. There was a significant ($P < 0.001$) reduction in body weight in diabetic control groups compared with control group at the end of 8 mo. However, no significant change in body weight was observed between drug treatment and diabetic control group (Table 1).

Blood glucose measurement. Blood glucose level was assessed on *day 1* and after 2, 4, 6, and 8 mo. Isophane insulin was administered to maintain blood glucose level of diabetic control and drug treatment groups between 300 and 400 mg/dl to prevent ketoacidosis. Hence, there was no significant difference in blood glucose level between diabetic control and treatment groups (data not shown).

Effect on renal function-related parameters. The diabetic rats had significantly increased levels of serum creatinine ($P < 0.01$), BUN ($P < 0.001$), and urinary albumin ($P < 0.001$), which indicates the development of diabetic nephropathy in rats (Table 2). Treatment with apigenin (20 mg/kg) and ramipril significantly normalized their levels compared with diabetic rats. Thus the highest dose of apigenin and ramipril preserved renal function, whereas the two lower doses of apigenin, i.e., 5 and 10 mg/kg, failed to exert any significant effect on nephrotoxic markers.

Effect on oxidative stress markers. In diabetic control group, there was a significant ($P < 0.001$) increase in the level of MDA, a marker of lipid peroxidation, and reduction in the level of antioxidants (GSH, SOD, and CAT) compared with the nondiabetic rats. Apigenin (20 mg/kg) and ramipril treatment significantly ($P < 0.01$) augmented the levels of the antioxidant enzymes and reduced the level of MDA in diabetic rats. The lower doses of apigenin (5 and 10 mg/kg) had no significant effect on the oxidant-antioxidant status of rat kidney (Table 3).

Effect on proinflammatory cytokines level. The cytokines TNF-α and IL-6 play an important role in the development and progression of diabetic nephropathy. The levels of these cytokines were measured using ELISA kits. In the diabetic control group, their levels were significantly increased compared with the control group. Although apigenin dose-dependently reduced the cytokine release, only the highest dose of apigenin, i.e., 20 mg/kg, and ramipril significantly inhibited the diabetes-mediated increase in the levels of TNF-α and IL-6 (Table 4).

Effect on fibrotic markers. The levels of fibrotic markers TGF-β1, FN, and type IV collagen were analyzed as these are associated with development of DN. In diabetic control rats, the levels of TGF-β1, FN, and type IV collagen were significantly ($P < 0.001$) elevated in comparison with nondiabetic control animals. Interestingly, apigenin (20 mg/kg) and ramipril treatment significantly reduced the level of fibrotic markers. Thus the renoprotective effect exerted by apigenin treatment may be attributed to its antifibrotic effect, which prevented the collagen deposition in the renal tissue (Table 4).

Effect on morphological changes in diabetic rats. Morphological changes in renal tissue were determined by H&E, MT, and PAS staining (Fig. 1). The glomerular mesangial area was estimated on PAS stain sections using ImageJ software under light microscope. Mean mesangial area of the glomerulus of all of the groups was calculated. The normal mesangial area was graded as 1, then up to 2 times increase in mesangial area was

Table 1. *Body weight changes in different experimental groups*

Group	*Day 1*	After 2 mo	After 4 mo	After 6 mo	After 8 mo
Control	155.5 ± 6.26	194 ± 7.20†	244.17 ± 8.39§	305 ± 8.84§	340.67 ± 9.88§
Dia-control	163.83 ± 5.92	155 ± 7.02*	145.5 ± 5.73*	136.67 ± 4.01*†	121 ± 3.13*§
Dia+APG 5	164.67 ± 5.79	151.33 ± 4.87	141.17 ± 5.63†	133 ± 2.37‡	124.5 ± 5.09‡
Dia+APG 10	170.5 ± 5.52	156.16 ± 6.26	146.33 ± 5.11†	134.17 ± 4.04§	125.83 ± 5.07§
Dia+APG 20	164.17 ± 4.57	153.33 ± 4.73	143.5 ± 5.89	137.17 ± 6.21†	128.33 ± 6.07§
APG 20ps	166.5 ± 4.16	183.5 ± 3.42§	230.5 ± 8.05§	288.33 ± 6.53§	324.33 ± 10.51§
Dia+RAM	165.5 ± 5.08	154.67 ± 5.11	147.17 ± 5.67	139.17 ± 4.49‡	131 ± 4.87§

Data are presented as means ± SE; $n = 6$ in each group. Dia-control, diabetic control; Dia+APG 5, diabetes + 5 mg/kg apigenin; Dia+APG 10, diabetes + 10 mg/kg apigenin; Dia+APG 20, diabetes + 20 mg/kg apigenin; APG 20ps, 20 mg/kg apigenin per se; Dia+RAM, diabetes + 2 mg/kg ramipril. *$P < 0.001$ vs. control rats, †$P < 0.05$, ‡$P < 0.01$, §$P < 0.001$ vs. respective baseline values.

Table 3. *Effect of apigenin on oxidant-antioxidant parameters in STZ-induced diabetic nephropathy in rats*

Group	MDA, nmol/g Tissue	GSH, μmol/g Tissue	SOD, U/mg Protein	CAT, U/mg Protein
Control	56.39 ± 2.03	2.59 ± 0.13	6.21 ± 0.17	6.56 ± 0.18
Dia-control	88.69 ± 2.74†	1.72 ± 0.13*	3.75 ± 0.22†	4.15 ± 0.26†
Dia+APG 5	83.34 ± 2.52	2.05 ± 0.14	4.34 ± 0.27	5.13 ± 0.27
Dia+APG 10	79.52 ± 2.69	2.29 ± 0.13	4.66 ± 0.23	5.36 ± 0.21
Dia+APG 20	76.37 ± 2.62‡	2.39 ± 0.17‡	4.99 ± 0.24‡	5.60 ± 0.17§
APG 20ps	60.21 ± 2.08	2.54 ± 0.11	6.14 ± 0.25	6.13 ± 0.26
Dia+RAM	74.07 ± 2.33§	2.41 ± 0.15‡	5.27 ± 0.24§	5.68 ± 0.22§

Data are presented as means ± SE; $n = 6$ in each group. MDA, malondialdehyde; GSH, reduced glutathione; SOD, superoxide dismutase; CAT, catalase; Dia-control, diabetic control; Dia+APG 5, diabetes + 5 mg/kg apigenin; Dia+APG 10, diabetes + 10 mg/kg apigenin; Dia+APG 20, diabetes + 20 mg/kg apigenin; APG 20ps, 20 mg/kg apigenin per se; Dia+RAM, diabetes + 2 mg/kg ramipril. *$P < 0.01$, †$P < 0.001$ vs. control rats, ‡$P < 0.05$, §$P < 0.01$ vs. diabetic control rats.

graded as 2, and up to 3 times increase in mesangial area was graded as 3. Control and per se groups revealed normal glomeruli without any pathological changes. In diabetic control rats, there was glomerular hypertrophy, fibrosis, mesangial cell proliferation, mesangial matrix expansion, and infiltration of inflammatory cells. Also, the injury score of the diabetic control rats was the highest as per the above grading compared with control group. Furthermore, the rats treated with 5 and 10 mg/kg apigenin had glomerular hypertrophy, mesangial matrix expansion, and inflammation similar to diabetic control group. However, the rats treated with 20 mg/kg apigenin and ramipril exhibited a decrease in the extent of glomerular hypertrophy, fibrosis, mesangial matrix expansion, and inflammation. Furthermore, injury score in the 20 mg/kg apigenin and ramipril group was significantly lower compared with diabetic control group.

Thus, on the basis of the above findings, it was observed that the renoprotective effect was significant at the highest dose of apigenin (20 mg/kg) and ramipril. Hence, these groups were further used for TUNEL, IHC, and Western blot analysis.

Effect on apoptotic changes in diabetic rats. To confirm the presence of apoptosis in diabetic rats, Bcl-2, Bax, and caspase-3 expressions were studied with IHC. In comparison with control rats, there was increased expression of proapoptotic proteins (Bax and caspase-3) and decreased expression of antiapoptotic protein (Bcl-2) in the diabetic rats. On the contrary, Bax and caspase-3 expressions were decreased and Bcl-2 expressions were increased in the treatment group (Fig. 2).

Our IHC findings were further corroborated by performing TUNEL assay in the renal tissue. Similar to IHC results, there was an increased number of TUNEL-positive nuclei in the diabetic control group compared with the control rats. In contrast, fewer TUNEL-positive cells were observed in rats treated with apigenin (20 mg/kg) and ramipril. Hence, there was marked reduction in apoptosis in rats treated with either apigenin (20 mg/kg) or ramipril (Fig. 2).

Effect on various protein expressions in diabetic rats. NF-κB is a transcription factor that causes release of proinflammatory cytokines in the renal tissue. Hence, the protein expression of NF-κB was analyzed with Western blotting. There was an increased expression of NF-κBp65 in the diabetic rats compared with nondiabetic controls. The expression of NF-κBp65 in rats treated with 20 mg/kg apigenin and ramipril was significantly attenuated. This is suggestive of decreased inflammation in these treatment groups (Fig. 3).

It is well-known that inflammation and apoptosis are regulated by MAPK pathway. Hence, we assessed the role of apigenin and ramipril treatment on MAPK activation by determining the phosphorylation of ERK1/2, JNK, and p38 using Western blot analysis. Diabetes resulted in activation of MAPK subfamilies, i.e., there was increased expression of ERK1/2, JNK, and p38, and treatment with 20 mg/kg apigenin and ramipril prevented the phosphorylation and decreased the expression of ERK1/2, JNK, and p38 in renal tissue. Thus there was attenuation of apoptosis and inflammation in renal tissue of rats that were treated with either apigenin (20 mg/kg) or ramipril (Fig. 3).

DISCUSSION

In the present study, we observed that chronic uncontrolled hyperglycemia following STZ administration led to the development of diabetic nephropathy (DN). We have reported that DN was attenuated by both apigenin (20 mg/kg) and ramipril.

Table 4. *Effect of apigenin on serum proinflammatory cytokines and tissue fibrotic markers in STZ-induced diabetic nephropathy in rats*

Group	TNF-α, pg/ml	IL-6, pg/ml	TGF-β1, pg/mg Protein	FN, pg/mg Protein	Type IV Collagen, ng/mg Protein
Control	15.83 ± 1.15	8.83 ± 0.76	31.01 ± 2.68	88.61 ± 7.19	9.83 ± 2.21
Dia-control	49.58 ± 1.56*	22.72 ± 1.51*	62.46 ± 3.17*	148.98 ± 10.02*	19.42 ± 4.85*
Dia+APG 5	46.58 ± 1.79	19.85 ± 1.53	58.34 ± 3.02	133.03 ± 9.67	17.19 ± 2.56
Dia+APG 10	43.75 ± 1.59	17.95 ± 1.49	53.95 ± 2.59	122.37 ± 8.94	15.17 ± 1.85
Dia+APG 20	40.66 ± 1.70‡	15.93 ± 1.38†	49.04 ± 2.63†	108.96 ± 7.41†	13.23 ± 3.03‡
APG 20ps	19.33 ± 1.55	9.31 ± 0.85	32.48 ± 2.67	91.49 ± 8.86	10.13 ± 4.7
Dia+RAM	36.5 ± 2.00§	13.97 ± 1.36§	45.08 ± 2.53‡	103.33 ± 6.31‡	11.40 ± 1.47§

Data are presented as means ± SE; $n = 6$ in each group. TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; TGF-β1, transforming growth factor-β1; FN, fibronectin; Dia-control, diabetic control; Dia+APG 5, diabetes + 5 mg/kg apigenin; Dia+APG 10, diabetes + 10 mg/kg apigenin; Dia+APG 20, diabetes + 20 mg/kg apigenin; APG 20ps, 20 mg/kg apigenin per se; Dia+RAM, diabetes + 2 mg/kg ramipril. *$P < 0.001$ vs. control rats, †$P < 0.05$, ‡$P < 0.01$, §$P < 0.001$ vs. diabetic control rats.

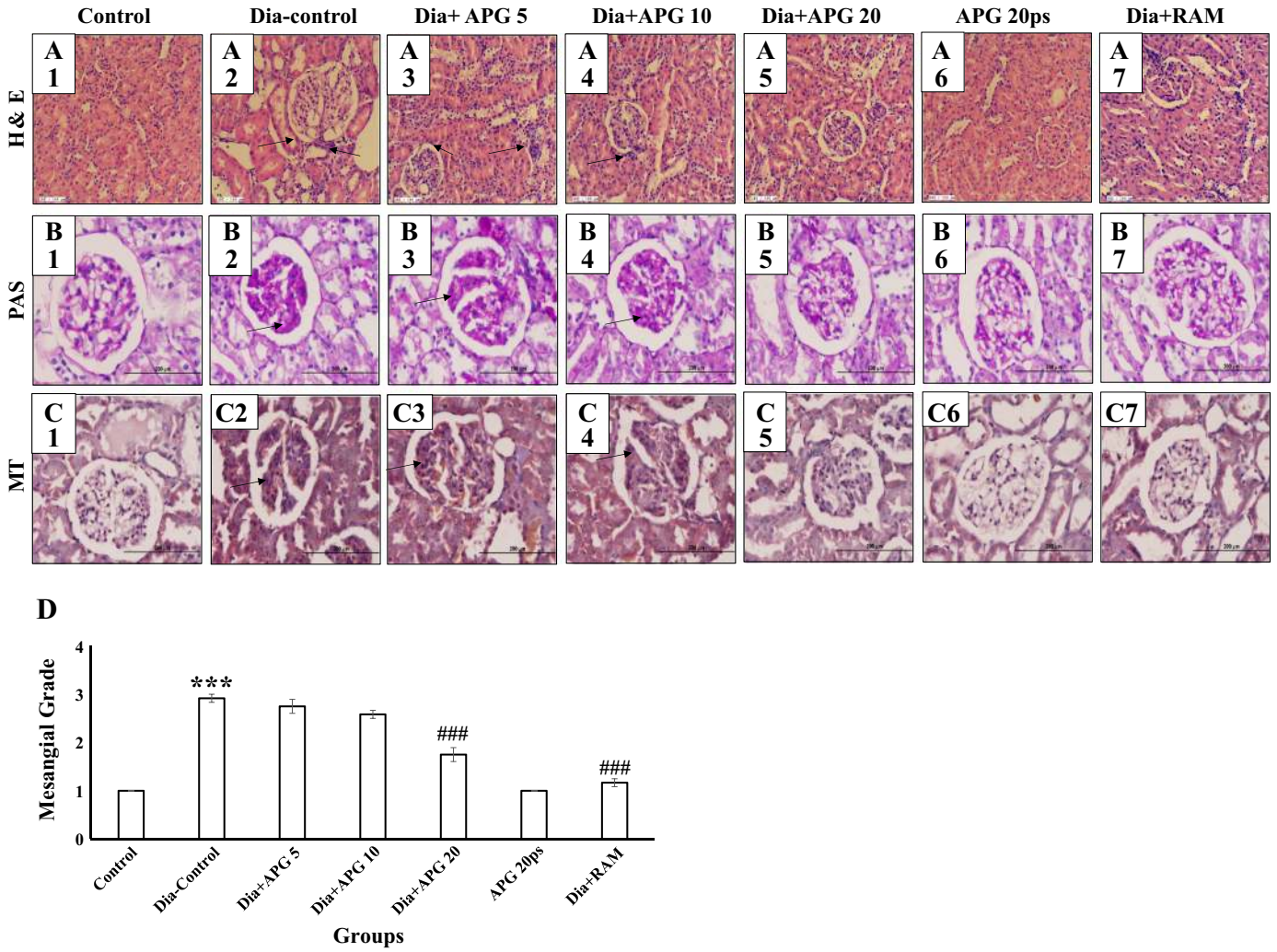

Fig. 1. Effect of apigenin on morphological changes (H&E stain: $n = 3$, ×20; PAS stain: $n = 3$, ×40; and MT stain: $n = 3$, ×40) in STZ-induced diabetic nephropathy in rats. *A1–C1*: control. *A2–C2*: diabetic control. *A3–C3*: diabetes + 5 mg/kg apigenin. *A4–C4*: diabetes + 10 mg/kg apigenin. *A5–C5*: diabetes + 20 mg/kg apigenin. *A6–C6*: 20 mg/kg apigenin per se. *A7–C7*: diabetes + 2 mg/kg ramipril. *D*: mesangial grade (→) glomerular damage. Data are presented as means ± SE; $n = 3$ in each group. ***$P < 0.001$ vs. control rats, ###$P < 0.01$ vs. diabetic control rats.

The renoprotective effect of these drugs was demonstrated by evaluating various factors such as renal function parameters and biochemical, histopathological, inflammatory, and apoptotic markers. Furthermore, the expression of MAPK was determined to delineate the mechanistic pathway responsible for its nephroprotection.

DN, a renal microvascular complication arising as a consequence of long-standing uncontrolled hyperglycemia, affects the renal glomeruli and tubules. There is expansion of mesangium and thickening of the basement membrane of glomeruli. The renal tubules exhibit hypertrophy initially in the early stages, but eventually interstitial fibrosis with tubular atrophy sets in, together with arteriolar hyalinosis along with infiltration with macrophages and T lymphocytes in the late stages. Ultrastructurally, there is loss of podocyte as well as reduced endothelial cell fenestration. Moreover, the structural change is also accompanied by functional loss. Functionally, there is glomerular hyperfiltration with increased albumin excretion in the early stage; and in the advanced stage, there is increased proteinuria with decline in glomerular filtration rate (38). Previous studies have established that the renal pathological and functional changes in STZ-induced diabetic rat model are very similar to the changes seen in human diabetic kidney (55). Thus, in the present study, we induced diabetes in rats with a single injection of STZ (55 mg/kg ip). These diabetic rats exhibited glomerulosclerosis, fibrosis, and infiltration of inflammatory cells. These histopathological renal changes were attenuated by treatment of rats with apigenin (20 mg/kg). Furthermore, in diabetic rats, the levels of serum creatinine, BUN, and urinary albumin were raised in comparison with the control rats. The treatment of rats with apigenin (20 mg/kg) significantly normalized serum creatinine, BUN, and urinary albumin level. Thus apigenin (20 mg/kg) treatment of diabetic rats preserved renal function. Our results are in accordance with a previously published study that had demonstrated that apigenin administration improved renal function in furan-induced toxicity in mice (49).

STZ-induced hyperglycemia causes an excessive production of reactive oxygen species (ROS) via exertion of heavy electron pressure on mitochondrial electron transport chain from

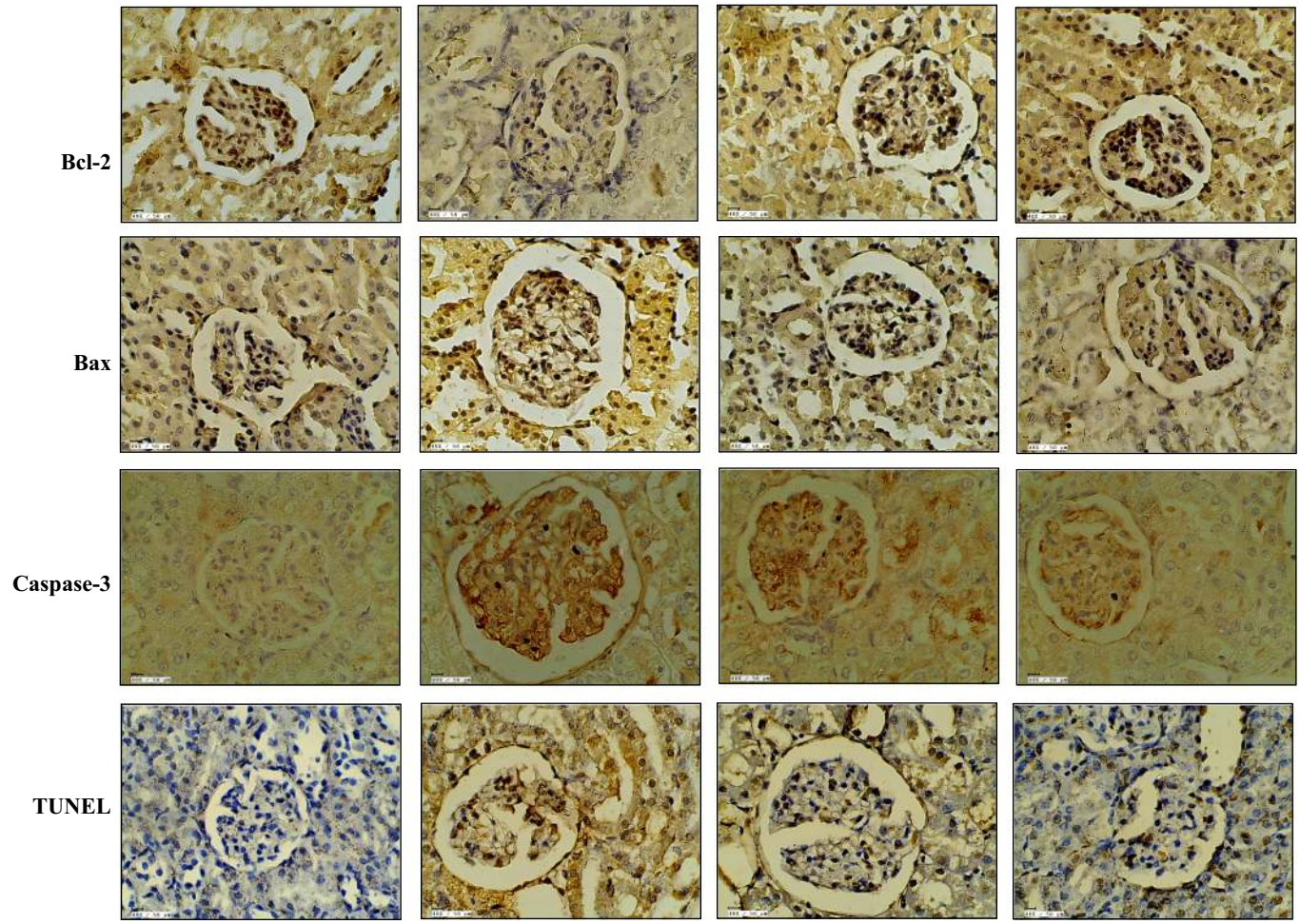

Fig. 2. Effect of apigenin on Bcl-2 immunohistochemistry, Bax immunohistochemistry, caspase-3 immunohistochemistry, and TUNEL positivity ($n = 3$, ×40) in STZ-induced diabetic nephropathy in rats.

oxidation of overproduced NADH (40). The balance between the formation of ROS, including superoxide anion ($O_2^{\cdot -}$) and the antioxidant defense system, which includes SOD, CAT, and GSH, reflects the extent of oxidative stress (39). There is substantial evidence from previous reports that oxidative stress is involved in the pathogenesis of DN (22). In the present study, we observed that in the diabetic control group, there was an increased ROS generation as evidenced by increased level of MDA, a marker of lipid peroxidation, and an increased consumption of free radical scavengers like SOD, CAT, and GSH. Apigenin (20 mg/kg) administration reduced ROS generation and restored antioxidant status, and these effects were more pronounced in the ramipril treatment group. This is in accordance with previous in vitro and in vivo studies that have also shown that apigenin has free radical scavenging properties (9, 23). Ramipril exerts renoprotection by blocking angiotensin type 1 receptor (AT_1R)-mediated oxidative stress. It is well-known that activation of AT_1R by angiotensin II (ANG II) leads to augmentation of oxidative stress in podocytes during DN (5).

ROS accumulation triggers apoptosis or programmed cell death, which has been implicated in the pathogenesis of various diseases, including diabetic nephropathy. Apoptosis may result as a consequence of direct ROS-mediated caspase release from mitochondria and via indirect ROS-mediated activation of downstream pathways leading to transcription of apoptotic protein genes (6). Hyperglycemia has been shown to stimulate caspase-3 cleavage and DNA fragmentation, and the resultant apoptosis causes mesangial cell loss in DN (31). Results from our study support previous studies as proapoptotic proteins were increased and antiapoptotic proteins were decreased in the diabetic control group. Treatment with 20 mg/kg apigenin reversed these changes with a near complete reversal seen in the ramipril treatment group. These findings support the antiapoptotic effect of apigenin documented in previous studies (15, 52).

Apart from apoptosis, oxidative stress also activates mitogen-activated protein kinases (MAPKs), which are serine/threonine kinases that comprise extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 MAPK, and c-Jun NH_2-terminal kinase (JNK; Ref. 30). MAPKs are activated by various stimuli, including oxidative stress. They have also been linked to the pathogenesis of DN, as increased phosphorylation of MAPKs has been detected in the kidney of STZ-induced diabetic rats (21). MAPK activation is associated with processes that are crucial to DN pathogenesis, including apoptosis, influx of inflammatory cells, and increased extracellular matrix (ECM) synthesis (13). There is strong evidence supporting the

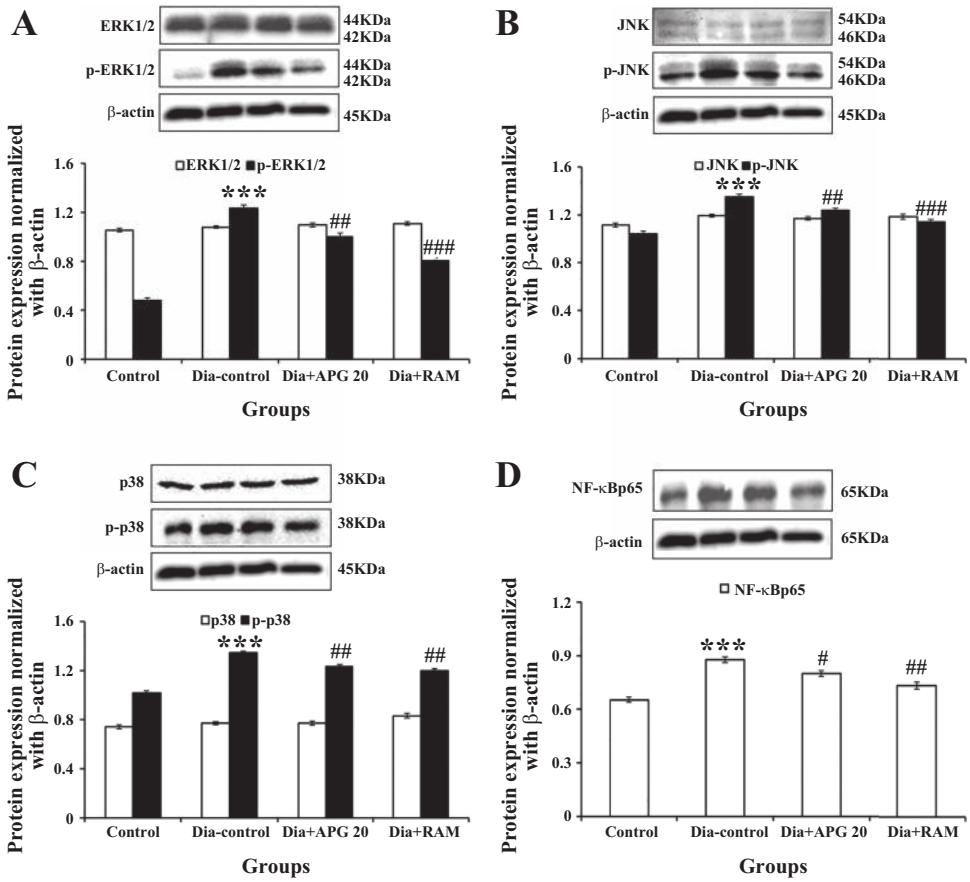

Fig. 3. Effect of apigenin on various protein expressions in STZ-induced diabetic nephropathy in rats. *A*: ERK1/2 and p-ERK1/2. *B*: JNK and p-JNK. *C*: p38 and p-p38. *D*: NF-κBp65. Dia-control, diabetic control; Dia+APG 20, diabetes + 20 mg/kg apigenin; Dia+RAM, diabetes + 2 mg/kg ramipril. Protein expressions are normalized with β-actin. Data are presented as means ± SE; $n = 6$. ***$P < 0.001$ vs. control rats, #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ vs. diabetic control rats.

pivotal role of the MAPK signaling pathway in hyperglycemia-induced cell damage (16). Recent data from studies suggest that hyperglycemia can activate MAPK signaling pathway by phosphorylation of MAPK. The phosphorylated MAPK can translocate to the cell nucleus and subsequently modulate the function of various transcription factors through phosphorylation at their serine and threonine residues. These transcription factors, in turn, modify the expression of genes, which ultimately result in a biological response (16). One such transcription factor activated by MAPK is nuclear factor-κB (NF-κB). NF-κB is expressed in various tissues and serves as an important molecule in mediating inflammation in DN (56). Its activation is mainly dependent on the alternate phosphorylation and degradation of IκB (17). The activation of NF-κB is evidently enhanced in the kidneys of diabetic animals, which augments expansion of mesangium (10). Activated NF-κB stimulates the transcription of inflammatory cytokines like TNF-α and IL-6 (45). Experiments in animal models of diabetes have demonstrated that TNF-α and IL-6 protein levels are increased in renal glomeruli and tubules (32). The phosphorylation of p38 MAPKs, which is seen to increase adhesion molecules and cytokines, has been shown to contribute to cell death in various disorders (30). In our study, there was increased phosphorylation of p38, JNK, and ERK1/2 in the diabetic control rats compared with the control rats with an associated increase in the levels of TNF-α and IL-6. The levels of these cytokines were normalized in the 20 mg/kg apigenin treatment group. The anti-inflammatory effect was, however, more pronounced in the ramipril group. The present study is also in keeping with other recent studies that have shown that apigenin reduces inflammation and modulates MAPK activity (53, 57). It is well-known that ANG II contributes to DN by increasing inflammation and MAPK activity. Thus administration of angiotensin-converting enzyme (ACE) inhibitor protects against development of DN in patients with diabetes (54). One of the downstream effector of MAPK in renal fibrosis is fibronectin. It is a major component of the ECM and is located on the mesangial matrix and glomerular basement membrane (GBM) where it mediates GBM attachment to glomerular cells (50). Fibronectin expression is increased during kidney injury. Its increased expression in DN has been previously documented (25). Since fibronectin is a downstream component of MAPK, activation of MAPK leads to induction of fibronectin gene (24). In our study, we observed increased expression of type IV collagen and fibronectin in the diabetic control group. The expression of these proteins was markedly reduced in rats treated with the highest dose of apigenin (20 mg/kg). Our results also corroborate well with another study in which apigenin was observed to suppress fibronectin expression (43).

In addition to fibronectin, TGF-β is another cytokine involved in the synthesis of ECM, and it is considered to be the most potent profibrotic molecule (7). Although three isoforms of TGF-β exist, TGF-β1 has an established role in kidney fibrosis. Studies have shown reciprocal relationship between oxidative stress and TGF-β. Oxidative stress can stimulate TGF-β and vice versa (26). TGF-β1 has been documented as an upstream regulator of MAPK (20). Recent studies have demonstrated the association between hyperglycemia and the

activation of the TGF-β1-p38 MAPK pathway in the kidney (50). We observed an increase in TGF-β1 expression in the diabetic control group, and this expression was attenuated by administration of 20 mg/kg apigenin. Also, a near-normal expression of TGF-β1 was observed in the ramipril treatment group. Our study validates the renal TGF-β1 suppression activity of apigenin, which has been documented previously (11). AT_1 receptors have been demonstrated in renal fibroblasts, which respond to ANG II stimuli by matrix expansion and fibronectin synthesis by a TGF-β-dependent mechanism (54). These facts provide an explanation for the near-normal ECM components observed in the ramipril group.

In conclusion, we have demonstrated that STZ administration resulted in renal dysfunction, oxidative stress, fibrosis, inflammation, apoptosis, and activation of MAPK pathway. Administration of 20 mg/kg apigenin attenuated oxidative stress, apoptosis, inflammation, and fibrosis via suppression of MAPK-NF-κB-TNF-α and TGF-β1-MAPK-fibronectin pathways. Thus apigenin has significant nephroprotective effect and good potential for alleviating DN. However, its use in human subjects requires further clinical evaluation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

D.S.A. conceived and designed research; S.M. performed experiments; K.S. and A.K.D. interpreted results of experiments; S.I.K. and J.B. drafted manuscript; K.K. and D.S.A. approved final version of manuscript.

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