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Protective effect of Forskolin on diabetes induced neprophaty via antioxidant activity

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ABSTRACT

The present study aimed to investigate the role of adenylyl cyclase activator in preventing diabetic nephropathy via antioxidant activity in rats. Biochemical parameters were performed to confirm Streptozotocin induced nephropathy in rats. Male Wistar rats were used in the present study to reduce the effect of estrogen. Rats were subjected to high fat diet (HFD) for two weeks followed by low dose of Streptozotocin (STZ) [35mg/kg, i.p.] to develop experimental diabetic nephropathy in eight weeks. Two weeks treatment with low dose of Forskolin (10mg/kg) reduced the level of diabetic nephropathy markers but results observed were not significant. Whereas, Forskolin intermediate dose (20mg/kg) and high dose (30mg/kg) treated rats significantly attenuated diabetes induced elevated renal function parameters and endogenous antioxidants enzymatic activities. High dose of Forskolin was found to be more effective in attenuating the renal structural and functional abnormalities. Forskolin prevented renal structural and functional abnormalities diabetic rats. In the present study, Glibenclamide (0.6mg/kg) and Atorvastatin (0.5mg/kg) were used as standard drugs. Our results demonstrated synergistic effects, when high dose of Forskolin was co-administered with standard drugs. In conclusion, treatment with adenylyl cyclase activator, Forskolin in diabetic rats reduced the oxidative stress, improved renal functions and enhanced level of endogenous antioxidants. Forskolin prevented renal functional abnormalities due to diabetes mellitus. Forskolin has a potential to prevent diabetic nephropathy, implicating direct renoprotective action in diabetic rats.

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Keywords
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INTRODUCTION

As per the World health organization report currently diabetes affects more than 170 million people worldwide and the number will increase up to 370 million by 2030. India is known as the diabetes capital of the world. Diabetes mellitus (DM) is increasing worldwide and particularly type 2 DM and the major complication of DM include diabetic nephropathy (DN). Chronic hyperglycaemia can affects kidneys, heart, eyes and nerves. The affected organs in DM are kidneys and it is the major cause of end stage renal disease worldwide and also the major cause of morbidity and mortality in DM [1].

Worldwide many pharmacological drugs are available to treat the DN, but it is still a matter of concern today, because these pharmacological drugs are less efficacious and are associated with side effects. There is still a need of new therapeutic drugs which not only prevent the development of DN by various metabolic and inflammatory pathways but on the other side are side effects free. The popularity of the com-

plementary medicines has increased nowadays. Hyperglycaemia leads to increased oxidative stress and activation of polyol pathway, which may cause inflammation and renal damage.

Mitochondrial dysfunction, advanced glycation end processes and others are believed to be the probable sources [2]. Increasing evidence indicates that the disruption of mitochondrial bioenergetics may be important in the development and progression of DN [3].

Forskolin (FSK) a diterpene isolated from the plant Coleus forskohlii, rapidly activate adenylyl cyclase (AC) which increases the cyclic Adenosine Monophosphate (cAMP) level by converting the Adenosine Triphosphate (ATP) into cAMP [4]. Previous studies have reported that FSK possesses several biological properties including antioxidant and anti inflammatory activities [5]. Beside its free radical scavenging property, it might be necessary to investigate its therapeutic potential in diabetic nephropathy which is associated with oxidative stress. Therefore, present study aimed to investigate the protective effect of FSK in diabetes induced nephropathy in experimental rats.

MATERIALS AND METHODS

Experimental Animals

Seventy, male Wistar rats, with an initial body weight of 180-220g were used in this study. All experimental procedures used in this study were approved by Institutional Animal Ethical Committee (IAEC) (RITS/IAEC/2016/08/08) as per the instructions of CPCSEA, Government of India (888/PO/Re/S/05/CPCSEA). All animals were housed in standard light/dark cycle with free access to standard high-fat diet and water ad libitum. The animals were housed in metabolic cages. A 24-h urine collection was obtained from each rat for laboratory investigations. The study was carried out for 12 weeks.

Drugs and Chemicals

STZ 35mg/kg was procured from Sigma Chemicals, St. Louis, USA. FSK (10, 20 and 30mg/kg, p.o.) was obtained from Bangladesh Petroleum Exploration and Production Company Ltd., Rajasthan, India. Glibenclamide (0.6mg/kg, p.o.) and Atorvastatin (0.5mg/kg/day, p.o.) were obtained from Sigma Aldrich [P] Ltd., Bangalore, was dissolved in distilled water. All other chemicals used in the present study were of analytical grade. FSK was dissolved in distilled water and then administered orally to the animals for 2 weeks after 8 weeks STZ administration. The three doses of FSK were selected on the basis of the acute oral toxicity studies reported in addition to the previous studies carried out on the FSK [6].

Induction and Assessment of Diabetes Mellitus

DM was induced by feeding HFD to rats for 2 weeks. Component of HFD (g/kg) were powdered normal pellet diet 365g, lard 310g, casein 250g, cholesterol 10g, vitamin and mineral mix 60g, DL-methionine 0.3g, followed by single low dose of STZ 35mg/kg, i.p. further followed by HFD for next 8 weeks [7]. After 1 week of STZ administration rats having blood glucose of >200mg/dl were considered as diabetic and selected in the present study. Blood samples were obtained from retero-orbital sinus and serum glucose level was determined by glucose oxidase-peroxidase (GOD-POD) method.

Experimental Protocol (n=7)

Ten groups were employed and each group consisting of seven rats. Glibenclamide and Atorvastatin have been reported to have anti-diabetic and lipid lowering compounds [8, 9]. Therefore, Glibenclamide and Atorvastatin have been employed as standard drugs in the present study. The three doses of FSK (10mg/kg, 20mg/kg and 30mg/kg) were selected on the basis of the acute oral toxicity studies reported in addition to the previous studies carried out on the FSK [10, 11].

Group-I (Normal control): Rats were maintained on standard food and water regimen and no treatment was given.

Group II (FSK per se): Normal rats were administered FSK [30mg/kg, p.o.] for two weeks.

Group III (Diabetic Control): Normal rats were fed HFD for 2 weeks, followed by single dose of STZ [35 mg/kg, i.p.] and further followed by HFD for another 10 weeks.

Group IV: Rats were administered FSK [10mg/kg, p.o.] for two weeks after 8 weeks of STZ administration.

Group V: Rats were administered FSK [20mg/kg, p.o.] for two weeks after 8 weeks of STZ administration.

Group VI: Rats were administered FSK [30mg/kg, p.o.] for two weeks after 8 weeks of STZ administration.

Group VII: Rats were administered Glibenclamide [0.6mg/kg, p.o.] for two weeks after 8 weeks of STZ administration.

Group VIII: Rats were administered Atorvastatin [0.5mg/kg/day, p.o.] for two weeks after 8 weeks of STZ administration.

Group IX: Rats were administered Glibenclamide [0.6mg/kg, p.o.] and FSK [30 mg/kg, p.o] for two weeks after 8 weeks of STZ administration.

Group X: Rats were administered Atorvastatin [0.5mg/kg, p.o.] and FSK [30mg/kg, p.o] for two weeks after 8 weeks of STZ administration.

Evaluation of Renal functional parameters

Serum creatinine, BUN and protein in urine level were determined in all serum samples by using standard diagnostic kits (Transasia Bio-Medicals Ltd., Baddi, India). 24 hours urine was collected from each rat accommodated individually in metabolic cages for urine analysis.

Assessment of Renal Hypertrophy

Renal hypertrophy (absolute kidney weight, kidney weight / body weight ratio) was estimated by Sinuani, 2006 method [12].

Assessment of Renal Collagen Content

The renal cortical tissue was used for the determination of kidney collagen content. Total collagen content of kidney was determined by analysis of hydroxyproline content. Hydroxyproline was determined colorimetrically in duplicates from 0.2g of renal cortical tissue using a modified method of Jamall et al., 1981 [13]. The tissue was homogenized in 4 mL of 6N Hcl and hydrolysed at 110oC for 16 hr. Dried hydrolysate was dissolved in 1000µL of 50% isopropanol and then to 30µL of aliquot, 1.2mL of 50% isopropanol was added and incubated with 0.2mL of 0.84% chloramine-T in acetate citrate buffer (Citric acid-5.5g; Sodium acetate-57g; Trisodium citrate $2H_2O$-37.5g; n-propanol-385mL make volume upto 1000 mL with distilled water) pH 6.0 for 10 minutes at room temperature. Then 1mL Ehrlich's reagent was added and the mixture was incubated at 60^0C for 25 minutes. The absorbance of the sample solution was measured at 560nm wavelength. The hydroxyproline content in 200mg of renal cortical tissue was calculated from standard curve of 4-hydroxy-L-proline. Hydroxyproline value was converted to collagen content by multiplying by a factor of 6.94 (as hydroxyproline represents approximately 14.4% of the amino acid composition of collagen) and expressed further as mg collagen per gram of wet tissue.

Assessment of Lipid Profile

In addition, diabetes mellitus induced lipid alterations (serum total cholesterol, LDL, VLDL and HDL) were estimated in serum samples by using standard diagnostic kits (Transasia Bio-Medicals Ltd., Baddi, India).

Assessment of Thiobarbituric Acid Reactive Substances (TBARS)

Diabetes induced renal oxidative stress were assessed by measuring TBARS level using Ohkawa, 1979 method [14].

Assessment of Kidney Antioxidant Parameters

Estimation of Reduced Glutathione (GSH): The procedure to estimate the reduced GSH followed the method described by Ellman, 1959 [15]. The homogenate (in 0.1M phosphate buffer, pH 7.4) was added with equal volume of 20% trichloroacetic acid containing 1mM EDTA to precipitate the tissue proteins. The mixture was allowed to stand for 5 min in prior to centrifugation for 10 min at 200 rpm. The supernatant (200µL) was then transferred to a new sets of test tubes and added 1.8mL of the Ellman's reagent (5, 5'-dithio bis¬2- nitrobenzoic acid) (0.1mM) was prepared in 0.3M phosphate buffer with 1% of sodium citrate solution. Then all the test tubes make up to the volume of 2mL. After completion of the total reaction, solutions were measured at 412nm against blank. Absorbance values were compared with a standard curve generated from standard curve from known GSH. The GSH level was calculated as micromole/g.

Estimation of Superoxide Dismutase (SOD): SOD activity was determined by the method of Misra and Fradovich, 1974 [16]. The reaction mixture consisted of 1.0mL carbonate buffer (0.2M, pH 10.2), 0.8mL KCL (0.015M), 0.1mL of plasma sample and water to make the final volume to 3.0mL. The reaction was started by adding 0.2mL of epinephrine (0.025M). The change in absorbance was recorded at 480nm at 15 second interval for one min at 25°C. Suitable control lacking enzyme preparation was run simultaneously.

One unit of enzyme activity is defined as the amount of enzyme causing 50% inhibition of auto oxidation of epinephrine.

Estimation of Catalase (CAT): CAT activity was measured according to procedure of Aebi, 1984 [17] at room temperature. 100µL plasma samples were placed on ice bath for 30 minutes at room temperature. 10µL Triton-X was added in each plasma containing test tube. In a cuvette, 200µL phosphate buffer (0.2M; pH 6.8), 20µL of sample and 2.53mL distilled water was added. The reaction was started by adding 250µL of H2O2 (0.066M in phosphate buffer) and decrease in optical density was recorded at 240nm wave length at every 15 second for one min. The molar extinction coefficient of 43.6 M cm-1 was used for determination of CAT activity.

One unit of enzyme activity was defined as the amount of enzyme that liberates half of the peroxide oxygen from H_2O_2 in one minute at 25°C.

Statistical Analysis

The results were expressed as mean ± standard deviation (SD). Results obtained from various groups were statistically analysed by One Way - ANOVA followed by Post hoc Tukey's test. $p<0.001$ was considered statistically significant.

RESULTS

All drugs were dissolved in drinking water and administered orally in normal and diabetic rats for two weeks after eight weeks of Streptozotocin (STZ) administration.

Effect of FSK on Body Weight

Diabetic rats subjected to high fat diet (HFD) significantly increased the body weight when compared to normal control group. Two weeks treatment with FSK (10, 20 and 30mg/kg), Glibenclamide (0.6mg/kg) and Atorvastatin (0.5mg/kg) reduced the body weight, however the results were not significant when compared with diabetic control group. Body weight was observed unaltered in treated rats and diabetic control group (Table 1).

Effect of FSK Serum Glucose, Urine Output, Serum Creatinine, Blood Urea Nitrogen and Protein in urine in Diabetic Rats

The concentration of serum glucose, urine output, serum creatinine, Blood Urea Nitrogen (BUN) and protein in urine were noted to be significantly increased ($P<0.05$) in HFD and low dose of STZ administered rats when compared with normal rats. All rats were individually housed in metabolic cages for 24 hours for urine collection. Treatment with low dose of FSK-10mg/kg slightly reduced the biomarkers of nephropathy but the results were not statistically significant. However, treatment with intermediate and high dose of FSK

Table 1. Effect of FSK on body weight, serum glucose, BUN, protein in urine and urine output

Groups	Serum Creatinine (mg/dL)	Serum Glucose (mg/dL)	BUN (mg/dL)	Protein in Urine (mg/24hr)	Urine output (mL/24 hrs.)
Normal Control	0.60 ± 0.12	105.8 ± 9.95	19.12 ± 7.47	11.66 ± 2.83	8.42 ± 2.63
FSK per se	0.58 ± 0.11	106.9 ± 10.23	19.93 ± 5.18	11.94 ± 2.31	8.43 ± 2.50
Diabetic Control	1.83 ± 0.07[a]	360.8 ± 16.90[a]	102.90 ± 7.39[a]	85.52 ± 3.33[a]	76.57 ± 4.61[a]
FSK-10 in Diabetic group	1.72 ± 0.07[a]	336.6 ± 17.00[a]	94.57 ± 6.79[a]	78.43 ± 4.46[a]	69.29 ± 4.64[a]
FSK-20 in Diabetic group	1.42 ± 0.06[b]	278.6 ± 13.22[b]	76.89 ± 6.29[b]	67.69 ± 3.73[b]	62.29 ± 4.53[b]
FSK-30 in Diabetic group	1.05 ± 0.08[bc]	203.9 ± 15.03[bc]	60.41 ± 6.90[bc]	54.16 ± 4.30[bc]	50.43 ± 4.72[bc]
Glibenclamide in Diabetic group	0.86 ± 0.06[bd]	169.4 ± 12.62[bd]	46.84 ± 6.68[bd]	43.69 ± 5.42[bd]	36.00 ± 3.87[bd]
Atorvastatin in Diabetic group	1.61 ± 0.11[b]	345.0 ± 13.71[a]	87.85 ± 5.89[b]	72.53 ± 4.97[b]	67.14 ± 4.94[b]
Glibenclamide+FSK-30 in Diabetic group	0.69 ± 0.04[be]	131.6 ± 9.67[be]	32.08 ± 4.59[be]	32.28 ± 3.96[be]	23.71 ± 4.60[be]
Atorvastatin + FSK-30 in Diabetic group	0.98 ± 0.07[bf]	234.7 ± 17.26[bf]	56.63 ± 8.11[bf]	56.63 ± 4.94[bf]	47.71 ± 3.94[bf]

Values are expressed as Mean ± SD (n = 7 per group).
[a] = p < 0.05 vs normal control, FSK per se group
[b] = p < 0.05 vs diabetic control & FSK-10mg/kg treated group
[c] = p< 0.05 vs FSK-20mg/kg treated group
[d]= p<0.05 vs FSK-30mg/kg treated group
e= p<0.05 vs GB-0.6mg/kg treated group
[f]= p<0.05 vs Atorvastatin-0.5mg/kg treated group.

(20mg/kg and 30mg/kg) significantly attenuated the levels of these markers in dose dependent manner.

Moreover, treatment with Glibenclamide-0.6mg/kg significantly attenuated diabetes induced elevated biomarkers when compared with FSK-30mg/kg treated group. Further, treatment with the combination of FSK-30mg/kg and Glibenclamide-0.6mg/kg treated group exhibited marked reduction in renal dysfunction parameters in treated rats when compared with Glibenclamide-0.6mg/kg treated group.

Furthermore, treatment with Atorvastatin-0.5mg/kg alone did not affect the serum glucose level. However, Atorvastatin-0.5mg/kg in combination with FSK-30mg/kg significantly reduced the serum glucose level and other renal dysfunction parameters when compared with diabetic control and Atorvastatin-0.5mg/kg alone treated group (Table 1 & 2).

Effects of FSK on Renal Hypertrophy Parameters

The level of renal hypertrophy (absolute kidney weight, kidney weight/body weight ratio) and renal collagen content were noted to be markedly increased (P<0.05) in diabetic rats after eight weeks of STZ administration when compared with normal rats. Treatment with low dose of FSK-10mg/kg for two weeks did not produce any significant effect on renal hypertrophy in treated rats when compared with diabetic control group.

However, treatment with FSK-20mg/kg and FSK-30mg/kg demonstrated significant effects in dose dependent manner. Moreover, treatment with Glibenclamide-0.6mg/kg significantly attenuated diabetes induced elevated renal hypertrophy parameters when compared with diabetic control group and FSK-30mg/kg treated group. Further, treatment with the combination of FSK-30mg/kg and Glibenclamide-0.6mg/kg treated group exhibited marked reduction in renal hypertrophy parameters when compared with Glibenclamide-0.6mg/kg treated group.

However, treatment with Atorvastatin-0.5mg/kg significantly reduced the renal hypertrophy when compared diabetic control group. Treatment with the combination of Atorvastatin 0.5mg/kg and FSK-30mg/kg exhibited marked reduction in absolute kidney weight, kidney weight/body weight and renal collagen content when compared with diabetic control group and Atorvastatin-0.5mg/kg alone treated group (Table 2).

Effects of FSK on Lipid Profile

Diabetic rats subjected to HFD significantly (P<0.05) increased the serum total cholesterol, Low-Density Lipoprotein (LDL) and Very Low-Density Lipoprotein (VLDL) level whereas High-Density Lipoprotein (HDL) level was found to be significantly reduced. Treatment with FSK 10mg/kg did not significantly affected the dyslipidaemia. However, two weeks treatment with FSK-20mg/kg and FSK-30mg/kg significantly reduced the elevated level of total cholesterol, LDL and VLDL and increased the level of HDL in treated rats when compared with diabetic control group.

Treatment with Glibenclamide-0.6mg/kg significantly improved the lipid profile when compared with diabetic control group. Moreover, treatment with combination of Glibenclamide-0.6mg/kg with FSK-30mg/kg markedly im-

Table 2. Effect of FSK on serum creatinine, absolute kidney weight, kidney weight/body weight %, total renal collagen content and serum total cholesterol

Groups	Body Weight (g)	Absolute Kidney Weight (g)	Kidney weight/Body weight %	Total Renal Collagen Content (mg/g)	Serum Total Cholesterol (mg/dL)
Normal Control	210.1 ± 14.80	0.42 ± 0.06	0.61 ± 0.04	2.32 ± 0.21	62.51 ± 6.79
FSK per se	215.0 ± 13.80	0.45 ± 0.11	0.58 ± 0.04	2.48 ± 0.24	60.18 ± 6.12
Diabetic Control	328.0 ± 19.03[a]	1.35 ± 0.09[a]	1.15 ± 0.06[a]	5.95 ± 0.35[a]	165.7 ± 6.77[a]
FSK-10 in Diabetic group	312.1 ± 18.68[a]	1.25 ± 0.06[a]	1.08 ± 0.06[a]	5.46 ± 0.27[a]	154.3 ± 8.93[a]
FSK-20 in Diabetic group	321.0 ± 18.85[a]	1.11 ±0.09[b]	1.01 ± 0.05[b]	4.82 ± 0.24[b]	127.9 ± 4.88[b]
FSK-30 in Diabetic group	315.0 ± 16.83[a]	0.93 ± 0.06[bc]	0.90 ± 0.04[bc]	3.94 ± 0.27[bc]	108.0 ± 7.09[bc]
Glibenclamide in Diabetic group	311.3 ± 19.64[a]	0.77 ± 0.07 [bd]	0.79 ± 0.05[bd]	3.21 ± 0.30[bd]	151.1 ± 7.73[b]
Atorvastatin in Diabetic group	310.6 ± 14.23[a]	1.13 ± 0.10[b]	0.99 ± 0.04[b]	4.55 ± 0.28[b]	84.55 ± 8.16[bd]
Glibenclamide + FSK-30 in Diabetic group	307.6 ± 12.34[a]	0.61 ± 0.06[be]	0.69 ± 0.04[be]	2.74 ± 0.27[be]	103.1 ± 4.22[be]
Atorvastatin + FSK-30 in Diabetic group	308.4 ± 12.73[a]	0.80 ± 0.55[bf]	0.81 ± 0.05[bf]	4.08 ± 0.34[bf]	69.78 ± 8.38[bf]

Values are expressed as Mean ± SD (n = 7 per group).
[a] = p < 0.05 vs normal control, FSK per se group
[b] = p < 0.05 vs diabetic control & FSK-10mg/kg treated group
[c] = p< 0.05 vs FSK-20mg/kg treated group
[d]= p<0.05 vs FSK-30mg/kg treated group
e= p<0.05 vs GB-0.6mg/kg treated group
[f]= p<0.05 vs Atorvastatin-0.5mg/kg treated group.

proved the lipid profile when compared to Glibenclamide 0.6mg/kg treated group.

However, treatment with Atorvastatin-0.5mg/kg significantly attenuated diabetes induced dyslipidaemia when compared with diabetic control group and FSK-30mg/kg treated group. Further, co-administration of Atorvastatin-0.5mg/kg with FSK-30mg/kg group exhibited marked reduction in total cholesterol, LDL and VLDL and increased HDL level when compared with Atorvastatin-0.5mg/kg treated group (Table 2 & 3).

Effects of FSK on Renal Thiobarbituric Acid Reactive Substances Level

Thiobarbituric Acid Reactive Substances (TBARS) level was significantly increased in diabetic control group (P<0.05) as compared to normal control group. Treatment with FSK-10mg/kg did not produce any significant effects on TBARS level. However, two weeks treatment with FSK-20mg/kg and FSK-30mg/kg significantly reduced the elevated level of TBARS level in dose dependent manner when compared with diabetic control group.

Treatment with Glibenclamide-0.6mg/kg significantly reduced the elevated TBARS level in treated rats. Moreover, treatment with combination of Glibenclamide-0.6mg/kg with FSK 30mg/kg markedly reduced the diabetes induced elevated TBARS when compared to Glibenclamide-0.6mg/kg treated group.

However, treatment with Atorvastatin-0.5mg/kg significantly attenuated diabetes induced elevated TBARS when compared with diabetic control group. Further, co-administration of Atorvastatin-0.5mg/kg with FSK-30mg/kg group exhibited marked reduction in TBARS when compared with Atorvastatin-0.5mg/kg treated group (Table 4).

Effects of FSK on Antioxidants Enzymes Activities (Glutathione, Superoxide Dismutase and Catalase)

A marked decrease (P<0.05) in antioxidant enzymes [Glutathione (GSH), Superoxide Dismutase (SOD) and Catalase (CAT)] activity was noted in diabetic rats when compared with normal rats. Treatment with FSK-10mg/kg improved the enzymatic activities but results obtained were not statistically significant. Treatment with intermediate and high dose of FSK (20mg/kg and 30mg/kg) significantly improved the levels of these markers when compared with diabetic control group.

However, treatment with Glibenclamide-0.6mg/kg significantly improved the level of these markers in treated rats. Moreover, treatment with combination of Glibenclamide-0.6mg/kg with FSK-30mg/kg markedly enhanced the diabetes induced reduced antioxidants activities when compared with Glibenclamide-0.6mg/kg treated group.

However, treatment with Atorvastatin-0.5mg/kg significantly raised the activities when compared with diabetic control group.

Further, co-administration of Atorvastatin-0.5mg/kg with FSK-30mg/kg group exhibited marked increased in levels of these antioxidant enzymes when compared with Atorvastatin-0.5mg/kg treated group (Table 4).

Table 3. Effect of FSK on HDL, LDL and VLDL

Groups	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)
Normal Control	8.64 ± 0.37	37.54 ± 6.12	15.76 ± 2.27
FSK per se	8.24 ± 0.50	37.71 ± 5.71	14.99 ± 1.99
Diabetic Control	3.25± 0.42[a]	170.8 ± 5.23[a]	43.87 ± 3.12[a]
FSK-10 in Diabetic group	3.85 ± 0.42[a]	156.1 ± 8.31[a]	40.29 ± 2.68[a]
FSK-20 in Diabetic group	4.83 ± 0.47[b]	129.0 ± 9.62[b]	35.15 ± 1.92[b]
FSK-30 in Diabetic group	6.03 ± 0.40[bc]	107.1 ± 7.55[bc]	29.88 ± 2.44[bc]
Glibenclamide in Diabetic group	4.31 ± 0.54[b]	147.7 ± 12.46[b]	37.75 ± 2.67[b]
Atorvastatin in Diabetic group	6.78 ± 0.45[bd]	70.41 ± 6.20[bd]	22.57 ± 2.07[bd]
Glibenclamide + FSK-30 in Diabetic group	6.55 ± 0.31[be]	101.2 ± 8.81[be]	27.88 ± 2.25[be]
Atorvastatin + FSK-30 in Diabetic group	7.90 ± 0.43[bf]	39.58 ± 6.23[bf]	17.74 ± 1.62[bf]

Values are expressed as Mean ± SD (n = 7 per group).
[a] = p < 0.05 vs normal control, FSK per se group
[b] = p < 0.05 vs diabetic control & FSK-10mg/kg treated group
[c] = p< 0.05 vs FSK-20mg/kg treated group
[d]= p<0.05 vs FSK-30mg/kg treated group
e= p<0.05 vs GB-0.6mg/kg treated group
[f]= p<0.05 vs Atorvastatin-0.5mg/kg treated group.

Table 4. Effect of various pharmacological interventions on TBARS, GSH, SOD and CAT

Groups	TBARS (nmol/mg)	GSH	SOD (µmol/min/mL)	CAT (nmol H_2O_2 consumed/min/mL)
Normal Control	0.60 ± 0.09	232.6 ± 11.83	14.22 ± 0.85	7.61 ± 0.57
FSK per se	0.62 ± 0.06	232.9 ± 10.75	13.29 ± 1.11	7.17 ± 0.51
Diabetic Control	1.77 ± 0.12[a]	97.71 ± 10.95 [a]	3.64 ± 0.94[a]	2.51 ± 0.44[a]
FSK-10 in Diabetic group	1.66 ± 0.09[a]	106.6 ± 10.39[a]	4.62 ± 0.63[a]	3.02 ± 0.29[a]
FSK-20 in Diabetic group	1.47 ± 0.08[b]	136.4 ± 9.88[b]	9.14 ± 0.69[b]	4.37 ± 0.39[b]
FSK-30 in Diabetic group	1.17 ± 0.07[bc]	162.90 ± 12.54[bc]	10.71 ± 0.78[bc]	5.24 ± 0.33[bc]
Glibenclamide in Diabetic group	0.99 ± 0.09[bd]	197.0 ± 12.94[bd]	12.25 ± 0.94[bd]	6.14 ± 0.36[bd]
Atorvastatin in Diabetic group	1.57 ± 0.08[b]	139.3 ± 13.05[b]	9.00 ± 0.81[b]	4.31 ± 0.56[b]
Glibenclamide + FSK-30 in Diabetic group	0.75 ± 0.09[be]	230.6 ± 16.25[be]	13.79 ± 0.69[be]	6.98 ± 0.40[be]
Atorvastatin + FSK-30 in Diabetic group	1.02 ± 0.10[bf]	175.90 ± 10.88[bf]	12.00 ± 1.00[bf]	5.58 ± 0.29[bf]

Values are expressed as Mean ± SD (n = 7 per group).
[a] = p < 0.05 vs normal control, FSK per se group
[b] = p < 0.05 vs diabetic control & FSK-10mg/kg treated group
[c] = p< 0.05 vs FSK-20mg/kg treated group
[d]= p<0.05 vs FSK-30mg/kg treated group
e= p<0.05 vs GB-0.6mg/kg treated group
[f]= p<0.05 vs Atorvastatin-0.5mg/kg treated group.

DISCUSSION

Metabolic disturbance, oxidative stress, and podocyte injury play important role in the progression of DN. Meanwhile, FSK was first demonstrated to be a hypotensive agent with anti-spasmolytic and cardiotonic activity [18]. In the present study, we investigated the effect of FSK on DN. Our results indicated that FSK can be a potential drug that can prevent renal dysfunction, dyslipidaemia, oxidative stress and DN in HFD and STZ administered wistar rats.

The various studies in the DN shows that there is a reduction in the body weight of the diabetic control animals due to the increased muscle wasting and loss of tissue proteins in diabetes [19]. In our study, the body weight was significantly increased in the diabetic rats when compared with normal rats due to free access to HFD. It has been reported that rats fed with HFD followed by STZ-low dose develops Type-II DM [20]. No significant effect on body weight was observed when diabetic rats were treated with different dose of FSK alone or in combination with Glibenclamide and Atorvastatin for two weeks after eight weeks of STZ administration.

Altered glomerular filtration rate is the main indicator of DN and many studies have reported that the administration of STZ in the rats elevate the serum renal markers [21, 22]. Elevated level of serum creatinine and proteinuria is an indication of renal dysfunction [23, 24]. To evaluate the renal functions, various biochemical parameters were performed in blood serum, urine and in tissue homogenate like serum glucose, serum creatinine, BUN, protein in urine, absolute kidney weight, renal hypertrophy, total renal collagen content were performed.

Eight weeks after STZ administration, significantly increased concentration of biochemical parameters were observed which indicated development of DN [25]. Reversed

effects were observed when diabetic rats were treated with intermediate and high dose of adenylyl cyclase activator, FSK (20mg/kg and 30mg/kg). Moreover, FSK treatment improved the structure and functions of kidney and prevented the development of DN and concurrently reduced the glucose level.

Moreover, absolute kidney weight and ratio of kidney weight/body weight was noted to be significantly elevated when compared with normal rats. These changes confirmed the development of renal structural abnormalities and nephropathy in diabetic kidney after eight weeks of STZ administration. Structural and functional changes were significantly prevented in diabetic rats when treated with FSK 30mg/kg. On the basis of above discussion it may be concluded that FSK 30mg/kg without affecting the body weight, prevented the development of DN in rats subjected to HFD.

There is a strong correlation between insulin deficiency and insulin resistance with dyslipidaemia. Dyslipidaemia has been reported to play critical role in the development and progression of DN in rats [26]. Studies have reported that the increased level of lipids in diabetes increases the risk of DN [27]. Elevated level of lipids leads to glomerular and tubular dysfunction in diabetes due to increased intracellular concentration of fatty acids [28]. According to the survey by the National Health and Nutrition Examination Survey, 61% of patients with type 2 diabetes were obese and 46% of diabetic patients had lipid abnormalities [29].

Lipid profile was estimated by measuring serum total cholesterol, LDL, HDL and VLDL level. HDL concentration was noted to be significantly reduced and level of serum total cholesterol, LDL and VLDL were noted be increased in diabetic rats. Treatment with FSK (20mg/kg and 30mg/kg) significantly reversed the level of HDL and total cholesterol, LDL and VLDL. Combinatorial effects of Atorvastatin 0.5mg/kg with FSK 30mg/kg presented synergistic results when compared with diabetic rats treated with Atorvastatin 0.5mg/kg or FSK 30mg/kg alone.

Kidneys are the most susceptible organs to oxidative damage caused by the free radicals. Hyperglycaemic conditions promote the oxidative stress resulting from the increased generation of reactive oxygen species, plays important role in pathophysiology of DN [30]. In DN patients, there is an increased production of free radicals which leads to lipid peroxidation. It has also been reported that free radical generation also decrease the activity of antioxidant enzymes due to auto oxidation and non-enzymatic glycosylation [31]. GSH, SOD and CAT are the enzymes which scavenge the free radicals by destroying the peroxides and provide the antioxidant defence mechanism [32, 33].

Oxidative stress was assessed by measuring the level of TBARS and level of endogenous antioxidant enzymes were analysed by evaluating the activity of GSH, SOD and CAT in diabetic rats. Two weeks treatment with FSK decreased TBARS level and increased the level of endogenous antioxidants activities (GSH, SOD and CAT) in diabetic rats in dose dependent manner when compared with untreated rats.

FSK directly activates AC, which increases intracellular cAMP levels [34]. Further, it has been reported that Adenosine Monophosphate Activated Protein Kinase (AMPK) may be a key factor that regulate lipid metabolism, antioxidant, anti-inflammatory activity and inhibits Tumor Necrosis Factor (TNF)-α, 1β, 6 and 8 [35]. The activation of the cAMP-dependent protein kinase (PKA) may inhibit TNF-α and nuclear factor-kappa B (NF-κB) which is implicated in inflammation & oxidative stress. NF-κB is produced by almost all cell types and is activated by a wide variety of cell-stress stimuli including hyperglycaemia, obesity, increased plasma free fatty acids, oxidative stress, hypertension, proteinuria and renal fibrosis etc. [36, 37].

In the present study, we analysed the effect of FSK on biomarkers of DN, oxidative stress and concluded that FSK employed renoprotective effects by improving kidney functions partly by reducing hyperglycaemia and partly by increasing intracellular cAMP level by enhancing the PKA and cAMP response element-binding protein (CREB) phosphorylation activation in diabetic rats.

CONCLUSION

On the basis of our study, it may be concluded that in diabetic condition marked increase in the biomarkers of renal hypertrophy, oxidative stress and dyslipidaemia play pathologic role in the development of nephropathy in rats. Our study demonstrated that Forskolin has potential to prevent diabetes induced renal hypertrophy, oxidative stress and dyslipidaemia. Treatment with Forskolin reduced the progression of DN by preventing kidney functional and structural abnormalities. Moreover, FSK has a potential to enhance the effect of lipid lowering and anti-diabetic activity of standard drugs.

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CONFLICTS OF INTEREST

The author(s) declare(s) that there is no conflict of interest regarding the publication of this article

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