

## Research Article

# Forskolin Modulate Silent Information Regulator 1 (SIRT1) gene Expression and Halts Experimentally-Induced Acute Kidney Injury

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**Abstract.** Acute kidney injury is a very serious medical condition; change of the normal physiological oxidant-antioxidant balance has been reported as a major cause for renal injury. Silent information regulator 1 (Sirt1) is a nicotinamide adenine dinucleotide-(NAD<sup>+</sup>-) dependent deacetylase that has nephro-protective effect against ischemia or injury by toxic substances by increasing cell resistance to oxidative stress. Forskolin is derived from plant *Coleus forskohlii* and has been used to treat the heart disease, hypertension, diabetes and asthma. This study was done to investigate the possible protective role of forskolin against glycerol-induced acute nephrotoxicity and also to study the possible mechanisms underlying this action. In the present study rats were randomly divided into four groups. Rats in the control group received distilled water orally for 15 days, four days before scarification they received half the dose of saline (10 ml/kg) in each hind limb muscle; rats in the FSK group received 500 mg/kg per day, orally for 15 days; those in the glycerol group (AKI) received half the dose of glycerol (10 ml/kg, 50% v/v in sterile saline) in each hind limb muscle; rats in the FSK + glycerol (AKI) group received FSK 500 mg/kg per day, orally 12 days before glycerol injection and continued for three days after glycerol administration with a total period of 15 days, all rats were deprived of water for 24 h before glycerol injection. Parameters tested in this study were kidney function tests (urea, creatinine), oxidative stress parameters (MDA, GST), anti-inflammatory marker (TNF- $\alpha$ ), anti-apoptotic marker (caspase-3), SIRT gene expression detected by RT-PCR and histopathological study. Results: Glycerol administration caused significant increase in all tested parameters except SIRT gene expression which decreased with glycerol administration. Pretreatment with forskolin caused significant decrease of levels of urea, creatinine, MDA, TNF- $\alpha$  and also decreased activity of caspase-3 and GST, with significant improvement of SIRT expression. Histopathological examination revealed that the glycerol caused severe kidney damage in the form of hemorrhage, inflammatory cell infiltration and intra-tubular cast formation compared to normal renal histology and architecture of the control and forskolin groups. Forskolin pretreatment of glycerol induced AKI caused marked improvement of histological picture which exhibited mild edema and tubular vacuolization compared to the control group. In conclusion the possible beneficial effect of forskolin in protection against nephrotoxicity is due to its ability to modulate the disrupted expression of SIRT gene as well as its anti-oxidant, anti-inflammatory and anti-apoptotic properties. This may open a new therapeutic window for renal patient.

**Keywords:** Forskolin, glycerol, nephrotoxicity, SIRT 1.

## 1. Introduction

Nephrotoxicity is one of the most common kidney problems. Nephrotoxicity may be temporary with a transient elevation of laboratory values of urea and/or

creatinine, this may be due to a simple cause such as dehydration or it may an indicator of a developing serious renal failure [1]. If the cause of the increased BUN and/or creatinine levels is determined early, and an appropriate intervention was done, permanent

kidney problems can be avoided. Nephrotoxicity can occur by multiple mechanisms as increased oxidative stress, inflammation and apoptosis [2].

Silent information regulator 1 (Sirt1) is a nicotinamide adenine dinucleotide- (NAD<sup>+</sup>-) dependent deacetylase that was first discovered in the 1970s with a higher expression of SIRT1 in renal inner medullary mesenchymal cells [3]. SIRT1 is highly expressed in medullary tubular cells and moderately expressed in cortical proximal tubular cells that indicate the renal protective effect of SIRT1 against the ischemia or the injury by toxic substances by enhancing cell resistance to oxidative stress [4].

SIRT1 by deacetylation of target proteins through coenzyme NAD<sup>+</sup> which is strongly linked to cellular energy metabolism and the redox state through a signal pathway [4]. Under different stress conditions, such as metabolic, oxidative stress or hypoxia the SIRT1 deficiency is linked to pathophysiology of renal diseases. In the kidneys, SIRT1 may inhibit renal cell apoptosis, inflammation and fibrosis, the activation of SIRT1 in the kidney may be therapeutic target to overcome the development of kidney diseases [5].

Sirt1 exerts a renal protective effect through deacetylation and regulation of various factors, and it plays an important role in the repression of inflammation in tissues and cells as macrophages, which indicate the role of SIRT1 in suppressing the activity of nuclear factor (NF- $\kappa$ B) which is considered as central regulator of cellular inflammatory response [6].

Over expression of SIRT1 leads to reduced NF- $\kappa$ B activity and vice versa, down-regulation of SIRT1 enhances lipopolysaccharide stimulated TNF $\alpha$  (tumor necrosis factor  $\alpha$ ) secretion [7].

The generation of ROS by ischemia reperfusion injury (IRI) is the cause of AKI through oxidative stress-mediated damage of the kidney [8]. During the ischemic phase of IRI there is production of low levels of ROS in the mitochondria, the higher levels of ROS are generated rapidly by mitochondria, during reperfusion phase [9], the strong oxidative stress is produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, or inflammatory cells, so for treating the pathogenesis and progression of kidney diseases suppression of oxidative stress is considered an important therapeutic strategy [10].

The imbalance between oxidant and antioxidant system with increased production of reactive oxygen species (ROS) and lipid peroxidation products that accumulate in kidneys are main mechanisms of AKI [11]. Within the cell the conversion of glycerol into

a highly reactive molecule by interacting with thiol-containing antioxidant glutathione leads to decreased glutathione and increased oxidative stress within the cells [12].

Forskolin is derived from plant *Coleus forskohlii*, it increases intracellular cAMP levels in a variety of cells by stimulating adenylcyclase [13]. Many studies have shown that forskolin pre-treatment, decreases lipid peroxidation significantly [14]. The increase in cAMP by forskolin improves cytotoxicity and apoptosis [15]. Also, previous studies have shown that forskolin had an antagonistic role on tumor necrosis factor alpha, and it reduced the levels of interleukins 1 $\beta$ , 6, and 8 [16]. So, these antioxidant and anti-inflammatory actions of forskolin had been used to treat the heart disease, hypertension, diabetes and asthma.

Glycerol also called glycerin is a colorless, odorless, viscous liquid that is sweet-tasting. The glycerol backbone is found in all lipids known as triglycerides. It is widely used in the food industry as a sweetener and in pharmaceutical formulations. Glycerol has three hydroxyl groups that are responsible for its solubility in water and its hygroscopic nature. Glycerol-induced renal lesions can have many causes, including increased oxidative stress and inflammation [17]. It is characterized by intense cortical acute tubular necrosis and inflammatory cell infiltration [18].

This study was done to investigate the possible protective role of forskolin against glycerol-induced acute nephrotoxicity and also to study some possible mechanisms underlying this action.

## 2. Materials and Methods

**2.1. Animals.** Thirty two adult male albino rats (at 11–13 weeks old) weighing 220–250 g were used in this study after acclimatization for one week. We get the rats from Helwan farm (Holding Company for Biological Products and Vaccines; VACSERA, Giza, Egypt). The rats were kept under 12 hour light/dark cycles and were fed on the standard laboratory diet and water. The experimental protocol was approved by the Animal Research Ethics Committee of the Faculty of Medicine, Benha University, Egypt.

**2.2. Drugs.** Forskolin and glycerol were obtained from Sigma-Aldrich Chemical Co. (St Louis, Missouri, USA). All drugs were freshly prepared just before use.

2.3. *Experimental design.* Rats were randomly divided into four groups. Rats in the control group received distilled water orally for 15 days, four days before scarification they received half the dose of saline (10 ml/kg) in each hind limb muscle; rats in the forskolin (FSK) group received 500 mg/kg per day, orally for 15 days [19]; those in the AKI group received half the dose of glycerol (10 ml/kg, 50% v/v in sterile saline) in each hind limb muscle [20]; rats in the FSK + AKI group received forskolin 500 mg/kg/day orally for 12 days before glycerol injection and three days after glycerol injection with a total period for 15 days. Three days after glycerol injection, all rats were sacrificed under general anesthesia using intraperitoneal injection of pentobarbital sodium (150 mg/kg. [20]. Blood was collected via heart puncture and the right kidney was excised immediately and stored in  $-80^{\circ}\text{C}$  for biochemical analysis. Then, the left kidney was fixed in 10% neutral buffered formalin for histological and immuno-histochemical studies.

2.4. *Sample preparation and biochemical assays.* Blood was collected via cardiac puncture; the serum was quickly separated through centrifuge. Serum biochemical parameters of urea and creatinine levels were measured ( $n = 8$  per group) using the commercially present kits. Whole blood (4 ml) was taken in plain test tubes without anticoagulant. The samples were allowed to clot for 30 mins at room temperature, and then centrifuged for 15 mins at  $1000\times g$ . The serum was removed for estimation of urea and creatinine levels using the Diamond D-P International-Germany kits.

The kidneys were isolated, then rinsed with ice-cold saline solution and then homogenized in 0.1 M Tris-HCl buffer. The homogenate was first centrifuged at  $10,000 g$  for 20 min and the supernatants then was centrifuged at  $100,000 g$  for 60 min. The resulting supernatant was used for the measuring the enzymatic activities and lipid peroxidation. MDA Sigma-Aldrich Company, USA, GSH Ray Biotech, Inc. USA, Caspase-3 USA & Canada | R&D Systems, Inc and TNF alpha USA & Canada | R&D Systems, Inc.

2.5. *Histopathological examination.* After scarification and dissection, kidneys of the different groups were fixed in 10% buffered formalin solution for one day, dehydrated in alcohol and then were put in paraffin. After that the kidney tissues were cut into  $5\text{-}\mu\text{m}$  thick sections then stained with hematoxylin & eosin stain.

2.6. *Immuno-histochemical staining.* Kidneys were fixed in formalin and then put in paraffin according to the usual histological technique. Immunohistochemistry was used to determine the caspase-3 antigen. Caspase-3 antibodies (Santa Cruz Biotechnology Inc.) were stored at  $4^{\circ}\text{C}$  overnight. The suitable horseradish peroxidase conjugated secondary antibodies were added and the samples were kept for 60 min at normal room temperature. Then we did counter staining by using hematoxylin and eosin, the sections were examined by using light microscope. Using the digital camera, quantification data of five randomly selected samples, at a power of  $400\times$  magnification, were used using a special program of image analysis.

2.7. *RT-qPCR.* Total RNA was extracted from blood by using RNA Mini Kit (Qiagen, USA) by using manufacturer's instruction where the quantity and quality of RNA were determined by Nano drops (Thermo Fisher Scientific – NanoDrop products Wilmington, Delaware USA). Ratio of readings at 260 nm and 280 nm provide an estimate of purity of RNA pure RNA had A 280/260 ratio of 1.9–2.3.

In ABI 7900HT fast real time PCR (applied Biosystem USA) RNA ( $5\ \mu\text{g}$ ) was then reversed transcribed by using revert aid TM first strand cDNA synthesis kit then cDNA was then amplified with the Syber Green PCR Master Kit in a 48-well plate, PCR was done in a  $30\ \mu$  reaction volume containing  $5\ \mu\text{l}$  cDNA,  $2.5\ \mu\text{l}$  forward primers,  $2.5\ \mu\text{l}$  reverse primers, sequences were designed as follows: Sirt1, forward TGGACGAGCTG ACCCTTGA and reverse TCCTGCGGATGTGGAGATT: and GAPDH was used as an internal control and the primer sequence was: forward CAGTGCCAGCCTCGTCTCAT and reverse AGGGGCCATCCACAGTCT TC.  $5\ \mu\text{l}$  of DNA polymerase,  $7.5\ \mu$  syber green and  $7.5\ \mu$  buffer. PCR amplification conditions were done in one step real time PCR instrument. Initial denaturation at  $94^{\circ}\text{C}$  for 5 min, then 30 s at  $94^{\circ}\text{C}$ , 35 s at  $54^{\circ}\text{C}$ , and 2 min at  $72^{\circ}\text{C}$  for a complete 35 cycles and a terminal extension at  $72^{\circ}\text{C}$  for 10 min [21, 22]. Relative quantification of Sirt1 was calculated using the equation  $2^{-\Delta\Delta\text{CT}}$  [23]. Technique, and analyzed by ABI prism 7500 the Boxplot software and results were normalized to the quantity of GAPDH.

### 3. Statistical Analysis

The collected data were tabulated and analyzed using SPSS version 16 software (Spss Inc, Chicago, ILL Company) data were expressed as mean  $\pm$  standard

deviation and range. Data were tested for normality using Shapiro-Wilks test, assuming normality at  $P > 0.05$ . Differences between groups were tested using ANOVA test for normally distributed variables or Kruskal Wallis test for non-parametric variables. Significant ANOVA or Kruskal Wallis were followed by post hoc multiple comparisons using Bonferroni test to detect the significant pairs. ROC curve analysis was used to detect cutoff values of the studied genes with optimum sensitivity and specificity in prediction of cardio, hepato and nephrotoxicity. The accepted level of significance in this work was stated at 0.05 ( $P < 0.05$  was considered significant).

## 4. Results

Concerning kidney functions: There was no significant difference in serum urea and creatinine levels between the control and FSK groups. Glycerol injection significantly increased serum urea, and creatinine level in glycerol (AKI) group compared to the control group. Forskolin treatment resulted in a significant reduction in urea, creatinine levels compared to the glycerol (AKI) group.

About oxidative stress markers: There was no significant difference in MDA level and GST activity between the control and FSK groups. Glycerol injection significantly increased MDA level and GST activity in glycerol (AKI) group compared to the control group. Forskolin treatment resulted in a significant reduction in MDA level and GST activity compared to the glycerol (AKI) group. About TNF- $\alpha$  and caspase-3, there was no significant difference in TNF- $\alpha$  level and caspase-3 activity between the control and FSK groups. Glycerol injection significantly increased TNF- $\alpha$ , and caspase-3 in glycerol (AKI) group compared to the control group. Forskolin treatment resulted in a significant reduction in TNF- $\alpha$  and caspase-3 compared to the glycerol (AKI) group.

This table showed that there is significant downregulation in SIRT1 mRNA expression in glycerol group. On the other hand there is significant upregulation in SIRT1 mRNA expression with forskolin treatment that indicates renal protective effect of forskolin.

*4.1. Effects of forskolin on renal histopathology.* Glycerol exposure in AKI group caused severe hemorrhage, inflammatory cell infiltration and edema compared to normal renal histology and architecture of the control and forskolin groups. Forskolin pretreatment of glycerol

induced AKI caused marked improvement of histological picture which exhibited mild edema and tubular vacuolization compared to the control group.

*4.2. Effects of forskolin on the apoptosis marker caspase-3.* Exposure to glycerol in AKI group caused in a significant increase in caspase-3 expression compared to the control and forskolin groups. Pretreatment with forskolin caused a significant decrease in caspase-3 expression in the FAS + AKI group compared to glycerol alone.

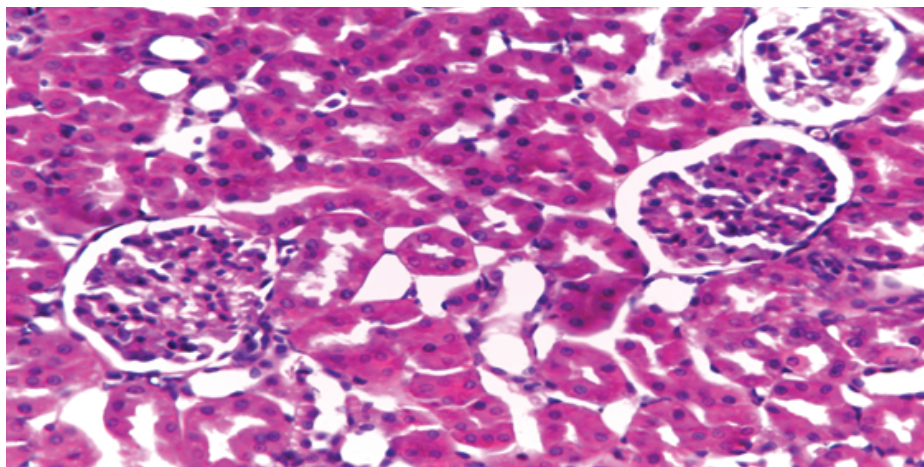
## 5. Discussion

Renal dysfunction is a very serious medical problem; change of the normal physiological oxidant-anti oxidant balance was reported as a main cause for kidney injuries [24]. Sirtuins (SIRT1s), present in the mitochondria, play an essential role in energy metabolism, oxidative stress and DNA repair, Sirtuins induce deacetylation and influence oxidation of fatty acid, ischemia/reperfusion (I/R) injury, oxidative stress, apoptosis, tumor necrosis factor (TNF)- $\alpha$ , induce the generation of ROS and the resulting apoptosis [25].

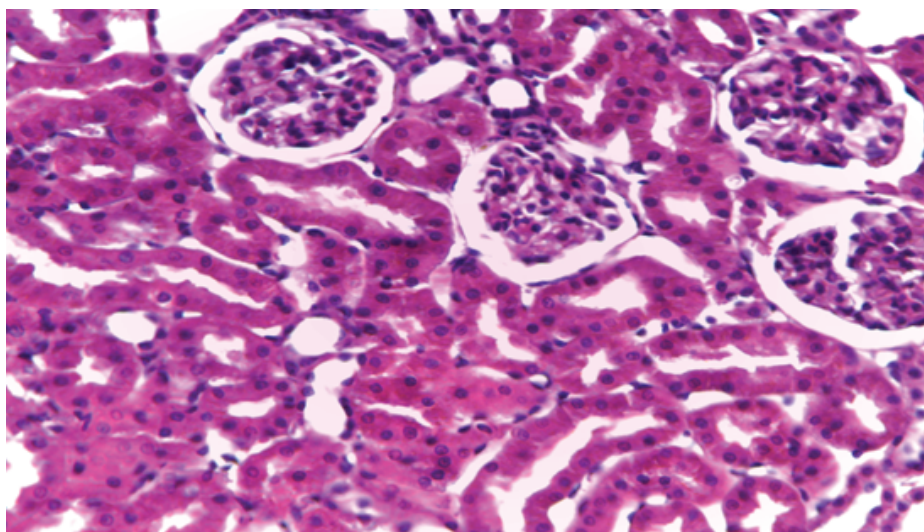
SIRT1 activators, FSK plays a reno-protective effect by increasing cAMP & cellular resistance to stress, inhibiting glomerular and tubular cell inflammation and apoptosis [26]. In the current study glycerol injection led to marked down regulation of SIRT1 gene expression as well as aggressive increase in urea, creatinine, GST, MDA, TNF- $\alpha$ , Caspase-3 levels and histopathological abnormalities. This was explained by [27] who stated that glycerol-induced nephrotoxicity is mediated by renal ischemia that induces oxidative stress and lipid peroxidation of the proximal tubular cells, triggering the release of a group of mediators, including caspases and cytokines, leading to activation of the leukocytes leading to tubular necrosis in the renal cortex [28] and histological changes as tubular injury which are in line with the problems observed clinically [29].

In the present work administration of forskolin improves the abnormalities of the tested parameters as well as increase SIRT1 expression. Also, decrease the levels of urea, creatinine, MDA, TNF- $\alpha$  and decrease the activities of GST, Caspase-3, improve the histopathological changes. Forskoline was found to protect against various oxidative stress injuries by its antioxidant, anti-apoptotic and anti-inflammatory effects [15].

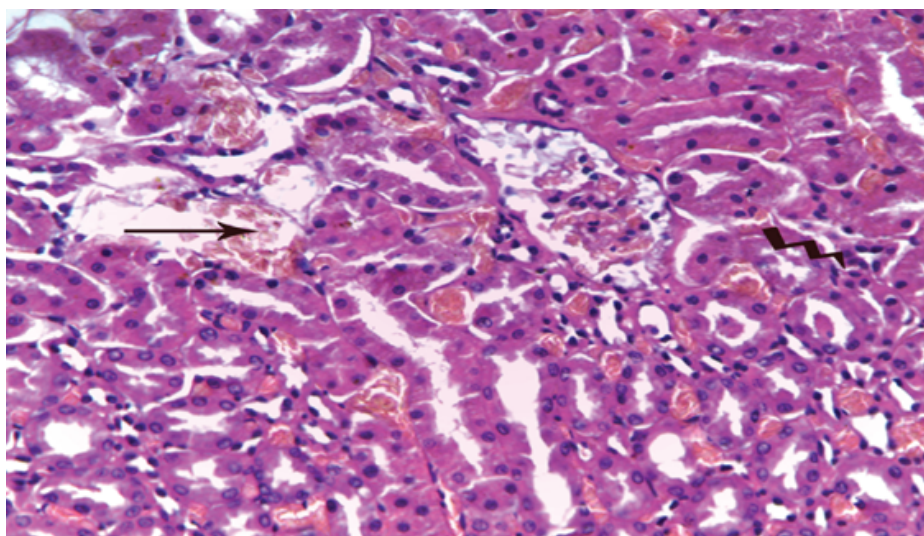




**Figure 1:** A photomicrograph of a cut section in the kidney of a control rat showing normal kidney architecture. (H × & E × 400).



**Figure 2:** A photomicrograph of a cut section in the kidney of a forskolin group showing normal kidney architecture. (H × & E × 400).



**Figure 3:** A photomicrograph of a cut section in the kidney of AKI group showing severe hemorrhage (straight arrow), edema and inflammatory cell infiltration (curved arrow). (H × & E × 400).

Table 1: The effects of forskolin and / or glycerol on kidney functions in studied groups.

| Variable           | Control (n = 8) |       | FSK (n = 8) |       | AKI (n = 8) |       | FSK + AKI (n = 8) |       |
|--------------------|-----------------|-------|-------------|-------|-------------|-------|-------------------|-------|
|                    | Mean ± SD       |       | Mean ± SD   |       | Mean ± SD   |       | Mean ± SD         |       |
| Urea (mg/dL)       | 38.3            | 2.16  | 37.6        | 2.16  | 91.3*†      | 12.65 | 55.6*†‡           | 4.17  |
| Creatinine (mg/dL) | 1.07            | 0.103 | 1.07        | 0.151 | 2.95*†      | 0.187 | 1.58*†‡           | 0.172 |

\*→Significant in comparison with control

†→Significant in comparison with FSK

‡→Significant in comparison with +AKI.

Table 2: The effects of forskolin and / or glycerol on oxidative stress, apoptotic and inflammatory markers in studied groups.

| Variable                  | Control (n = 8) |      | FSK (n = 8) |      | AKI (n = 8) |      | FSK + AKI (n = 8) |      |
|---------------------------|-----------------|------|-------------|------|-------------|------|-------------------|------|
|                           | Mean ± SD       |      | Mean ± SD   |      | Mean ± SD   |      | Mean ± SD         |      |
| MDA (nmol/ml)             | 39.0            | 3.68 | 39.3        | 3.50 | 93.0*†      | 6.32 | 57.0*†‡           | 4.24 |
| GST (mg/g)                | 5.7             | 1.00 | 5.4         | 1.34 | 9.6*†       | 3.40 | 6.0‡              | 0.70 |
| Capase -3 (pg\mg protein) | 0.90            | 0.14 | 0.91        | 0.14 | 12.0*†      | 3.16 | 6.5*†‡            | 1.51 |
| TNF alpha (pg\mg protein) | 6.0             | 1.41 | 6.0         | 1.41 | 170.0*†‡    | 9.89 | 70.0*†            | 6.32 |

\*→Significant in comparison with control

†→Significant in comparison with FSK

‡→Significant in comparison with AKI.

These results suggest that FSK decreases oxidative stress and improves the acute kidney injury of glycerol, this is in agree with [30] that reported the protective effect of chronic administration of forskolin on glycemia and oxidative stress in rats with and without induced diabetes. [31, 32] reported that cAMP also increased renal blood flow and glomerular filtration rate suggesting that an intracellular accumulation of cAMP by forskolin may be responsible for the afferent arteriolar dilatation which improves the kidney function. Forskolin increased the indirect anti-oxidant capacity by activating the endogenous anti-oxidant enzymatic defense system [33], and by increasing the expression of SIRT1 gene, which increases its antioxidant target genes so reduces the ROS in the kidney. By decreasing the oxidative stress and cellular damage, forskolin improved the GFR and consequently improved plasma urea and creatinine concentrations [31]. This is in agreement with previous studies which reported that forskolin decreased the lipid peroxidation in pre-treated erythrocytes and concluded that the antioxidant effect of forskolin could be compared with the effects of probucol and vitamin E [34]. It has been observed that in vitro forskolin increased the level of the antioxidants [19] Also there is anti-inflammatory effect of forskolin

which is related to its antagonistic effect on TNF- $\alpha$  and also by decreasing the level of inflammatory mediators as histamine, interleukins (IL-1 $\beta$ , IL-4, IL-6, IL-8), leukotriene (LT-4), antigens [30].

SIRT1 depends on NAD<sup>+</sup> through the acetylation or phosphorylation of different substrates. It has a regulatory effect on gene silencing; apoptosis and cell proliferation. SIRT1 is involved in the pathogenesis of many kidney problems, SIRT1 can inhibit the apoptosis produced by injury of kidney cell, reduce kidney inflammation, improve function of the mitochondria, and decrease oxidative stress, so, SIRT1 can protect the kidney from acute injury [35]. SIRT1 activators, FSK exerts a protective effect by inhibiting the effects of cellular stresses, reducing glomerular and tubular cell inflammation and apoptosis. SIRT1 reduces mesangial cell apoptosis that is triggered by oxidative stress by decreasing the activity of p53 activity [36] and promoting TGF- $\beta$ -induced apoptotic signaling of glomerular mesangial cells so it protect from oxidative stress, and TGF- $\beta$  dependent apoptosis. TGF- $\beta$  and oxidative stress are responsible for many renal diseases, so chemical activators of SIRT1 improve the cell inflammation was by NF- $\kappa$ B deacetylation [37].



Table 3: SIRT1 expression in studied groups.

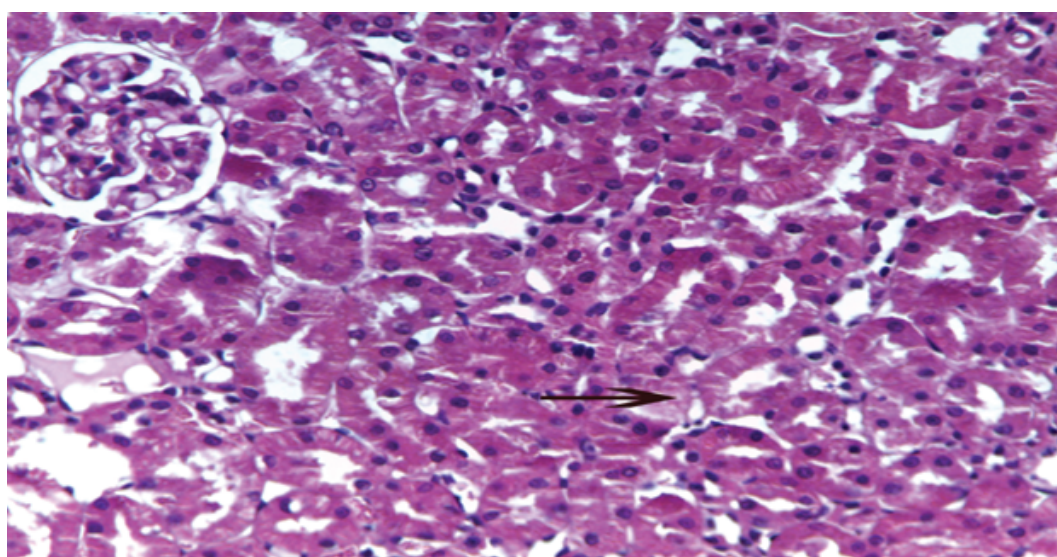
|               | Mean $\pm$ SD              | Anova   |
|---------------|----------------------------|---------|
| Control group | 5.38 $\pm$ 0.68            |         |
| FSK           | 5.38 $\pm$ 0.68            | KW = 14 |
| AKI           | 0.17* $\dagger$ $\pm$ 0.01 |         |
| FSK + AKI     | 4.86 $\ddagger$ $\pm$ 1.06 |         |

KW  $\rightarrow$  Kruskal Wallis test was used

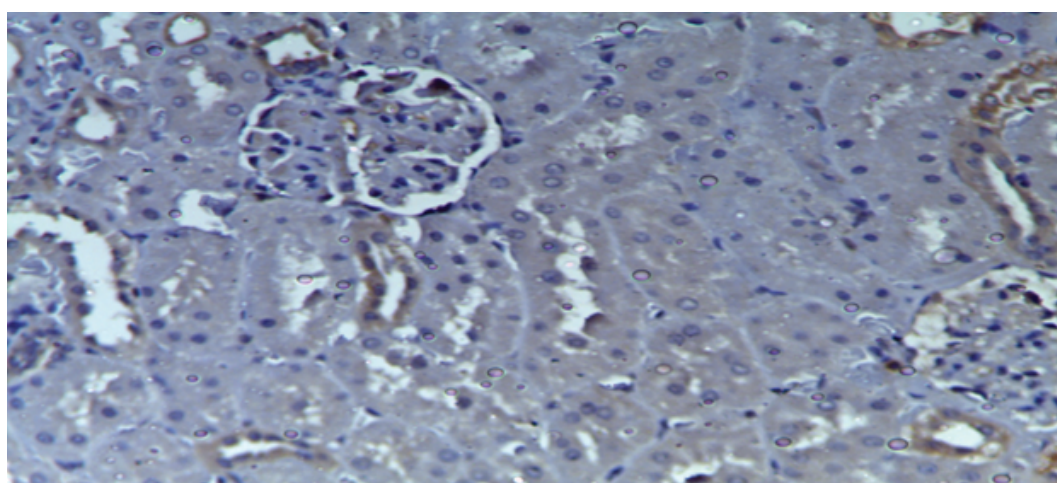
\*  $\rightarrow$  Significant in comparison with controls

$\dagger$   $\rightarrow$  Significant in comparison with FSK

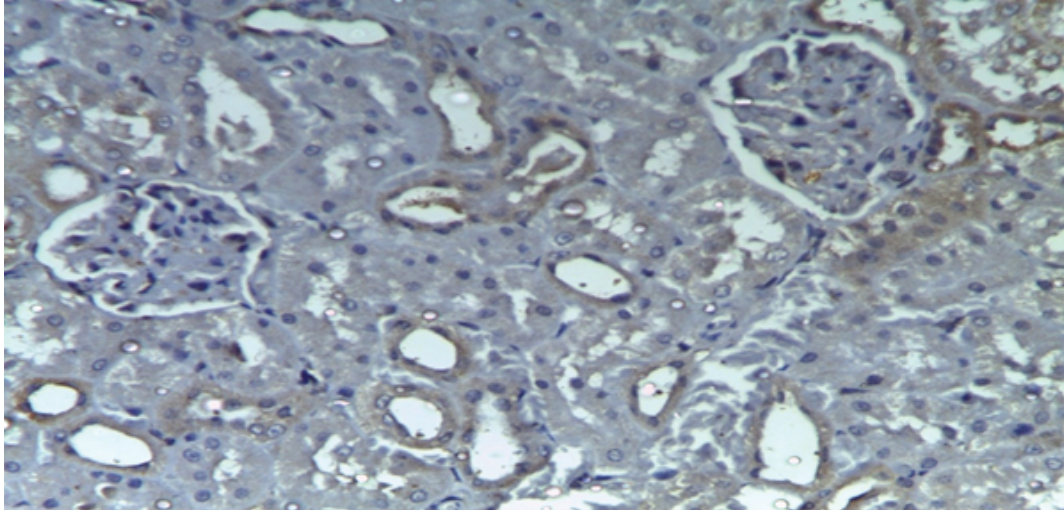
$\ddagger$   $\rightarrow$  Significant in comparison with AKI



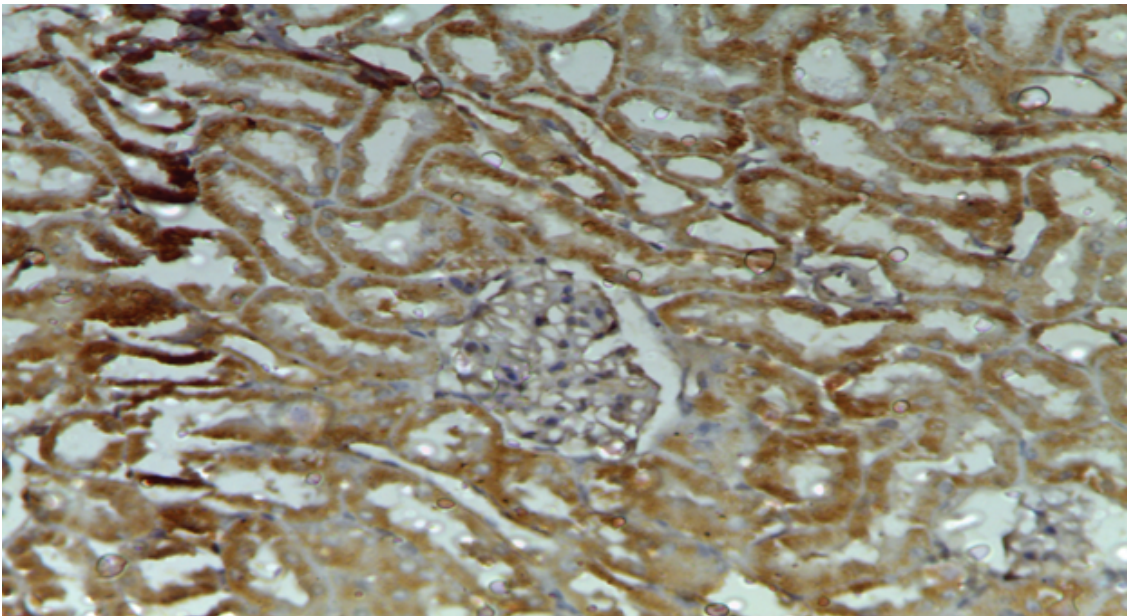
**Figure 4:** A photomicrograph of a cut section in the kidney of FSK + AKI group showing mild edema and tubular vacuolization (straight arrow) (H  $\times$  & E  $\times$  400).



**Figure 5:** A photomicrograph of a cut section in the kidney of a control group with minimal expression of caspase-3.



**Figure 6:** A photomicrograph of a cut section in the kidney of a control group with minimal expression of caspase-3.



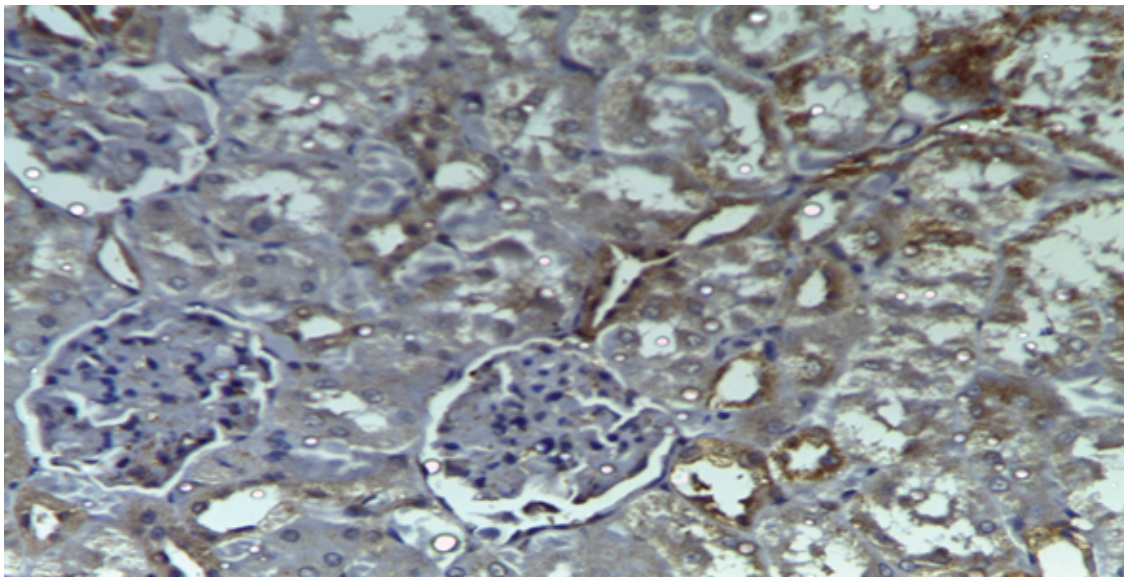
**Figure 7:** A photomicrograph of a cut section in the kidney of AKI group showing marked expression of caspase-3.

Production of oxygen free radicals by glycerol enhances oxidative damage in the mitochondria. That leads to mitochondrial vacuolation and increases production of oxygen free radical and the apoptosis of renal tubular cells, resulting in AKI [38] through up-regulation of SIRT1 expression by FSK it protects against the acute renal injury induced by glycerol by two mechanisms. First mechanism, SIRT1 up-regulation can degrade excessive free radicals, so increase purine degradation, and lead to ATP generation thus can ameliorate AKI by inhibition of oxidative stress and the another mechanism, SIRT1 by up-regulating the

number and function of mitochondria that have a direct effect on cell apoptosis, ATP synthesis, and maintains the normal cell and organ functions. Disturbance in Mitochondrial is an important factor for acute kidney disease that is caused by toxic substances or ischemia [39]. FSK significantly increased the SIRT1 mRNA in kidneys of rats. The relative amounts of SIRT1 mRNA were higher in rats treated with FSK compared to untreated rats.

This is in agreement with [40] who reported that the oxidative stress is a major cause and aggravating factor for kidney diseases. Oxidative-stress molecules,





**Figure 8:** A photomicrograph of a cut section in the kidney of a FSK + AKI group showing moderate expression of caspase-3.

as reactive oxygen species, increase in the kidneys of rat models for acute kidney injury, pharmacological up-regulation of sirt1 activity in the renal tubules significantly decreases acute kidney injury and prevents its progression to a chronic kidney disease (CKD) by ameliorating oxidative stress [22].

In conclusion the produced beneficial effect of forskolin in protection against nephrotoxicity is due to its ability to modulate the disrupted expression of SIRT1, the pharmacological enhancement of SIRT1, exerts cyto-protective effects through its anti-apoptotic, anti-oxidative, and anti-inflammatory effects, **simultaneously** with up-regulation of mitochondrial biogenesis so forskolin may become a new therapeutic target of kidney disease.

2

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### Competing Interests

3

The authors declare no competing interests.

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## Comments

1. Please check missing parenthesis.
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