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ORIGINAL ARTICLE

Anti-cancer effect Shikonin on Ferric Nitrilotriacetate-induced Renal Cancer Rats Apoptosis Mediating PEG2/NF-κB Signaling Pathway

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ABSTRACT

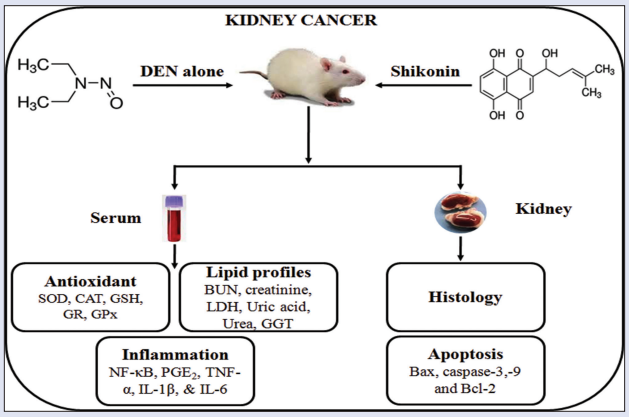
Background: Shikonin (SKN) is a widely used Chinese Traditional Medicine with anti-inflammatory and anti-arthritic properties. **Objectives:** The research aimed to study the anti-cancer effect of SKN in diethylnitrosamine (DEN) and ferric nitrilotriacetate (Fe-NTA)-induced renal carcinoma in rats. **Materials and Methods:** The experimental animal groups consisted of Group I (Control), Group II (DEN + Fe-NTA), Group III (DEN + SKN), and Group IV (SKN alone). Retroperitoneal infusions were given for 24 weeks. Subsequently, tissues and blood samples were tested for biochemical, histopathologic, enzyme-linked immunosorbent assay, and western blot tests. **Results:** After 24 weeks, the antioxidant enzymes were found to increase in the SKN-treated groups. Histopathology revealed normal tissue morphology with a significantly reduced inflammatory response in SKN-treated animal groups compared to the control group. The serum levels of necrosis factor kappa B, PGE_2, interleukin-1β, interleukin-6, and tumor necrosis factor-alpha were also down-regulated in the SKN-treated animal groups. The apoptotic proteins (Caspase-3, -9, and Bcl-2-associated X protein) were higher confirming SKN-induced apoptosis. **Conclusion:** Overall evidence suggests that SKN exhibits a reno-protective anti-cancer effect against DEN + Fe-NTA induced renal carcinoma in rats. Thus, SKN emerges as a therapeutic agent for kidney cancer therapy.

Key words: Apoptosis, diethyl Nitrosamine, inflammation, kidney cancer, Shikonin

SUMMARY

- SKN inhibits the NF-κB, PGE_2, IL-1β, IL-6, and TNF-α through DEN-induced renal cancer rats.
- SKN has exhibited its reno-protective anti-cancer effect against the DNE and Fe-NTA-induced renal carcinoma rats.
- Anti-cancer effect against the DNE and Fe-NTA-induced renal carcinoma rats.

Abbreviations used: Bax: Bcl-2-associated X protein; TNF-α: Tumor Necrosis Factor-alpha; Bcl-2: B-cell lymphoma 2; CAT: Catalase; DEN: Diethylnitrosamine; GGT: γ-Glutamyltranspeptidase; RCC: Renal cell carcinoma; GPx: Glutathione Peroxidase; NF-κB: Nuclear Factor kappa B; GR: Glutathione Reductase; SKN: Shikonin; GSH: Glutathione; GST: Glutathione S-transferase; IL-1β: Interleukin-1β; NTA: Nitrilotriacetic acid; IL-6: Interleukin-6; LDH: Lactate Dehydrogenase; QR: Quinone Reductase; SOD: Superoxide Dismutase.



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INTRODUCTION

Renal cell carcinoma (RCC) is the commonest cancer of the genitourinary tract with a higher mortality rate.[1,2] It accounts for 90% of all kidney neoplasms and 3% of adult malignancies.[3,4] About 1.02 million deaths and 2.09 million new cases of RCC are recorded every year.[5] Over the decade, RCC incidence has been rising steadily in the European Union and the USA.[5] Patients with RCC have an average survival rate of 4 months in its metastatic state.[6] Only about 10% of RCC-affected individuals survive for 1 year.[6] RCC has seven different subtypes based on pathology; clear cell (70–80%), papillary (15–20%), chromophobe (4–5%), clear cell papillary (2–4%), unclassified (2%), collecting duct (1%), and medullary (1%).[7,8] RCC mostly originates from the proximal tube epithelium.[7,8] Despite advanced diagnostic modalities (CT scan and X-ray) available. There are multiple treatment strategies available for RCC. However, it remains resistant to chemotherapy, radiation, and hormonal treatment.[9] Although novel therapeutics have been developed due to the understanding of the underlying pathophysiology of RCC, it remains incurable and lethal.[6] Surgical resection is effective in 70% of the early diagnosed and localized RCC patients. Response to immunotherapy is partial and poor (10–15%).[10]

Diethylnitrosamine (DEN) has been reported for its carcinogenic properties. DEN finds application in plastic, rubber, latex, and cosmetic materials. It

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can also induce hepatic and renal toxicity by generating reactive oxygen species. Thus, DEN has been known to affect the endogenous antioxidant system. Thus, it causes oxidative stress and organ toxicity in humans.[11-13] Nitrilotriacetate (NTA) is an environmentally toxic synthetic tricarboxylic acid heavily used in detergents. It is often washed into the water.[14,15] Along with the Fe^{3+} ion, it forms ferric nitrilotriacetate (Fe-NTA), which is a potential nephrotoxic carcinogenic agent. Intra-peritoneal dose of NTA has also been reported to convert the necrotic renal tubule cells to carcinoma cells. Along with NTA, DEN pretreatment with DEN tends to enhance tumor incidence and decrease cancer induction time. This has been widely used in experimental animals such as Wistar rats.[16,17] The primary mechanism for tumor induction is oxidative stress leading to lipid peroxidation and deoxyribonucleic acid (DNA) damage.[18]

Multiple studies have used DEN and Fe-NTA to induce acute and sub-acute renal proximal tubular damage and subsequent development of RCC at high incidence rates (60–92%) in rats and mice. Moreover, Fe-NTA administration can specifically cause allelic loss of the p16 tumor suppressor gene in renal tubular cells.[19] Fe-NTA induces renal toxicity and renal tumor formation by inducing oxidative stress, DNA damage, cellular proliferation, and inflammation.[20-22] Thus, DEN and Fe-NTA have been clinically approved. The experimental design and the initiator DEN + Fe-NTA dose for our study was selected from Gravis *et al.*, 2016 study.[23] The study successfully used DEN + Fe-NTA for tumorigenesis in rats.

Shikonin (SKN) has been used in Chinese medicine. It is well known for its anti-inflammatory and anti-cancer properties. It is extracted from the roots of Lithospermum erythrorhizon Sieb Et Zucc. It belongs to the Boraginaceae family.[24] SKN has exerted anti-cancer effects over different cancer cells and showed potent anti-arthritic activity.[25] SKN was given to rats in different doses to study the acute and chronic effects. No changes were noted in weights, food consumption, clinical signs, and tissue morphology in the acute stage. However, in the chronic stage glossiness of the coats and the activities of rats were reduced with doses of 18–180 g.[26]

This study aims to examine the nephroprotective effect of SKN *in vivo* in the DEN and Fe-NTA-induced renal carcinoma in rats. We used histopathology, biochemical evaluation, enzyme-linked immunosorbent assay (ELISA), and western blot assay to study the effect of SKN. Pathological disease progression was monitored to assess the potential of SKN as a potent therapeutic medicine for renal carcinoma.

MATERIALS AND METHODS

Chemicals and reagents used

SKN: #54952-43-1, ≥98% [high performance liquid chromatography (HPLC)], DEN, ferric nitrate, and NTA, including other chemicals and reagents. Analytical grade chemicals and reagents were procured from Sigma-Aldrich, USA.

Animals

About 6–8 weeks old male Wistar rats were used in the study. Rats were maintained at the animal facility. The room temperature was maintained. Rats were exposed to alternative 12-hourly cycles of light and dark. They were provided with a standard diet and tap water. The study was conducted as per Organization for Economic Cooperation and Development (OECD) guidelines for experimental studies. Prior approval from the Animal Ethics Committee (IEAC) of Xi'an International Medical Center Hospital was taken (IEAC approval No. 7854XI).

DEN and Fe-NTA solution preparation

DEN solution was prepared and dissolved in pH 4.5 phosphate buffer saline (PBS).[12] The Fe-NTA solution was prepared using 0.64 mM disodium salt of NTA (fourfold excess) and ferric nitrate (0.16 mM).[22] The solution was adjusted to the pH of 7.4 using sodium bicarbonate. The reagents and solutions were prepared fresh before the intraperitoneal administration in rats.

Experimental design

Four animal groups were studied. Each group consisted of six animals. Group I contained the control animals. Group II (negative group) was administered intraperitoneal injections (i.p.) of DEN (200 mg/kg bw) + Fe-NTA (9 mg/kg bw). Group III was given DEN (200 mg/kg bw), Fe-NTA (9 mg/kg bw) intraperitoneally and SKN (100 mg/kg bw) through oral gavage. Group IV was administered with SKN (100 mg/kg bw) twice a week for 16 weeks.

At the end of 24 weeks, the animals were sacrificed by cervical dislocation. Their kidneys were quickly removed and preserved in 10% neutral buffered formalin for histopathological studies. Haematoxylin and eosin (H&E) preparations of processed sections were prepared for microscopic examination. Blood was collected for biochemical and serological studies.

Levels of stress markers

The levels of oxidative stress markers were evaluated using biochemical assays. The effect of SKN on the oxidation stress markers such as superoxide dismutase (SOD),[27,28] catalase (CAT),[29] glutathione (GSH),[29] glutathione reductase (GR),[30] glutathione peroxidase (GPx),[31] glutathione S-transferase (GST),[32] and quinone reductase (QR)[33] was evaluated. Lipid peroxidation levels of malonaldehyde (MDA) were assessed according to the protocol of Erdelmeier *et al.*, 1998.[34]

Biochemical evaluation of renal toxicity markers

Renal toxicity markers were evaluated to study the effect of SKN. Markers such as blood urea nitrogen (BUN),[35] creatinine,[36] uric acid,[37] urea,[38] lactate dehydrogenase (LDH),[39] and γ-glutamyltranspeptidase (GGT)[40] were assessed according to the modified protocol. All the protein estimations were done according to Lowry *et al.*, 1951.[41]

Histopathologic assessment

The kidney tissues isolated from the animals were washed with PBS and fixed with 10% formalin for 24 h. Thereafter, the specimens were dehydrated and fixed in paraffin wax. The tissues were then sliced in uniform thickness of about 5 μm and subjected to permeabilization with H and E stains.

Levels of inflammatory markers

The cytokines' [IL-1β, IL-6, TNF-α, PGE_2 (Prostaglandin), and *nuclear factor kappa B* (NF-κB)] levels were analyzed using ELISA kits obtained from Cayman Chemicals, USA.

Protein expression levels apoptotic markers

The proteins were isolated and transferred to the polyvinylidene fluoride (PVDF) membrane using sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE). The percentage of the SDS-PAGE used was 4–16%. Dilutions of primary and secondary antibodies were used at 0.02% with 1 h incubation. The blot was subjected to probing overnight with anti-Bcl-2-associated X protein (Bax), caspase-3, -9, and B-cell lymphoma (Bcl-2). Added primary antibodies subsequently treated with secondary antibodies for 1 h. Detection of bands was done by applying chemiluminescence reagent followed by blot densitometry analysis. The blot was normalized against β-actin.

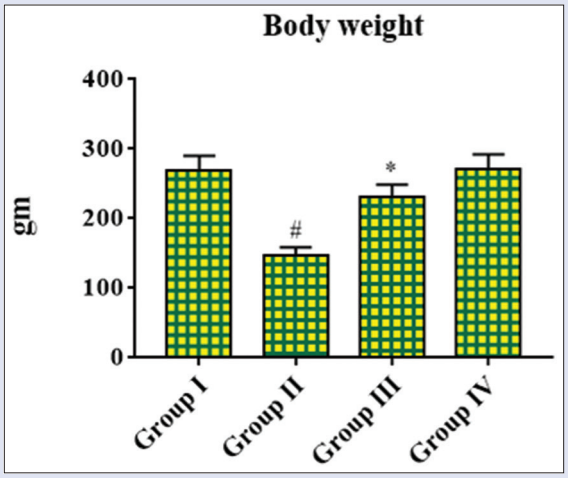

Figure 1: Body weight of control and experimental rats. Data represents the mean ± SEM ($^{\#}P < 0.05$; $^{*}P < 0.01$)

Statistical analysis

Overall data were expressed as mean ± standard error of mean (SEM). One-way analysis of variance and Student's unpaired t-test were used for comparison between groups and within groups. The Statistical Package for Social Sciences version-11 was used to carry out statistical analysis. A *P* value of < 0.05 ($P \leq 0.05$) was considered statistically significant and a *P* value of < 0.01 (≤0.01) was considered highly significant.

RESULTS

Effect of SKN on body weight

The body weight was measured at the beginning and after 24 weeks. The change in the mean body weights for the four groups was compared using an unpaired *t*-test. There was a significant decrease in the body weight (P <.05) of Group II rats (DEN + Fe-NTA). Group IV rats (SKN orally 100 mg/kg bw) showed a significant increase in body weight ($P < 0.05$). Thus, the results indicate bodyweight improvement with SKN [Figure 1].

Effect of SKN on antioxidant enzymes and oxidative stress markers

Oxidative stress is one key factor contributing to the proceeding of cancer pathogenesis. The antioxidant enzymes—SOD, CAT, GSH, GR, and GPx—were compared in the four groups using the Student's unpaired *t*-test [Figure 2]. The levels of these enzymes were significantly lowered in the Group II animals indicating increased stress as a contributing factor to the disease. In contrast, the animal groups treated with SKN (Group III and IV) showed a significant increase in the levels of the antioxidant enzymes compared to the control group.

The oxidative stress markers—GST and MDA—were significantly increased ($P < 0.05$) for Group II animals and decreased for Group III and IV animals as compared to the control group. In contrast, QR level was significantly decreased (P < 005) in Group II animals and remained the same for Group III and IV animals compared to the control group. Thus, the results highlight the antioxidant properties of SKN [Figure 3].

Effect of SKN on renal toxicity markers

The levels of the nephrotoxic markers—BUN, creatinine, LDH, uric acid, urea, and GGT—were analyzed in the animals. These nephrotoxic markers' levels were found to be significantly increased ($P < 0.05$) in the Group II animals treated with Fe-NTA. In contrast, the levels of the nephrotoxic markers were reduced in animal groups treated with SKN (Group III and IV). [Figure 4] Thus, the results suggest the reno-protective effect of the SKN against renal carcinoma.

Effect of SKN on renal histopathology analysis

Histopathological analysis revealed that standard tissue architecture with intact Bowman's capsule, normal glomerulus, and collecting duct was there in the tissues isolated from the control group, SKN-treated group, and SKN alone group [Figure 5]. Group II animals developed changes in the normal tissue architecture with increased inflammatory cells. Their tissues also revealed increased necrosis, glomerular degeneration, and swollen renal tubules along with signs of inflammation. In contrast, the Group III animals treated with Fe-NTA + SKN maintained an excellent tissue architecture with much lesser inflamed blood vessels and Bowman's capsule. Animals from Group IV treated with SKN alone showed a close resemblance to the control group.

Effect of SKN on inflammatory mediators

The inflammatory mediators' (IL-1β, IL-6, TNF-α, PGE_2, and NF-κB) levels were found to be significantly increased in Group II animals ($P < 0.05$). The animals of Group III and IV animals showed a decrease in the serum levels of inflammatory mediators [Figure 6]. Levels of the aforementioned antioxidant enzymes were decreased in the Group II carcinogenic animals, which on the administration of SKN increased significantly.

Effect of SKN on apoptosis-related proteins

Western blot analysis was done to evaluate the effect of SKN on apoptotic markers; Bax, caspase-3, -9, and Bcl-2 [Figure 7]. The apoptosis markers' levels were significantly raised in all other groups compared to the Group II animals ($P < 0.05$). Thus, indicating that SKN potential to trigger the release of the apoptosis-related proteins. Moreover, Bcl-2 was significantly down-regulated in the SKN-treated rats compared to the Group II animals indicating a protective effect ($P < 0.05$).

DISCUSSION

The increased prevalence of the RCC could be attributed to the challenges in diagnosis and resistance to therapeutics. Treatment modalities also remain limited. Thus, there is a need for exploring effective alternative therapeutics to combat the commonest and the most lethal genitourinary carcinoma, RCC.[42] SKN is a Chinese traditional medicine with known potent anti-inflammatory and anti-arthritic activities.[43] With this background, we studied the reno-protective effects of SKN in the experimental animal models using four groups.

Kidneys play a crucial role in the disposal of various toxic substances and are more susceptible to damage to various drugs and toxins. As an outcome of oxidative stress, the levels of the MDA tend to increase. In our study, we found increased levels of MDA and GST. We also found lowered levels of antioxidant enzymes in the chemically induced tumorigenesis animal group. Our finding is in agreement with the previous reports.[20,42] The anti-oxidant enzyme levels were increased in the animal groups treated with SKN. SKN administration also augmented the levels of GST and QR. Lipid peroxidation causing membrane damage has been implicated in several diseases, including renal conditions. The renal membranes integrity can be compromised by the serum enzyme raised levels,

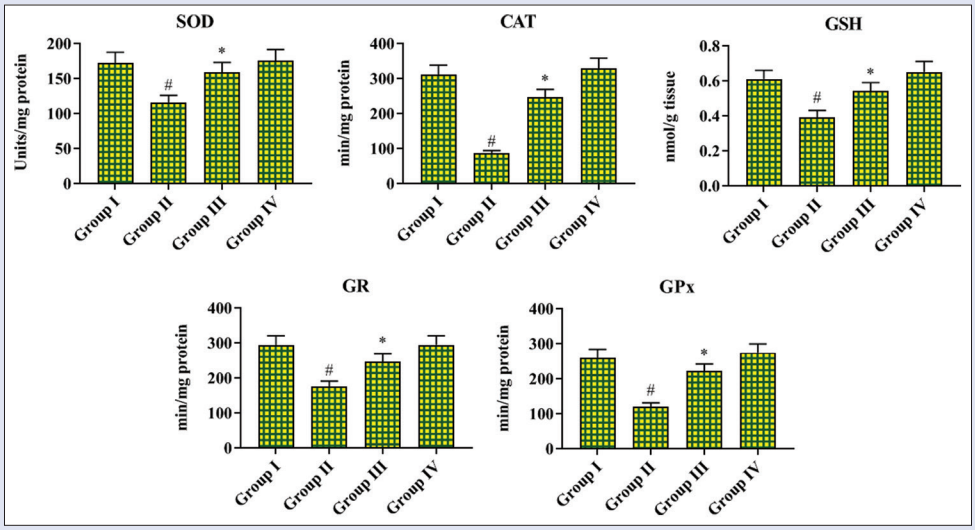

Figure 2: Biochemical evaluation of antioxidant enzymes. Data represents the mean ± SEM. (#$P < 0.05$; *$P < 0.01$)

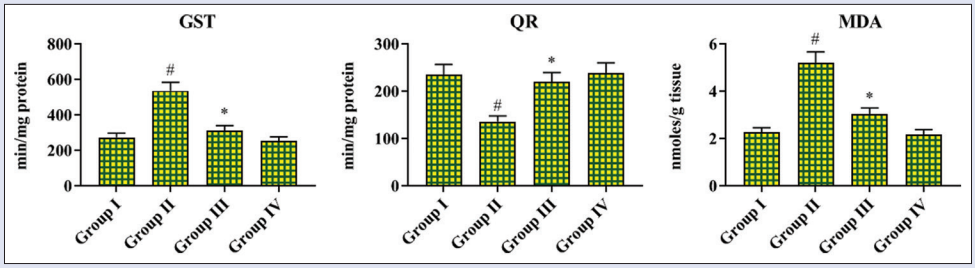

Figure 3: Effect of SKN on oxidative stress markers in experimental rats. Data represents the mean ± SEM (#$P < 0.05$; *$P < 0.01$)

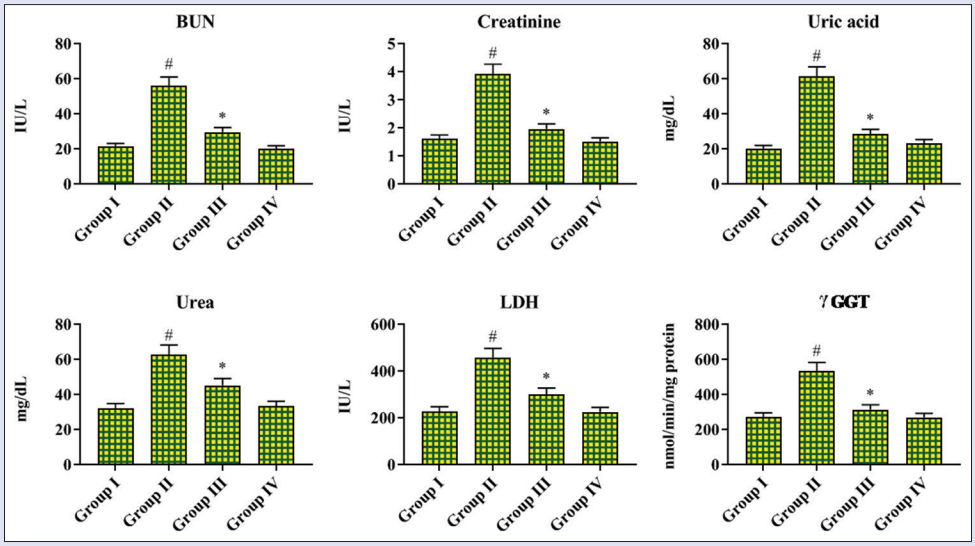

Figure 4: Levels of renal toxicity markers in experimental rats. Data represents the mean ± SEM (#$P < 0.05$; *$P < 0.01$)

indicating the leakage of cells. The levels of nephrotoxic serum markers BUN, creatinine, uric acid, urea, LDH, and GGT were significantly reduced in SKN-treated rats as compared to the negative control and control group. This exhibits the reno-protective effect of SKN against renal carcinogenesis in rats.

Histopathologic evaluation showed deceased cellular architecture in the Group II animals. SKN-treated groups showed good cellular architecture with intact Bowman's capsule, lesser infiltration of inflammatory cells, and normal morphology of the renal tubules in comparison to the disease-induced animals. This reflects the

ameliorating effect of SKN. Pro-inflammatory cytokines have been reported in a wide variety of autoimmune diseases and cancers. These serve as triggering agents for the multiple pathways affecting various downstream effectors. Of these, IL-1β, IL-6, and TNF-α have been predominantly and widely studied. NF-κB also serves as the focal hub for pathways governing cancer pathogenesis and tends to influence other cascades. NF-κB regulates the functions of the essential cell including cell cycle, cell survival, and differentiation.[39,40] PGE_2 is a major inflammatory mediator associated with the arachidonic acid pathway inducing severe inflammation.[44,45] In our study, the ELISA results reveal that all the mediators were significantly suppressed in the SKN-treated animal groups as compared to the control groups.

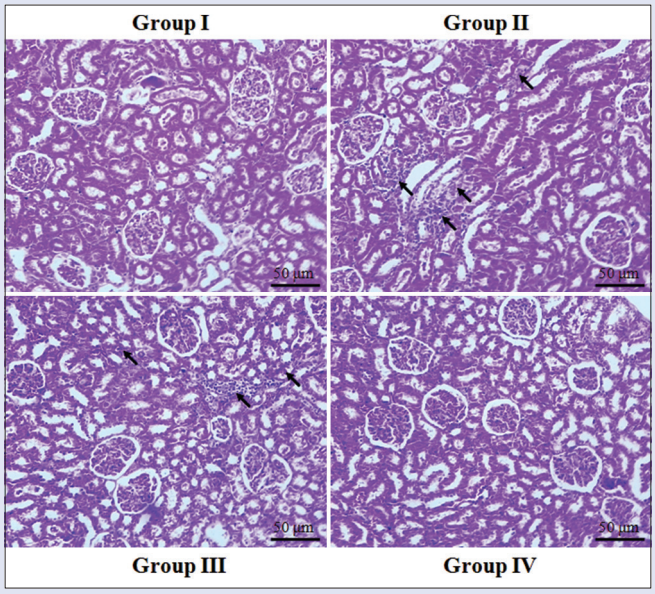

Figure 5: Comparison of histopathology findings of the four groups in the experimental animal model. Histopathology changes in the kidney tissues are indicated by arrow () of control and experimental rats (H&E 40×). Scale bar = 50 µm. Group I (Control) exhibiting normal tissue architecture; Group II showing increased necrosis, which circled by glomerular degeneration, swollen renal tubules with signs of inflammation; Group III (Fe-NTA + SKN); and Group IV SKN alone

Apoptotic proteins, including Bax, caspase-3, -9, and Bcl-2, play a crucial role in inducing apoptosis via the mitochondrial pathway. Bax is an apoptotic protein, and the Bcl-2 is an anti-apoptotic protein that plays an essential role in initiating apoptosis through several cascades. Mitochondria-mediated apoptosis pathways play a vital role in instigating apoptosis in cancer cells which progress through the effector proteins caspase-3 and -9. Caspase-3 is called an initiator protein. Caspase-9 is called the effector protein.[46,47] In our study, caspase-3, -9, and Bax were down-regulated, whereas Bcl-2 was up-regulated in the Group II animals. In the animals treated with the SKN, the caspase-3, -9, and Bax levels were up-regulated, and the Bcl-2 was down-regulated, triggering apoptosis. Thus, the results suggest that the SKN triggers apoptosis in the cancer cells in Fe-NTA-induced renal cancer rats.

CONCLUSION

SKN increased the antioxidant enzymes (SOD, CAT, GSH, GR, and GPx) in the rats. SKN inhibited the NF-κB, PGE2, IL-1β, IL-6, and TNF-α in the rats despite tumorigenesis Fe-NTA infusions. SKN also exhibited its reno-protective anti-cancer effect in the rats against the Fe-NTA-induced renal carcinoma. SKN was found to trigger apoptosis in the cancer cells of rats in the experimental animal model via caspase-3 and -9 up-regulation. Furthermore, the ELISA assay confirmed the down-regulation and effective inhibition of NF-κB, and PGE_2 levels in the renal carcinoma cells of rats infused with Fe-NTA. Thus, it makes SKN a potential therapeutic agent for renal carcinoma therapy.

Author contributions

Dong Wang and Yahong Lu conceived and designed the study; Wenjing Meng performed the experiments and wrote the original article; Zengyue Yang analyzed the review and editing. All authors read and approved the final manuscript.

Financial support and sponsorship

Nil.

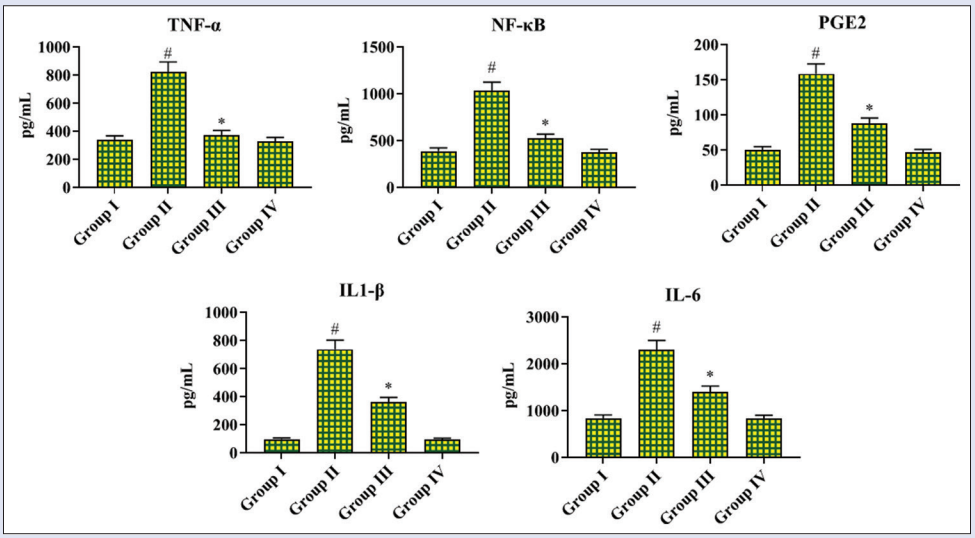

Figure 6: Serum levels of inflammatory mediators (TNF-α, NF-κB, PGE_2, IL-1β, and IL-6). Data represents the mean ± SEM ($^{\#}P < 0.05$; $^{*}P < 0.01$)

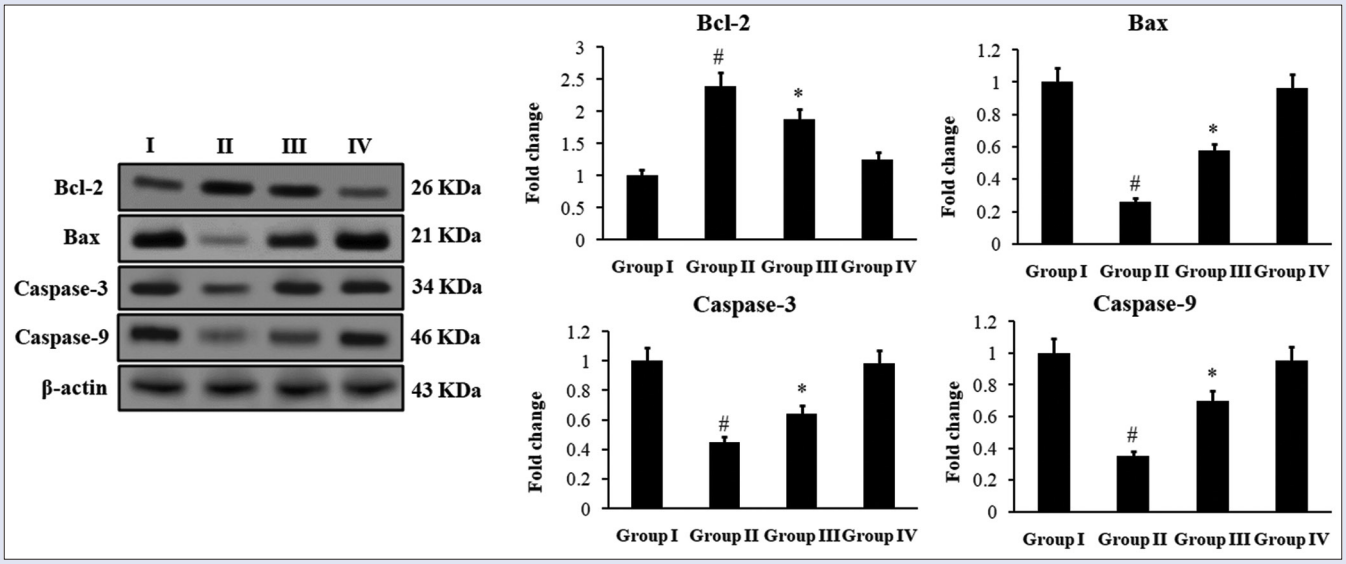

Figure 7: Effect of SKN on the apoptosis-related proteins. The protein expressions' levels of Bcl-2, Bax, caspase -3, and -9 of control and experimental rats. Data represents the mean ± SEM ($^{\#}P < 0.05$; $^{*}P < 0.01$)

Conflicts of interest

There are no conflicts of interest.

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