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Original Article

Visnagin Mitigates Glycerol-induced Acute Kidney Injury in Rats through Decreasing Inflammation, Oxidative Stress, and Renal Dysfunction Markers

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ABSTRACT

Background: Acute kidney injury (AKI) is a heterogenous condition, characterized by dysregulated kidney function along with reduced glomerular filtration, solute excretion, and urine output. **Objectives:** This work was focused to investigate the therapeutic roles of visnagin against the glycerol-induced AKI in rats via reducing the inflammation and oxidative stress responses. **Materials and Methods:** The AKI was induced in the Wistar rats by injecting 50% of glycerol (10 ml/kg). Then AKI rats were treated with 20 and 40 mg/kg of visnagin, respectively for 12 consecutive days. The rats were sacrificed followed at the end of experiments and blood and renal tissue samples were collected. The creatinine, BUN, urea, and LDH was quantified using assay kits. The nitric oxide (NO), MDA, and MPO were analyzed using assay kits. The antioxidants such as GSH, SOD, GSH, GPx, GR, and CAT activities were assessed by the standard methods. The IL-1β, IL-6, TNF-α, TGF-β1, MCP-1, and ICAM-1 levels were measured using assay kits. The histopathological analysis was done on the renal tissues. **Results:** The 20 and 40 mg/kg of visnagin treatment reduced the relative kidney weight of AKI rats. The urea, creatinine, BUN, and LDH levels were diminished by the visnagin. The 20 and 40 mg/kg of visnagin treated AKI rats appreciably reduced the MDA, MPO, and NO levels and augmented the GSH, SOD, GPx, GR, and CAT activities in the renal tissues. The IL-6, IL-1β, TNF-α, and TGF-1β levels were reduced by the visnagin treatment. The MCP-1 and ICAM-1 status were also diminished by the visnagin. The findings of the histopathological analysis revealed that visnagin attenuated the glycerol-induced AKI in rats. **Conclusion:** In conclusion, the outcomes of this study revealed that the visnagin demonstrated nephroprotective effects against glycerol-induced renal damage in rats. The ability of visnagin to ameliorate inflammatory response, oxidative stress, and renal dysfunction make it a potential candidate for treating AKI.

Keywords: Glycerol, Renal dysfunction, Creatinine, Inflammation, Visnagin, Rhabdomyolysis.

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INTRODUCTION

Acute Kidney Injury (AKI) is a heterogeneous condition, which arises due to the dysregulated renal function that further results in the changes in electrolyte levels, fluid volume, and waste product retention.[1] The pathogenesis of glycerol-induced AKI is a multifaceted process, which comprises excessive renal inflammation, acute hypoxia, oxidative stress, ischemia, myoglobin toxicity, hypercoagulation, venous congestion, and apoptosis.[2] AKI also involves infiltration of inflammatory cells and acute tubular necrosis. AKI is mainly diagnosed by the increased creatinine level and/or reduced urine excretion.[3] International Society of Nephrology has recorded that the AKI cases was reaches 13.3 million each year and 1.7 million mortalities were caused by the AKI annually worldwide. There are no effective treatments in the clinic to revert the kidney damage caused by the AKI.[4]

The rhabdomyolysis is the major pathophysiological manifestation of the AKI, which triggers severe kidney toxicity due to the increased renal inflammation, oxidative damage, endothelial dysfunction, edema, ischemia, apoptosis, and vasoconstriction. Rhabdomyolysis-induced AKI signifies 10-40% of all diagnosed AKI incidences.[5] Glycerol is the most extensively employed chemical agent to initiate the AKI in animals, as it triggers rhabdomyolysis, which attributes to the degradation of skeletal muscle with myoglobin migration into the bloodstream. Glycerol-induced rhabdomyolysis subsequently AKI, is a well-known study model utilized to study the pathogenesis of AKI.[6] The AKI is characterized by renal vasoconstriction, tubular

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obstruction, and the release of myoglobin. Though the precise underlying mechanisms that participated in the AKI are unknown, numerous factors are proposed to perform major functions such as renal inflammation, oxidative stress, disruption of the cellular detoxifying mechanisms, and activation of apoptosis.[7]

Inflammation plays a major role in both primary and secondary injury of AKI.[8] The inhibition of kidney inflammatory responses can attenuate renal injury by reducing the infiltration of inflammatory cells and cytokine generation. IL-6, IL-1β, and TNF-α is a major inflammatory cytokines and is participated in several inflammatory reactions, which play a major role in inflammatory kidney diseases.[9] Furthermore, oxidative stress also performs a vital functions in the development of AKI. The SOD, CAT, and GSH are the primary antioxidants that give protection against oxidative stress and are often reduced in AKI conditions due to the increased ROS production and lipid peroxidation.[10] Some studies suggested that the AKI can be attenuated by antioxidants and anti-inflammatory agents.[11,12] Hence, the inhibition of inflammatory response and oxidative stress might be a hopeful approach to treating and preventing AKI.

Visnagin is an active compound extensively found on the *Ammi visnaga* plant. *A. visnaga* is a biennial herb found throughout Asia, Europe, and North Africa. A previous study reported that it prevents the renal epithelial cells[13] and acts as a diuretic infusion against bladder and kidney stones.[14] It was reported that the visnagin has demonstrated the cardioprotective effects[15] and vasodilator effects.[16] Visnagin was found effective against urolithiasis and hypertriglyceridemia[17] reducing apoptosis in the follicular tissues of rat ovaries,[18] and decreasing inflammatory responses in LPS-activated BV-2 microglial cells.[19] However, the effects of visnagin against the AKI are not systematically analyzed yet. Hence, here we focused to examine the therapeutic roles of visnagin against the glycerol-induced AKI in rats.

MATERIALS AND METHODS

Chemicals

The glycerol, visnagin, and other chemicals were obtained from Sigma-Aldrich, USA. The ELISA assay kits for the assessment of oxidative stress, inflammatory markers, and angiogenic protein markers were procured from the Thermofisher and R&D Systems, Minneapolis, USA, respectively.

Experimental Rats

Wistar albino rats weighing above 210 ± 30g were employed in this study and the same was housed in infection-free polypropylene cabins and maintained at standard circumstances with 22–26°C of temperature, 50–60% of humidity, and 12 hr of dark/light sequences. The rats were fed a standard diet with pure drinking water during the study.

Experimental Design

The one acclimatized experimental rats were distributed into four groups with six rats in each (n=6) as follows: Group I: control rats orally treated with 0.9% NaCl for 12 consecutive days. Group II: rats were intramuscularly administered with 50% of glycerol (10ml/kg) after being deprived for 24 hr to induce the AKI. Group III and IV: The AKI was induced as described in Group II and then treated with 20 and 40 mg/kg of visnagin, respectively for 12 consecutive days. After the completion of the experiments, all rats from each group were anesthetized and sacrificed by the cervical decapitation method. Then the renal tissues were taken out and weighed to measure the changes in relative kidney weight. The remaining tissue and blood samples were utilized for the additional assays.

Determination of Kidney Dysfunction Markers in the Experimental Animals

The collected blood samples were utilized to prepare the serum and utilized for the biochemical studies. The contents of creatinine, blood urea nitrogen (BUN), urea, and lactate dehydrogenase (LDH) were quantified with the help of assay kits (Thermofisher, USA).

Determination of Nitric Oxide (NO), Malondialdehyde (MDA), and Myeloperoxidase (MPO) in the Renal Tissues Homogenates

The excised renal tissues were homogenated using buffered saline and centrifuged at 4°C using at 10,000 rpm for 15 min. Then collected supernatant was utilized to examine the levels of oxidative stress markers. The levels of NOS, MDA, and MPO were assessed using assay kits as per the recommended protocols by the manufacturer (Thermofisher, USA).

Determination of Antioxidant Biomarker Levels in the Experimental Rats

The antioxidants such as SOD, GSH, GPx, GR, and catalase (CAT) were quantified in the renal tissue homogenates of the experimental rats. The GSH level, SOD, GPx, CAT, and GR activities were examined by using assay kits (Thermofisher, USA).

Measurement of Pro-inflammatory Cytokines in the Experimental Rats

The contents of interleukin-1β (IL-1β), IL-6, TNF-α, and TGF-β1 in the kidney tissue homogenates of the experimental rats were measured by assay kits using the described guidelines by the manufacturer (R&D Systems, Minneapolis, USA).

Measurement of MCP-1 and ICAM-1 Levels

The contents of angiogenic protein markers i.e., MCP-1 and ICAM-1 in the kidney tissues homogenates in the experimental rats were estimated by using the assay kits by the protocols recommended by the manufacturer (R&D Systems, USA).

Histopathological Analysis

The excised renal tissues from the experimental rats were processed with a 10% of formalin solution. After that, the kidney tissues were paraffinized and cut into 5mm sizes. Then tissue sections were stained with hematoxylin-eosin (H&E) and finally, alterations in the histopathology of renal tissues were assessed using an optical microscope at 40× magnification.

Statistical Analysis

All the values are determined by the GraphPad prism software and outcomes were demonstrated as mean±SD of triplicates. The results are analyzed using one-way ANOVA and DMRT assay. The significance between experimental groups was fixed at $p<0.05$.

RESULTS

Effect of Visnagin on the Relative Kidney weight of Experimental Rats

The relative renal weight of both control and visnagin treated experimental rats were studied and outcomes were revealed in Figure 1. The glycerol-treated AKI rats demonstrated a considerable increment in the kidney weights. However, the 20 and 40 mg/kg of visnagin treatment considerably decreased the kidney weight of the kidneys in the AKI rats. The treatment with 40 mg/kg of visnagin effectively decreased the kidney weight of the AKI rats (Figure 1).

Effect of visnagin on the renal dysfunction marker levels in the experimental rats

The kidney dysfunction marker levels such as creatinine, urea, BUN, and LDH were determined in the serum of experimental rats, and outcomes were revealed in Figure 2. The levels of creatinine, urea, BUN, and LDH activity were considerably elevated in the serum of AKI rats. Remarkably, the treatment with the 20 and 40 mg/kg of visnagin effectively decreased the creatinine, urea, BUN, and LDH activity in the AKI rats (Figure 2). The 40 mg/kg of visnagin treatment appreciably decreased the creatinine, urea, BUN, and LDH levels in the serum of AKI rats than the 20 mg/kg treatment.

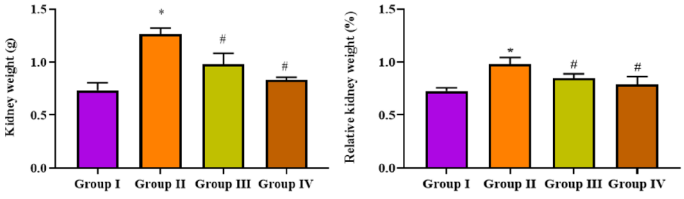

Figure 1: Effect of visnagin on the relative kidney weight of experimental rats.
Each bar depicts the mean±SD of triplicate values analysed by the one-way ANOVA consequently DMRT. Note: '#' indicates that data are significant at $p<0.05$ from control; '##' indicates that data are significant at $p<0.01$ from AKI-induced rats.

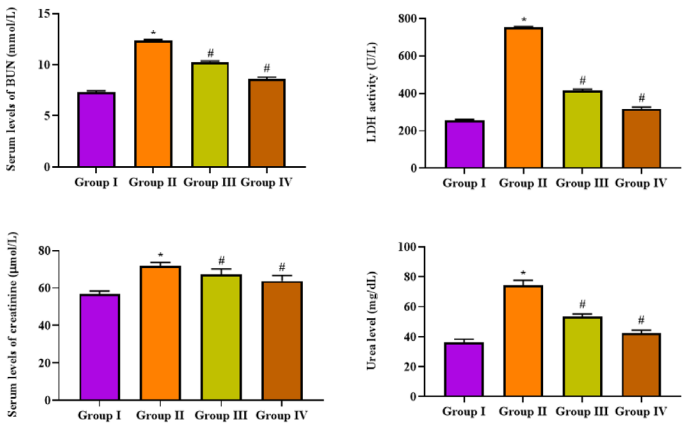

Figure 2: Effect of visnagin on the renal dysfunction marker levels in the experimental rats.
Each bar depicts the mean±SD of triplicate values analysed by the one-way ANOVA consequently DMRT. Note: '#' indicates that data are significant at $p<0.05$ from control; '##' indicates that data are significant at $p<0.01$ from AKI-induced rats.

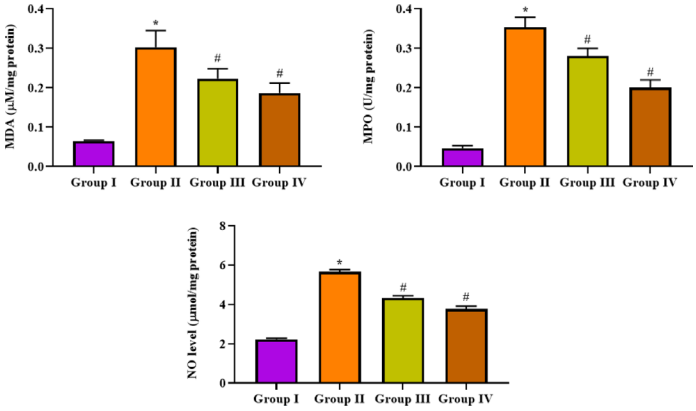

Figure 3: Effect of visnagin on the oxidative stress markers in the renal tissues of experimental rats.
Each bar depicts the mean±SD of triplicate values analysed by the one-way ANOVA consequently DMRT. Note: '#' indicates that data are significant at $p<0.05$ from control; '##' indicates that data are significant at $p<0.01$ from AKI-induced rats.

Effect of visnagin on the oxidative stress markers in the renal tissues of experimental rats

Figure 3 elucidates the MDA, MPO, and NO levels in the kidney tissue homogenates of the experimental rats. The increased status of MDA, MPO, and NO was noted in the kidney tissue homogenates of the AKI rats. Remarkably, the appreciable decrement in the contents of MDA, MPO, and NO was noted in the kidney tissue homogenates of the 20 and 40 mg/kg of visnagin treated AKI rats, which is in contrast to the glycerol-alone treated AKI rats (Figure 3). The treatment with 40 mg/kg of visnagin remarkably depleted the MDA, MPO, and NO levels in the kidney tissues of AKI rats than the 20 mg/kg treatment.

Effect of visnagin on the levels of antioxidant biomarkers in the experimental rats

The antioxidant biomarkers such as GSH level, SOD, CAT, GPx, and GR activities were analyzed in the kidney tissue homogenates of the experimental rats, and outcomes were depicted in Figure 4. The considerable decrement in the GSH level, SOD, CAT, GPx, and GR activities were noted in the renal tissues homogenates of the AKI rats. Nonetheless, the 20 and 40 mg/kg of visnagin treatment effectively augmented the GSH, SOD, CAT, GPx, and GR in the renal tissue homogenates of the glycerol-induced AKI rats (Figure 4). These antioxidants were considerably augmented in the kidney tissues of AKI rats by the 40 mg/kg of visnagin treatment than the 20 mg/kg of visnagin.

Effect of visnagin on the inflammatory biomarkers in the experimental rats

The levels of IL-6, IL-1β, TNF-α, and TGF-β1 were examined in the kidney tissue homogenates of the experimental rats, and the results were revealed in Figure 5. As a consequence of the serious inflammatory response, the glycerol-induced AKI rats exhibited an elevation in IL-6, IL-1β, TNF-α, and TGF-β1 levels in the renal tissues. Interestingly, the IL-6, IL-1β, TNF-α, and TGF-β1 contents were appreciably diminished by the treatment with 20 and 40 mg/kg of visnagin to the glycerol-induced AKI rats (Figure 5). The 40 mg/kg of visnagin treatment remarkably depleted the IL-6, IL-1β, TNF-α, and TGF-β1 levels in the kidney tissues of AKI rats than the 20 mg/kg treatment.

Effect of visnagin on the MCP-1 and ICAM-1 levels in the experimental rats

Figure 6 reveals the contents of MCP-1 and ICAM-1 in the renal tissues homogenates of the experimental rats. The glycerol-induced AKI rats revealed the increased status of MCP-1 and ICAM-1 in the kidney tissue homogenates when compared with control. A considerable decrease in the MCP-1 and ICAM-1 content was noted in the kidney tissue homogenates of the 20 and 40 mg/kg of visnagin treated AKI rats (Figure 6). The treatment with the 40 mg/kg of visnagin remarkably decreased the IL-6, IL-1β, TNF-α, and TGF-β1 levels in the kidney tissues of AKI rats than the 20 mg/kg of visnagin.

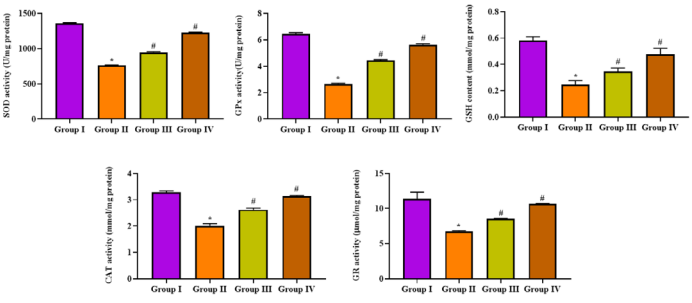

Figure 4: Effect of visnagin on the levels of antioxidant biomarkers in the experimental rats.
Each bar depicts the mean±SD of triplicate values analysed by the one-way ANOVA consequently DMRT. Note: '#' indicates that data are significant at $p<0.05$ from control; '##' indicates that data are significant at $p<0.01$ from AKI-induced rats.

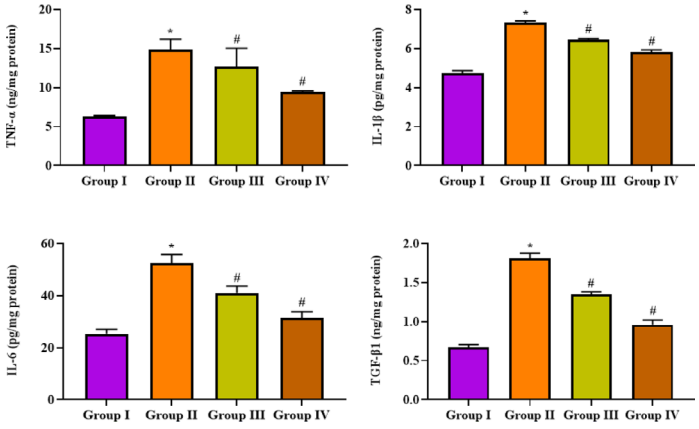

Figure 5: Effect of visnagin on the inflammatory biomarkers in the experimental rats.
Each bar depicts the mean±SD of triplicate values analysed by the one-way ANOVA consequently DMRT. Note: '#' indicates that data are significant at $p<0.05$ from control; '##' indicates that data are significant at $p<0.01$ from AKI-induced rats.

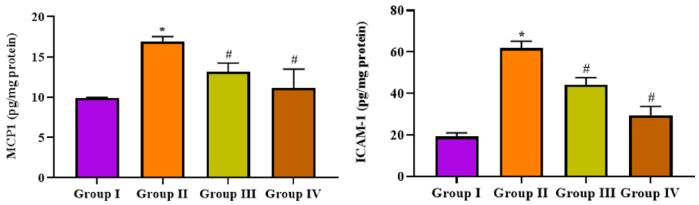

Figure 6: Effect of visnagin on the MCP-1 and ICAM-1 levels in the experimental rats.
Each bar depicts the mean±SD of triplicate values analysed by the one-way ANOVA consequently DMRT. Note: '#' indicates that data are significant at $p<0.05$ from control; '##' indicates that data are significant at $p<0.01$ from AKI-induced rats.

Effect of visnagin on the kidney histopathology of the experimental rats

The histopathological analysis of kidney tissues of experimental rats was performed and the outcomes were illustrated in Figure 7. The control rats revealed normal cell arrangements in the renal tissues. However, the glycerol-induced AKI rats demonstrated extensive tissue injury in both medullar and cortex regions. The histopathological alterations include the vacuolation and tubular dilatation, loss of microvilli, tubular necrotic lysis, and severe tubular injury were noted. Interestingly, the concomitant treatment with 20 and 40mg/kg of visnagin considerably attenuated the glycerol-induced histopathological changes (Figure 7). The 40 mg/kg of visnagin treatment considerably attenuated the glycerol-induced histopathological alterations in the kidney tissues of AKI rats than the 20 mg/kg of visnagin treatment.

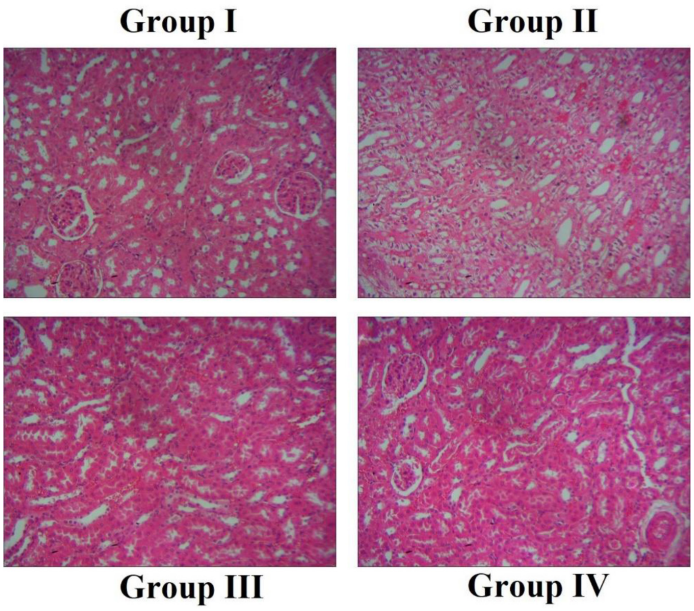

Figure 7: Effect of visnagin on the kidney histopathology of the experimental rats.
Group I: The control rats revealed a normal architectures in the kidney tissues. Group II: The glycerol-induced AKI rats demonstrated the vacuolation and tubular dilatation, loss of microvilli, tubular necrotic lysis, and severe tubular injury in the renal tissues. Group III and IV: The treatment with 20 and 40mg/kg of visnagin, respectively attenuated the glycerol-induced histopathological alterations in the renal tissues.

DISCUSSION

Rhabdomyolysis is a condition of skeletal muscle fiber destruction, where the myoglobin, creatinine, LDH, electrolytes, and aldolase contents were released into the bloodstream. These contents are filtered by glomeruli and speed up the AKI progression.[20] AKI is considered as most serious complication of rhabdomyolysis. The intramuscular administration of glycerol generates a myoglobinuric condition that is comparable to the clinical rhabdomyolysis that demonstrates increased creatinine and BUN levels due to the reduced glomerular filtration rate followed by the glycerol administration.[21] Glycerol-stimulated AKI is regulated by myoglobin nephrotoxicity and kidney ischemia. The clinical signs of glycerol-induced AKI like tubule damage are found like a human AKI. Glycerol is a well-known nephrotoxic chemical that interrupts the glomerular filtration, stimulates renal tubule injury, and prompts epithelial and stromal cell swelling, therefore elevating kidney weight. Furthermore, edema and kidney enlargement was developed in response to the elevated relative renal weight of glycerol-administered rats, which confirms toxic insult to the kidneys.[22] Similarly, we also noted the increased kidney weight in the glycerol-stimulated AKI rats. Interestingly, the visnagin treatment effectively decreased the kidney weight of the AKI rats.

Intramuscular administration of glycerol leads to the muscle cell breakdown and release of myoglobin and sarcoplasmic proteins. The excessive amounts of these compounds are highly nephrotoxic and stimulate inflammation in the kidney tissues.[23] Inflammation is a vital player in the development of renal ailments due to increased podocyte injury. AKI is characterized by increased inflammation in the renal tissues.[24] The elevated status of IL-6, IL-10, IL-1β, and TNF-α was positively correlated to the severity of renal inflammation. IL-6 and TNF-α are the critical inflammatory cytokines that are over-expressed at the tissue injury sites.[25] The increased expressions of IL-6 and TNF-α were already highlighted in the patients with AKI and chronic kidney failure.[26] Here, our findings revealed that the IL-6, IL-1β, and TNF-α status were considerably elevated in the renal tissue homogenates of the glycerol-induced AKI rats. Meanwhile, the visnagin treatment effectively diminished the IL-6, IL-1β, and TNF-α contents in the renal tissues of AKI rats. These outcomes confirmed that the visnagin has anti-inflammatory activity.

Oxidative stress is a condition of imbalance between the free radicals and antioxidant protection system that neutralizes the deleterious effects of free radicals, leading to irreversible tissue injury. In addition, oxidative stress could directly enhance the cell apoptosis thereby aggravating the AKI.[27] The kidney tissues are highly vulnerable to oxidative stress-mediated damage. Kidney injury can also result from the infiltration of large amounts of xenobiotics. The oxidative stress-mediated damage due to the increased ROS production is a vital cause of AKI pathogenesis.[28] The SOD, GPx, CAT, GR, and GSH are the first-line defenses against oxidative damage, which is decreased during AKI development. These antioxidants act by degrading free radicals thereby protecting the cells. The renal oxidative stress promotes the generation of mitochondrial ROS, triggers lipid peroxidation, and reduces the antioxidant activities. The reduced antioxidant levels in renal tissue followed by the glycerol administration were highlighted earlier.[29] In a similar manner, the antioxidants such as GSH and the SOD, CAT, GPx, and GR activities were considerably decreased in the renal tissues of AKI rats. However, the visnagin treatment appreciably improved these antioxidant activities in the AKI rats. These findings evidenced the strong antioxidant potential of the visnagin.

The creatinine, urea, and BUN are the well-known renal dysfunction markers that are considerably elevated in the serum of AKI rats. These biomarkers are the early predictors of kidney damage, as they all are excreted by the kidneys through urine.[30] The serum creatinine is the most sensitive biomarker of rhabdomyolysis and muscle damage, which is considerably elevated followed by the intramuscular administration of glycerol. The considerable elevation in the BUN and creatinine status in the serum indicates the occurrence of renal damage and dysregulated excretory ability of the kidneys.[31] Here, we noted that the creatinine, urea, and BUN were considerably elevated in the AKI rats. Interestingly, the elevations were reverted by

the visnagin treatment. These findings revealed that the visnagin improved the renal functions in the AKI rats.

MPO is an enzyme released from the immune cells in response to inflammation and oxidative stress. It catalyzes the generation of several reactive oxidant species, which play a vital role in the renal disease development.[32] Furthermore, MPO also regulates oxidative stress-mediated damages. MDA is an end product of lipid peroxidation, which is extensively utilized to detect oxidative stress levels and inflammation-mediated cell injury.[33] The neutrophil infiltrations into the inflammatory sites lead to the MPO release. MPO acts on nitrite to generate the nitrogen radicals to trigger lipid peroxidation. NO is a major player of renal oxidative stress and considerable elevation in NO level is connected with renal damage.[34] Here, we observed that the levels of MPO, MDA, and NO were drastically elevated in the AKI rats. However, the treatment with the visnagin considerably diminished the MPO, MDA, and NO in the AKI rats. These outcomes confirmed that the visnagin decreased oxidative stress in the AKI rats.

MCP-1 is the most sensitive kidney biomarker which is highly activated in inflammation and tissue injury sites and leads to macrophage recruitment. MCP-1 is the most investigated renal biomarker in recent times. The increased expression of MCP-1 occurs during the progression of renal diseases and inflammatory infiltration conditions.[35] ICAM-1 is an adhesion molecule that occurs in the endothelial and immune cell membranes. The inflammatory cytokines can trigger the generation of ICAM-1 and recruit immune and inflammatory cells from the bloodstream to the inflammatory sites.[36] Here, increased status of MCP-1 and ICAM-1 were observed in the kidney tissues of the AKI rats. Contrastingly, the visnagin treatment appreciably decreased the status of MCP-1 and ICAM-1 in the AKI rats. A previous study reported histological changes such as vacuolation and necrosis with luminal casts in the renal tissues of the glycerol-induced AKI rats.[37,38] Our findings revealed that the glycerol-induced AKI rats demonstrated vacuolation and tubular dilatation, tubular necrotic lysis, and severe tubular injury. Interestingly, the treatment with the visnagin effectively ameliorated the glycerol-induced histological changes in the kidney tissues.

CONCLUSION

In summary, the findings of this work propose a substantial therapeutic role of visnagin against glycerol-induced kidney damage. The visnagin-treated AKI rats demonstrate the considerable nephroprotective effects against AKI condition. The capacity of visnagin to alleviate inflammatory response, oxidative stress, and renal dysfunction makes it an ideal candidate for treating AKI. However, further studies are still required in the future to understand the precise therapeutic roles of visnagin.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

AKI: Acute Kidney Injury; **BUN:** Blood Urea Nitrogen; **LDH:** Lactate Dehydrogenase; **NO:** Nitric Oxide; **MDA:** Malondialdehyde; **MPO:** Myeloperoxidase.

SUMMARY

- Acute Kidney Injury (AKI) is a heterogenous condition, characterized by dysregulated kidney function along with reduced glomerular filtration, solute excretion, and urine output.
- Visnagin treatment appreciably decreased the status of MCP-1 and ICAM-1 in the AKI rats.
- The capacity of visnagin to alleviate inflammatory response, oxidative stress, and renal dysfunction makes it an ideal candidate for treating AKI.

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