Histopathological Changes of the Effect of Ketotifen in a Rat Model of Nephropathy

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Abstract: Acute kidney injury (AKI) remains a common clinical problem with serious consequences and unsatisfactory therapeutic options. Mast cells are distributed in the kidneys, have a role in the inflammation. Thus, a model of Acute kidney injury of rhabdomyolysis induced by glycerol was done in order to investigate the possible healing effect of Ketotifen, a selective stabilizer of mast cells and a histamine H_1 receptor antagonist, in rats.

Methods: Wister rats (250-350 g) were used. Renal failure was produced by rats deprived of water for 24 hours followed by i.m. injection with 50% (vol/vol) glycerol (10 ml/kg of body weight). After 30 min, Ketotifen was used at a dose of 2 mg/kg. Rats received treatment for 5 consecutive days**.** On the 6^{th} day, the rats were sacrificed; blood was obtained for blood urea and creatinine assays. The kidney tissue was used for the determination the histological injury.

Results: The levels of urea and creatinine were decreased significantly (P < 0.0001). The macroscopic and histopathological parameters were reduced significantly in the Ketotifen treated group to (P < 0.05) compared to the glycerol group.

Conclusions: The anti-inflammatory effects of Ketotifen in treating the AKI are due to its potential to reduce the urea and creatinine levels and improve the macroscopic, and histological markers.

Keyword: Ketotifen, Acute kidney injury, glycerol, histopathological.

INTRODUCTION

Glycerol-induced acute kidney injury (AKI) is a model of rhabdomyolysis-induced AKI. Rhabdomyolysis-induced AKI develops after skeletal muscle trauma related to physical, thermal, ischemic, infective, metabolic, or toxic causes, releasing toxic doses of myoglobin and other intracellular proteins into the circulation [1]. The main pathophysiological mechanisms of rhabdomyolysis-induced myoglobinuric AKI are blood volume reduction, renal vasoconstriction, intraluminal cast formation, and direct myoglobin induced cytotoxicity [2], and tubular damage associated with reactive oxygen species (ROS) production [3]. As another mechanism, Inflammation plays a pivotal role in the pathophysiology of RIAKI. Recent studies have shown the importance of leukocytes, especially macrophages, in the development of Rhabdomyolysis-induced AKI (RIAKI) [4].

Mast cells are ordinarily distributed in normal connective tissue, mainly located adjacent to blood and lymphatic vessels, nerves, and epithelial surfaces, as well as in the skin, in the gastrointestinal, and the respiratory system. Mast cells distribute very sparsely in the kidneys under normal circumstances, but the number of mast cells increases dramatically during renal ischemia/reperfusion injury. Under pathological conditions mast cells stimulate and release, numerous mediators, such as histamine, trypsin-like enzyme, chymase, heparin, and a host of cytokines, these mediators produce a wide array of biological effects. Mast cells can increase oxidative damage and promote the production of reactive oxygen species (ROS), which reduce the activity of SOD and increase MDA levels. Altering mast cell activity and function has proven to be a promising technique to reduce intestine, lung, heart, and brain ischemia reperfusion injury. Previous studies demonstrated that Ketotifen decreased intestinal ischemia reperfusion injury [5].

Ketotifen, a histamine H1-receptor antagonist, used in the management of allergic disorders, including bronchial asthma and atopic dermatitis [6]. The principal of pharmacological effects are to block the release of chemical mediators from mast cells and other inflammatory cells and to block their effects on targeted organs [7].

The present study was performed to evaluate cure effect of Ketotifen on kidney function, and oxidative damage in glycerol-induced experimental AKI. However, Ketotifen effect on the quality of experimental nephropathy healing has not been investigated previously.

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EXPERIMENTAL DESIGN AND TREATMENT PROTOCOL

Animals and Treatment

Wistar rats weighing (250-300g) were adapted for one week before any experimental procedures. The rats were fed with standard commercial rat pellets, and allowed water ad libitum. They were kept under controlled environmental conditions (temperature 23 ± 2°C , humidity 55 ± 15%, lighting regimen of 12-h light: 12-h dark). All methods performed in this study were in accordance with regulatory guidance on the care and use of experimental animals.

Rats were dehydrated for 24 h before glycerol injection. Rats were divided randomly into three groups (n = 6 for each group). The first group (untreated, N) was not injected with any treatment; the second and third (G) and (K) groups of rats were given intramuscular injections of 50% glycerol (surechem products LTD) (10 ml/kg) in their hind limbs [8]. The first and second groups received normal salin, and the third group received ketotifen fumarate obtained from Sigma-Aldrich Co. LLC, (2 mg/kg/day, suspended in normal saline), oral administertion was done using an intubation needle [9], once daily for 5 consecutive days, applied 30 min after glycerol injection.

An hour after the last dose, the rats were sacrificed under deep ethyl ether anesthesia (surechem products LTD). Blood samples and kidney tissues were harvested for future biochemical and pathology analyses.

The Assessments

Renal function was monitored by blood samples collected through heart puncture. Serum was separated for renal function tests (serum urea and creatinine concentrations).

Serum Creatinine Concentratoin

According to the manufacturer's instructions, creatinine concentrations in plasma samples were measured with a rate-blanked and compensated picric acid colorimetric assay (CREA, Roche/Hitachi Modular p analyzer). In this enzymatic method, creatinine is converted to creatine under the activity of creatininase. In alkaline solution, creatinine forms a yellow-orange complex with picrate. The absorbance of samples and standard were measured twice (after 30 sec (seconds) and 90 sec) spectrophotometrically (Hitachi U-1800) at 505 nm. The concentrations were calculated according to the manufacturer's protocol.

Serum Urea Concentration

The principle of the urea measurement, using the Roche/Hitachi Modular p analyzer kit, is based on the change in the intensity of staining ammonia compounds with sodium salicylate and sodium hypochlorite, which is directly proportional to the concentration of urea in the sample. The colored complex was measured by spectrophotometrically at 340 nm, according to the manufacturer's protocol.

Histological Examination and Grading

kidneys were preserved in 15% paraformaldehyde solution for 24 h, and washed with 70% ethanol. Tissues were then placed in small metal caskets, stirred by a magnetic stirrer, dehydrated using alcohol series from 70% to 100% alcohol and embedded in paraffin using an embedding machine. Paraffin blocks were sectioned using a rotary ultra microtome, distributed onto glass slides and then dried overnight. Slides were observed under a light microscope after being stained with hematoxylin and eosin (H&E) dyes and mounted.

Samples were blindly analyzed to determine the extent of kidney injury based on the technique outlined by Erdogan *et al.* [10]. The examinations focused on the kidney proximal tubules, were graded for the degree of renal damage, based on the following parameters: tubular cell necrosis and apoptosis, cytoplasmic vacuole formation, and tubular dilatation. Interstitial edema and medullary congestion were also assessed. The severity of these lesions was determined based on the percentage of involvement of the kidney. Higher scores represent more severe damage, with the maximum score being 4 : [0, histopathological changes <10%; 1, (10%–25%); 2, (25%–50%); 3, (50%–75%); and 4, (75%–100%)].The renal glomerular injury, hemorrhage, inflammation, fibrinoid and hyaline dystrophies were examined, where the presence of these injuries was grade (1); and their absence was grade (0). The mean score for each parameter was determined and subjected to statistical analysis.

Statistical Analysis

Results were expressed as (mean ± standard deviation SD). Statistical analysis was performed using the GraphPad Prism (Version 6) statistical package. Comparisons between the groups for parameter were performed using a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, for serum creatinine, and urea concentration;

statistical significance was set at p < 0.0001. Lesion score and histological score (non-parametric values) were analyzed using the Mann Whitney U test, and the frequency of categorical binary data was evaluated using fisher's exact test. P values <0.05 were considered as statistically significant.

RESULTS

Kidney Function Tests

Kidney function tests are presented in Table **1**. Glycerol injection caused a marked increase in serum creatinine, and blood urea levels (p < 0.0001). Ketotifen had a curative effect on serum creatinine and urea levels were significantly reduced to (p < 0.0001).

Macroscopic and Histological Results

Macroscopic Evaluation

In control group (N) Kidneys had normal macroscopic appearance and color; the same was in group (K). However, kidneys in injured group (G) were bigger than those in the control group, with different macroscopic morphology. They showed yellow cortex with dark brown medulla. Noticed congestion and edema (Figure **1**).

Histopathologic Evaluation

Light microscopy images of kidney sections are shown in Figure **7**. Tubular cell necrosis, cytoplasmic vacuoles, apoptotic morphology, and tubular dilatation were observed in histological specimens from glycerol group (Figure **7B** and **C**). Renal tubular scores were significantly higher compared to control group (N), (P<0.05). There was also mild to moderate medullary congestion and interstitial edema. The histological scores of these lesions were significantly higher than control group (P<0.05) (Figures **2-4**). The glomerular injuries were observed in this group represented as mesangial extracellular matrix deformation, necrosis and glomerular capillary congestion, these lesions' scores were significantly higher than those of the control group (N), (P<0.05). Hyaline dystrophies also observed in this group specimen (Table **2**), and were significantly different from the control group (N) (P<0.05). The signs of inflammatory lesions also observed in this group represented as mononuclear and polymorphonuclear leukocytes infiltration in the tubules and interstitium, these lesions were not significantly compared control group. Hemorrhage and coagulation in renal vessels were also observed in some samples. All these lesions were absent in the control group (Figure **7A**). Histological alterations were markedly reduced in specimens from the Ketotifen-treated groups (Figure **7D**) compared to (G) group. As compared to (G) group, the histological scores of these features for group (K) were significantly lower than of the injured group (G), (P<0.05).

Table 1: Renal Function: Serum Level of Urea and Creatinine Concentrations

Groups	Urea mmol/L	Creatinine µmol/L
Normal (N)	33.85 ± 9.237	0.398 ± 0.088
Glycerol (G)	111.7 ± 21.09 χ	1.87 ± 0.265 χ
Ketotifen (K)	61.67 ± 18.63 η	0.683 ± 0.079 η

χ p < 0.0001 vs. N; η p < 0.0001 vs. G.

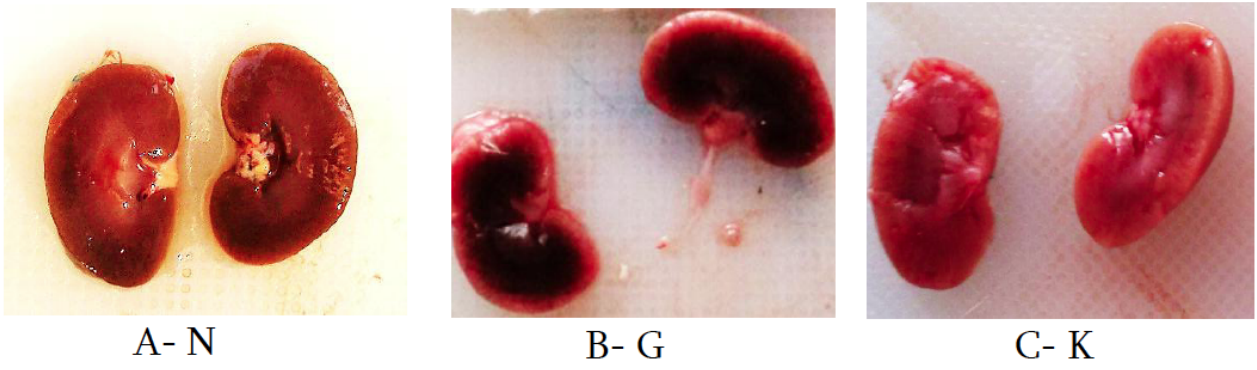

Figure 1: Representative macroscopic kidney changes: (N) normal group; (G) glycerin group; (K) Ketotifen group.

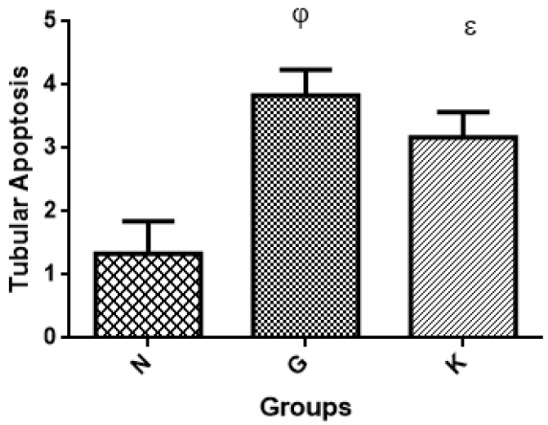

Figure 2: Effect of Ketotifen on histological score of damage on tubular apoptosis.

Each column represented as the mean±SD.

φ significant vs. N group P=0.0011<0.05; ε significant vs. G group P=0.040<0.05

N: control group; G: glycerol, K: Ketotifen.

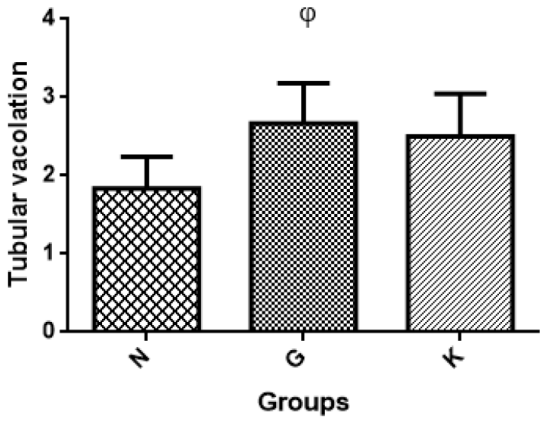

Figure 3: Effect of Ketotifen on histological score of damage on tubular vacuolation. Each column represented as the mean±SD.

φ significant vs. N group P=0.0227<0.05

N: control group; G: glycerol, K: Ketotifen.

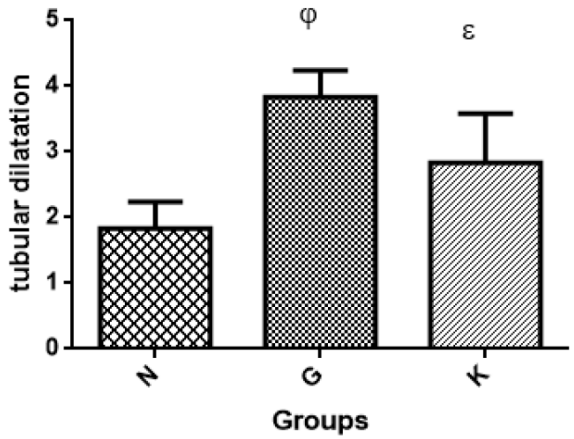

Figure 4: Effect of Ketotifen on histological score of damage on tubular dilatation. Each column represented as the mean±SD.

φ significant vs. N group P=0.0011<0.05; ε significant vs. G group P=0.0271<0.05. N: control group; G: glycerol, K: Ketotifen.

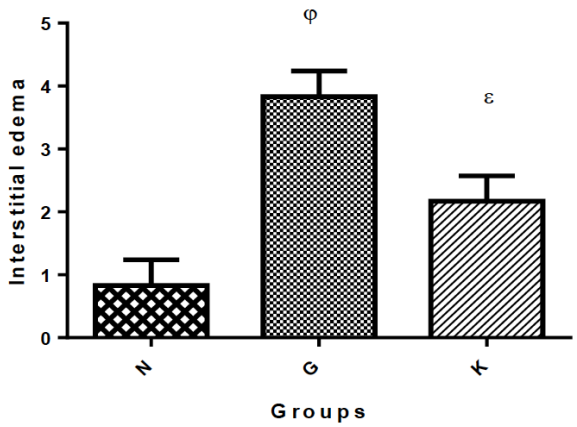

Figure 5: Effect of Ketotifen on histological score of damage interstitial edema.

Each column represented as the mean±SD.

φ significant vs. N group P=0.0011<0.05 ; ε significant vs. G group P=0.0022<0.05. N: control group; G: glycerol, K: Ketotifen.

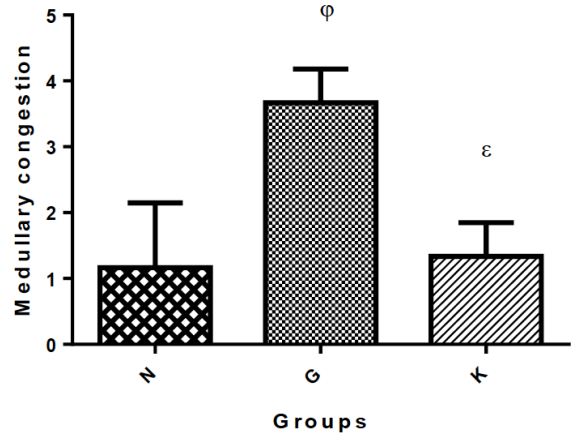

Figure 6: Effect of Ketotifen on histological score of damage on medullary congestion. Each column represented as the mean±SD.

φ significant vs. N group P=0.0011 <0.05; ε significant vs. G group P=0.0011 <0.05.

N: control group; G: glycerol, K: Ketotifen.

DISSCUSION

The present study showed that glycerol-induced ARF was associated with macroscopic, microscopic and biochemical changes compared to control group. Glycerol-induced ARF in rodents is mediated by renal ischemia and myoglobin nephrotoxicity [11]. The pathogenic mechanisms involved in glycerol-induced renal failure include ischemic injury, tubular nephrotoxicity caused by myoglobin, and the renal actions of cytokines released after rhabdomylosis [12].

Ischemia/reperfusion (I/R) injury involves the inflammatory response of both the innate and adaptive immune systems through exaggerated inflammatory cell infiltration and tubular epithelial cell activation. When renal I/R injury occurs, inflammatory cells initially

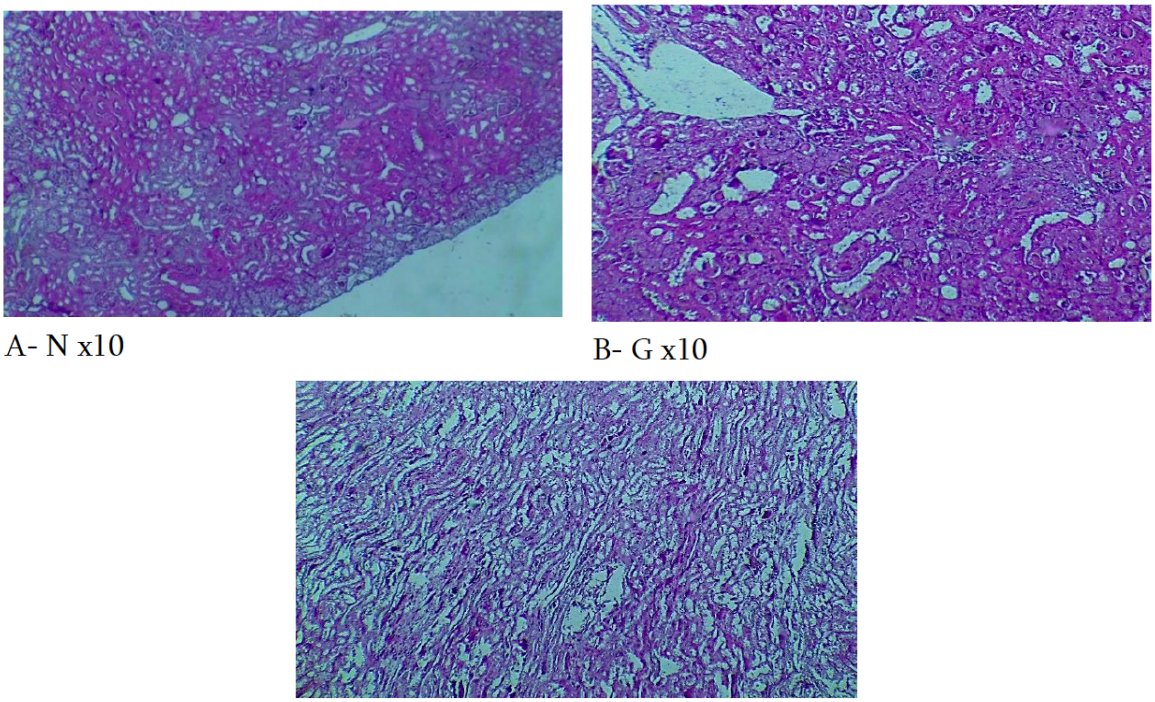

Figure 7: Representative histomorphological kidney changes: (N) normal group; (G) glycerine group; (K) Ketotifen group. All photomicrographs were taken at a magnification of 10x. A- (N) represent kidney section of a control rat showing normal architecture. B-(G) represent kidney section of a glycerol treated rats showing glomerular deformation, tubular dilatation, vacuolation, swelling and degeneration of their lined epithelial cells, vascular congestion, fibrinoid distrophy and hyaline dystrophy. C-(K) represent kidney section of Ketotifen treated rats showing the enhancement in tubular and glomrular injuries and other pathologic alterations.

Table 2: Effects of Ketotifen on Glomerular Injury, Fibrinoid Dystrophy and Hyaline Dystrophy, Expressed as Frequency of Injured Animals in each Group

Hyaline dystrophy			Fibrinoid dystrophy			Glomerular injury			feature
Significance	No injury	injury	Significance	No injury	injury	Significance	No injury	injury	Groups
	6	0		6	0		6	0	N
γ	0	6		6	0	θ	1	5	G
κ	6	0	κ	0	6		2	4	K

θ P=0.0076<0.05 as compared to N group; γ P=0.0011<0.05 as compared to N group; κ P=0.0011<0.05 as compared to G group. N: control group; G: glycerol, K: Ketotifen.

infiltrate into the damaged renal tissue and promote marked pro-inflammatory mediator secretion; the latter promotes the infiltration of inflammatory cells into the damaged tissues and further promotes inflammatory tissue responses. Furthermore, infiltrated inflammatory cells reduce renal blood flow, which leads to microcirculatory dysfunction [5].

Tong results showed that I/R injury induced the production of TNF-α, IL-6, and ICAM-1. Tissue histology showed significant pathology, with renal tissue bleeding, edema, and inflammatory cell infiltration. Serum urea and creatinine were also significantly increased [5]; as in fair agreement with this study, such as tubular cells necrosis, cytoplasmic vacuoles, apoptotic morphology, tubular dilatation, medullary congestion and interstitial edema, glomerular injury, urea and creatinine increased. The results were in agreement with many studies such as Stefanovic [13], and Manikandan [14].

Serum creatinine and urea concentration were blunted by repeated administration of Ketotifen. From a histological viewpoint, the deleterious effects of glycerol on the kidney were also reduced in animals repeatedly administered Ketotifen. Interestingly, Ketotifen treatment suppressed the pathological changes in the kidney to the same degree as the normal feature. It

showed a normal architecture similar to the control animals.

Kalia results suggest that endogenous mast cells are activated after renal ischemia reperfusion and subsequently release a large number of active substances such as histamine that damage kidney tissues. Histamine, one of the main active substances produced by mast cells, can increase vascular permeability, induce inflammatory cell infiltration, stimulate epithelial cells, and release cytokines, all of which cause or exacerbate tissue damage that can further induce mast cell activation [15].

In various experimental and clinical conditions, ketotifen was noted to reduce mast cell degranulation and to decrease the release of histamine, mast-cell proteases, myeloperoxidase, leukotrienes, platelet-activating factor (PAF) and various prostaglandins. Ketotifen also inhibits polymorphonuclear aggregation and migration, and attenuates inflammatory responses. It also directly reduces eosinophil function and viability [6].

Reports demonstrated that inhibition of inflammatory factors such as TNF-α, IL-6, and ICAM-1, as well as inhibition of lipid peroxidation can all protect kidneys against renal ischemia reperfusion-induced injury [16]. The administration of ketotifen reduced the level of TNF-α, IL-6 and downregulated the expression of ICAM-1 [5].

CONCLUSION

These results imply that antagonistic histamine effects can reduce glycerol-induced ARF injury. Demonstrated that 2 mg/kg of Ketotifen efficiently suppressed renal dysfunction and tissue injury, as histopathologic scores showed.

This study has investigated for the first time, the role of Ketotifen, by renal morphological changes, in attenuating glycerol-induced nephrotoxicity.

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