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Original article

Rosmarinic acid protection against glycerol-induced kidney damage in rats: a promising therapeutic approach for acute kidney injury

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Abstract

Objective: Rhabdomyolysis occurs when muscle fibers break down, releasing myoglobin and creatine kinase into the circulation, which can lead to acute kidney injury (AKI). Rosmarinic acid (RA) is a phytochemical that exerts inflammation-modulating and antioxidant properties. This research investigated the role of RA against AKI caused by rhabdomyolysis in rats.

Materials and methods: Six groups of male Wistar rats (n=6) were selected for the study: The control group received normal saline. Group 2 was given 50% glycerol (10 ml/kg, intramuscularly). Groups 3, 4, and 5 received different doses of RA (5, 10, and 25 mg/kg, intraperitoneally, for 4 days) with glycerol (given only on the first day), while group 6 received only RA (25 mg/kg). On day 5, serum and kidney tissues were isolated. Levels of serum creatinine (Cr) and blood urea nitrogen (BUN), renal malondialdehyde (MDA), glutathione (GSH), interleukin-1 beta (IL-1β), and lipocalin associated with neutrophil gelatinase (NGAL), and pathological alteration were evaluated.

Results: Glycerol injection induced pathological lesions and increased markers of kidney damage, such as Cr, BUN, MDA, IL-1β, and NGAL, and decreased GSH content. Administration of RA (25 mg/kg) reduced these indicators, suggesting a renoprotective role in rhabdomyolysis-induced AKI. Histopathological analysis showed increased tubular necrosis and myoglobin cast formation in the glycerol-treated group, which were diminished in the RA-treated animals. However, the decline was not statistical significant.

Conclusion: This study suggests that RA administration decreased renal disturbance in the glycerol-triggered AKI rat model through the reduction of oxidative damage and inflammatory responses.

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Introduction

Rhabdomyolysis arises in skeletal muscle injuries. It results in the leakage of cellular contents into the circulatory system. The release of cellular components can potentially damage various organs like the kidneys, liver, and heart (Văcăroiu et al. 2022). Although rhabdomyolysis is often associated with traumatic events, it can also be triggered by a range of medical conditions including severe physical activity, abrupt changes in body temperature, exposure to toxic agents, and infections (Reis et al. 2019). When muscle cells are damaged, they release their proteins and electrolytes, including myoglobin and creatine kinase (CK), into the circulatory system, which can cause symptoms like muscle weakness, cramping, and dark urine (Baeza-Trinidad 2021). Approximately 10–50% of patients with rhabdomyolysis can experience acute kidney injury (AKI) or death if left untreated. Identifying the underlying causes of rhabdomyolysis is pivotal for finding effective treatment and preventing the condition's progression (Melli et al. 2005; Bosch et al. 2009; Ward 1988). Researchers commonly use animal models of AKI caused by glycerol to study the mechanisms of rhabdomyolysis. In this model, injection of 50% glycerol into muscle tissue increases creatinine (Cr), blood urea nitrogen (BUN), and malondialdehyde (MDA) levels. It also releases myoglobin and other cellular contents into the bloodstream, leading to vascular occlusion, cell death, and inflammation, ultimately inducing AKI (Mousleh et al. 2018; Subeq et al. 2009; Plotnikov et al. 2009). The development of AKI resulting from rhabdomyolysis includes three distinct pathways: tubular obstruction, tubular damage from oxidant injury, and renal vasoconstriction (Holt and Moore 2001; Boutaud and Roberts 2011). It has recently been revealed that myoglobin redox cycling and subsequent lipid peroxidation are the primary contributors to oxidant injury in rhabdomyolysis-induced AKI. Furthermore, the lipid peroxidation catalyzed by myoglobin produces bioactive molecules, including F2-isoprostanes that induce renal vasoconstriction (Panizo et al. 2015; Boutaud et al. 2010). Myoglobin released into the bloodstream binds to Tamm-Horsfall protein, causing casts and tubule obstruction in the distal tubules, and ischemic and toxic damage in the proximal tubules due to high metabolic activity (Bosch et al. 2009). A different pathway involved in AKI caused by rhabdomyolysis is inflammation. Skeletal muscle fiber degeneration during rhabdomyolysis releases the immune-stimulating molecules. These molecules activate cytokines involved in inflammatory responses, such as IL-1β and tumor necrosis factor-alpha (TNF-α), ultimately causing kidney malfunction (Nishida et al. 2015; Grivei et al. 2020). Therefore, using natural compounds with antioxidant properties may hold promise in treating rhabdomyolysis-induced AKI by targeting inflammatory pathways and reducing oxidative stress (Tavafi et al. 2016; Palipoch 2013).

Rosmarinic acid (RA) is a phenolic substance from the Boraginaceae family (Gordo et al. 2012). Research projects proved that RA possesses anti-inflammatory (Luo et al. 2020), antioxidant (Noor et al. 2022), anti-diabetic (Ngo et al. 2018), anti-tumor (Cao et al. 2019), anti-viral (Sarkar and Das 2021), neuroprotective (Rahbardar and Hosseinzadeh 2020), and hepatoprotective (Komeili-Movahhed et al. 2022) properties. Among these, one of the significant effects of RA can be its renal protection against various toxins such as cadmium (Joardar et al. 2019) and cisplatin (Domitrović et al. 2014). The nephroprotective effect of RA is mainly due to its antioxidant properties that inhibit pro-inflammatory mediators and prevent apoptosis. Additionally, RA suppresses nuclear factor kappa B (NF-kB), a key modulator of inflammatory processes. Consequently, this causes a decrease in the levels of cytokines involved in

inflammatory responses and adhesion factors in the kidney (Domitrović et al. 2014). It also influences the mitogen-activated protein kinase (MAPK) signaling pathway which is associated with oxidative stress, inflammation, and apoptosis (Joardar et al. 2019). Moreover, RA increases renal oxidative capacity against various harmful chemical compounds through enhancing the expression of multiple antioxidants including glutathione (GSH), catalase, and superoxide dismutase (SOD). Overall, these mechanisms contribute to its protective effect against oxidative, inflammatory, and apoptotic kidney damage (Nunes et al. 2017; Tavafi and Ahmadvand 2011; Domitrović et al. 2014).

Considering the nephroprotective effects of RA, which have been proven in different studies, current research aims to examine whether RA provides protection in AKI induced through rhabdomyolysis with attention to its free-radical–scavenging and inflammation-suppressing activities.

Materials and Methods

Chemicals

RA was provided by Sigma-Aldrich, USA. Thiobarbituric acid (TBA), KCl, NaCl, glycine, sodium dodecyl sulfate (SDS), phosphoric acid, methanol, and N,N,N',N'-tetramethyl ethylenediamine (TEMED) were obtained from Merck, Germany. 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and Trichloroacetic acid (TCA) were provided by Sigma-Aldrich, Germany. Polyvinylidene difluoride (PVDF) was purchased from Bio-Rad, USA.

Experimental design

Thirty-six male Wistar rats, each weighing approximately 225 g on average, were used in this research. They were housed in an animal room at Mashhad University of Medical Sciences School of Pharmacy under controlled conditions, where they had unlimited access to food and water. The rats were maintained in a 12-hr light-dark cycle at a constant temperature of 22-25°C. The animal experiments were performed based on the rules of the Ethics Committee of Mashhad University of Medical Sciences (Ethical number: IR.MUMS.AEC.1401.074). We grouped the animals into six categories (n=6). The first group, serving as the control, was administered a single intramuscular injection and four intraperitoneal injections of normal saline. To induce kidney damage, the second set of animals was administered one intramuscular injection of a 10 ml/kg dosage of 50% glycerol, which was equally distributed between the muscles of both hind limbs (Abd-Ellatif et al. 2019). The next three groups (Groups 3–5) were designated as intervention groups; they were given glycerol in conjunction with a range of doses of RA (5, 10, and 25 mg/kg, intraperitoneally, for 4 days). The animals in the last group (Group 6) received RA without any additional intervention (25 mg/kg, IP) (Domitrović et al. 2014; Li et al. 2019) (Table 1).

Table 1. Study design

Groups	Day 1		Day 2	Day 3	Day 4	Day 5
	Zero time	½ hour later				
Control	Saline (IM)	Saline (IP)	Saline (IP)	Saline (IP)	Saline (IP)	Sacrificed
GLY 50%	GLY (IM)	-	-	-	-	
RA 5 mg/kg + GLY	GLY (IM)	RA 5 mg/kg (IP)	RA 5 mg/kg (IP)	RA 5 mg/kg (IP)	RA 5 mg/kg (IP)	
RA 10 mg/kg + GLY	GLY (IM)	RA 10 mg/kg (IP)	RA 10 mg/kg (IP)	RA 10 mg/kg (IP)	RA 10 mg/kg (IP)	
RA 25 mg/kg + GLY	GLY (IM)	RA 25 mg/kg (IP)	RA 25 mg/kg (IP)	RA 25 mg/kg (IP)	RA 25 mg/kg (IP)	
RA 25 mg/kg	-	RA 25 mg/kg (IP)	RA 25 mg/kg (IP)	RA 25 mg/kg (IP)	RA 25 mg/kg (IP)	

RA: Rosmarinic acid; GLY: Glycerol; IP: Intraperitoneal; IM: Intramuscular

Sample collection

After administering the compounds in accordance with ethical standards, ketamine-xylazine (ketamine at a 60 mg/kg dose administered alongside xylazine at 6 mg/kg) and CO_2 gas were used to reduce their consciousness before sacrificing them. Then, blood samples and tissue were collected. The right-sided kidney was preserved at a temperature of -80°C for additional assessment. Meanwhile, we maintained the left-sided kidney in 10% formalin to examine it according to pathological criteria.

Biochemical analysis

Kidney function was measured by collecting blood samples and separating the serum. The autoanalyzer was used to measure BUN and Cr levels.

Histopathological examination

The rat kidney tissues were conserved in a 10% formalin solution for histopathological analysis. The samples were subsequently embedded in paraffin and sliced into thin sections of approximately five microns. Staining of the tissue sections with hematoxylin-eosin was performed. They were later analyzed with a light microscope. The results were scored according to how severe the tubular necrosis was, and myoglobin casts were visible in the tissue. A score of 0 indicated a healthy sample with no lesions (-), while a score of 1 denoted a minor lesion affecting less than 5% of the kidney tissue section (+). A score of 2 signified lesions affecting between 5-25% of the tissue section (++), and a score of 3 corresponded to lesions covering 25-75% of the tissue section (+++). Finally, a score of 4 represented extensive lesions affecting over 75% of the examined kidney tissue section (++++) (Ramadhan et al. 2020).

Measurement of lipid peroxidation

MDA is a well-established biomarker for assessing lipid peroxidation and oxidative stress in biological systems, offering crucial data on lipid oxidation levels and damage due to free radicals (Del Rio et al. 2005). To measure MDA levels, the kidney tissue was first homogenized in a 1.15% potassium chloride (KCl) solution to make a 10% w/v homogenate. We combined 0.5 ml of this homogenate with 3 ml of 1% phosphoric acid and 1 ml of 0.6% TBA solution. After boiling and cooling this mixture, 4 ml of n-butanol was added. The sample was centrifuged, and the supernatant absorbance was read at 532 nm using a spectrophotometer. The MDA content was expressed in units of (nmol/g) (Ohkawa et al. 1979).

Determination of reduced glutathione (GSH) content

In the GSH assay, free sulfhydryl groups participate in a reaction with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) in an alkaline environment. This results in the formation of a yellow complex, which shows absorption at 412 nm. To create a 10% tissue homogenate, we initially homogenized the kidney tissue in phosphate buffer (pH 7.4). Then, the homogenate was with trichloroacetic acid (TCA) (1:1). After centrifuging, the upper phase was separated. It was then blended with 2.5 ml of a phosphate buffer (pH 8) and 500 µl of DTNB reagent. Finally, the spectrophotometer was used to measure the light absorbance at 412 nm. The GSH concentration is reported as nmol/g (Gheita and Kenawy 2014).

Western blotting

This study used previous methods to investigate the amount of IL-1β and NGAL in kidney tissue (Ramadhan et al. 2020; Del Rio et al. 2005). The protein extraction process involved degrading the kidney tissues using a buffer containing 50 mM Tris-HCl (pH 7.4), 2 mM ethylene glycol tetraacetic acid, 2 mM ethylenediaminetetraacetic acid, 1 mM Na3VO4, 10 mM NaF, 10 mM β-glycerol phosphate, 1 mM phenylmethylsulfonyl fluoride, 2 µl protease inhibitor cocktail,

10% v/v 2- mercaptoethanol, and 0.2% w/v sodium deoxycholate. We quantified the protein content using a Bio-Rad Protein Assay Kit, and then equal volumes of the supernatants were mixed with sodium dodecyl sulfate sample buffer, heated, and loaded onto 12% SDS-PAGE gels. Following protein separation and transfer, membranes were blocked with Tris-buffered saline with Tween 20 containing 5% dry skim milk for 2 hr at room temperature. Subsequently, the membranes were washed with Tris-buffered saline, 0.1% Tween 20 After running and transferring the proteins, the membranes were blocked in TBST with 5% dry skim milk for 2 hr. Next, they were rinsed with TBST and incubated with primary antibodies, including IL-1β (Abcam#9722), NGAL (Abcam#216462), and β-actin (Cell signaling, #3700S), according to standard protocols. After washing the blots with TBST, secondary antibodies with a dilution of 1:3000 were used for 2 hr at room temperature to incubate the blots. Membrane development was performed using ECL Western blotting substrate and imaged with the Alliance 4.7 gel documentation system (UK). Quantitative analysis of band intensities was conducted using UVtec software (UK) with normalization to the corresponding β-actin expression level.

Statistical analysis

The statistical software GraphPad Prism 8 was utilized for statistical calculations. Data is displayed as mean ± SD (mean ± standard deviation), and one-way ANOVA with Tukey-Kramer post-test was used to compare different groups. Histopathological results were reported as median with an interquartile range and to check for statistical differences among these results, a Kruskal-Wallis non-parametric test and Dunn's post-test were performed. A $p<0.05$ was considered statistically significant.

Results

Effect of RA on kidney functional impairment caused by glycerol

A 10 ml/kg intramuscular injection of 50% glycerol significantly raised serum Cr and BUN levels in the group that received only glycerol ($p<0.001$). In groups that were treated with 10 and 25 mg/kg doses of RA, serum Cr levels ($p<0.05$ and $p<0.01$, respectively) significantly decreased, while administration of RA 25 mg/kg reduced BUN levels ($p<0.001$). The levels of the mentioned factors remained unchanged after the administration of RA alone (25 mg/kg) (Figure 1A, B).

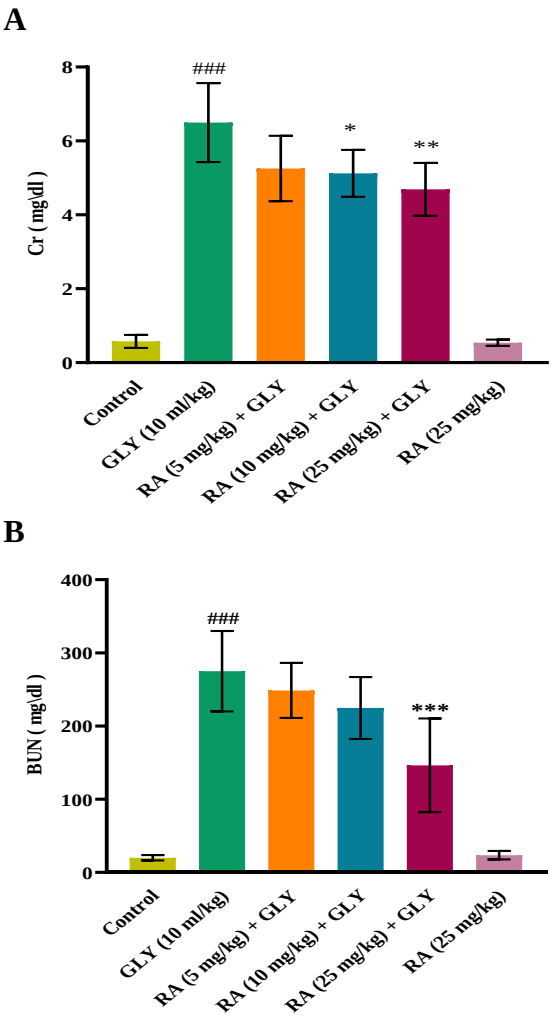

Figure 1. (A) The effect of RA on glycerol-induced changes in Cr. (B) The effect of RA on glycerol-induced changes in BUN. The data are presented as mean ± SD (n=4-6). One-way ANOVA and Post-test Tukey-Kramer were used to examine statistical differences. ###$p<0.001$ vs. control group, ***$p<0.001$, **$p<0.01$ and, *$p<0.05$ vs. glycerol group. RA: Rosmarinic acid, GLY: Glycerol

The role of RA on glycerol-induced renal tissue damage

A 10 ml/kg injection of 50% glycerol solution caused myoglobin cast formation and tubular necrosis within the renal tissue (Table 2). Compared to the group that received only glycerol, 5 and 10 mg/kg doses of RA did not significantly affect pathological alterations in the kidney. The injection of RA 25 mg/kg following glycerol injection exhibited a reduction in both tubular necrosis and myoglobin cast formation. Compared to the control group, histological examination did not reveal significant changes in the renal tissues of animals that received only RA 25 mg/kg (Table 1 and Figure 2).

Table 2. Histopathological alterations in different groups

Groups	Treatment	Myoglobin cast	tubular necrosis
1	Control	0 (0)	**0 (0)**
2	GLY(10 ml/kg)	4 (0) ###	4 (0) ###
3	GLY + RA (5 mg/kg)	3.5 (1)	3.5 (1)
4	GLY+ RA (10 mg/kg)	3 (0.5)	3 (1)
5	GLY + RA (25 mg/kg)	2 (1.25)	2 (0.5)
6	RA (25 mg/kg)	0 (0)	0 (0)

Tissue lesions including myoglobin cast and tubular necrosis in rat kidney caused by injection of 50% glycerol and RA administration. The data are median with interquartile range (n=4-6). A Kruskal-Wallis non-parametric test and Dunn's post-test were used to check the statistical differences. ### p<001 vs. control group.
RA: Rosmarinic acid, GLY: Glycerol

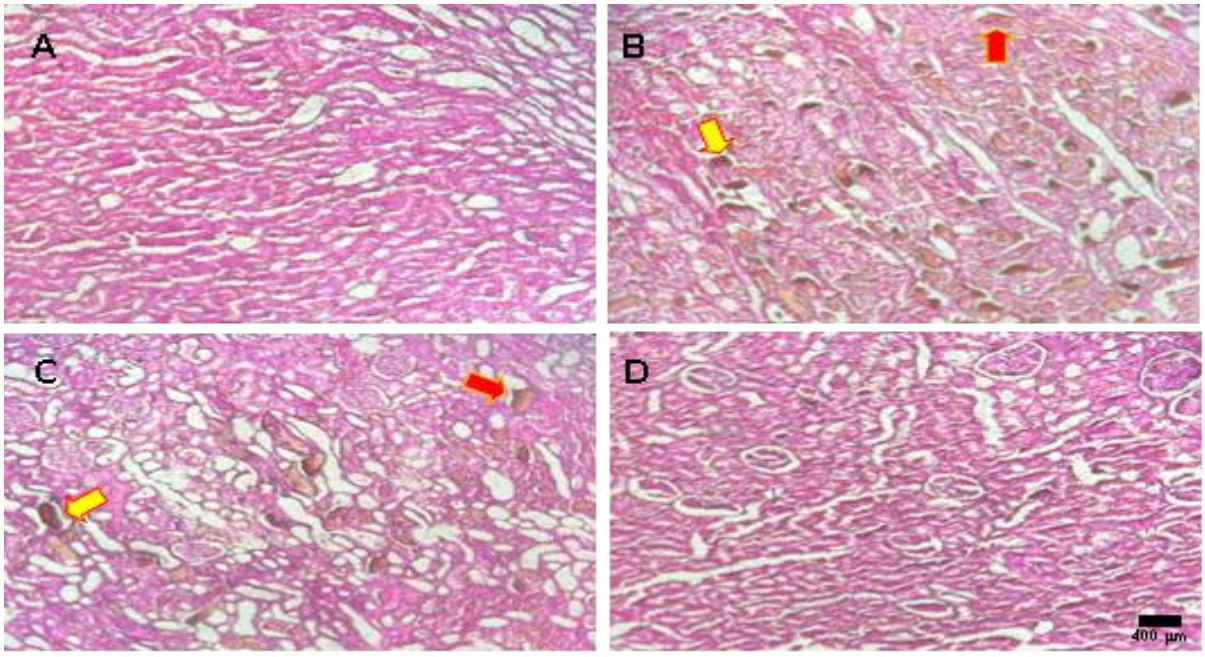

Figure 2. The impact of 50% Glycerol (10 ml/kg) and RA on renal tissue histopathology. The presented sections are representative of kidney histopathology observed through hematoxylin and eosin staining at 400x magnification. (A) control group, (B) glycerol, (C) glycerol + RA (25 mg/kg), and (D) RA (25 mg/kg). The red arrow indicates myoglobin casts and the yellow arrow denotes tubular necrosis.

Impact of RA on glycerol-induced lipid peroxidation

To evaluate lipid peroxidation in kidney tissue, we considered the amount of MDA as a crucial biomarker. Observations from the study revealed a notable increase in MDA levels following an intramuscular glycerol injection (10 ml/kg, 50%), indicating a spike in lipid peroxidation ($p<0.001$ vs control). When RA was administered at 25 and 10 mg/kg ($p<0.001$), as well as 5 mg/kg ($p<0.05$) simultaneously with glycerol, kidney MDA levels showed a considerable decrease. There was no significant alteration in the amount of MDA following treatment with 25 mg/kg of RA (Figure 3A).

Impact of glycerol and RA on renal GSH levels

The impact of a one-time 50% glycerol administration (10 ml/kg, IM) on GSH content of kidney tissue is shown in Figure 3B. The finding exhibited a notable decline in the amount of GSH ($p<0.001$) in the group that received only glycerol. However, in animals that received 25 mg/kg of RA (4 days, IP) simultaneously with glycerol, the amount of GSH increased ($p<0.01$). GSH levels in rats treated solely with RA (25 mg/kg) remained unchanged (vs control).

Impact of glycerol and RA on the level of IL-1β and NGAL proteins in renal tissue

Administration of 10 ml/kg of 50% glycerol (IM) to rats significantly elevated kidney tissue levels of IL-1β and NGAL protein ($p<0.01$). In contrast, treatment with RA (25 mg/kg, IP, for 4 days) significantly reduced the amount of these proteins compared to animals given only glycerol ($p<0.05$ for IL-1β and $p<0.01$ for NGAL). Interestingly, following administration of only RA (25 mg/kg vs the control group), the amount of IL-1β and NGAL was not changed (Figure 4).

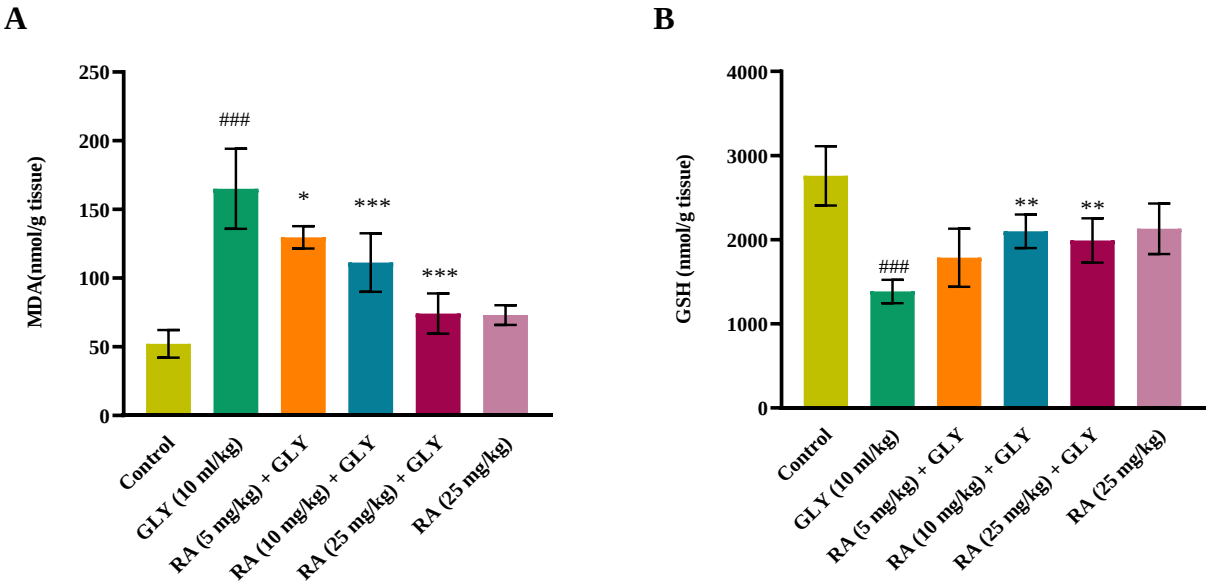

Figure 3. The effect of glycerol 50% (10 ml/kg) and RA on the levels of (A) MDA (malondialdehyde) and (B) GSH (glutathione) in kidney tissue. Data are expressed as mean±SD (n=6). One-way ANOVA and Post-test Tukey-Kramer were used to assess statistical differences. ###$p<0.001$ vs. control group, ***$p<0.001$, **$p<0.01$ and, *$p<0.05$ vs. glycerol group. RA: Rosmarinic acid, GLY: Glycerol.

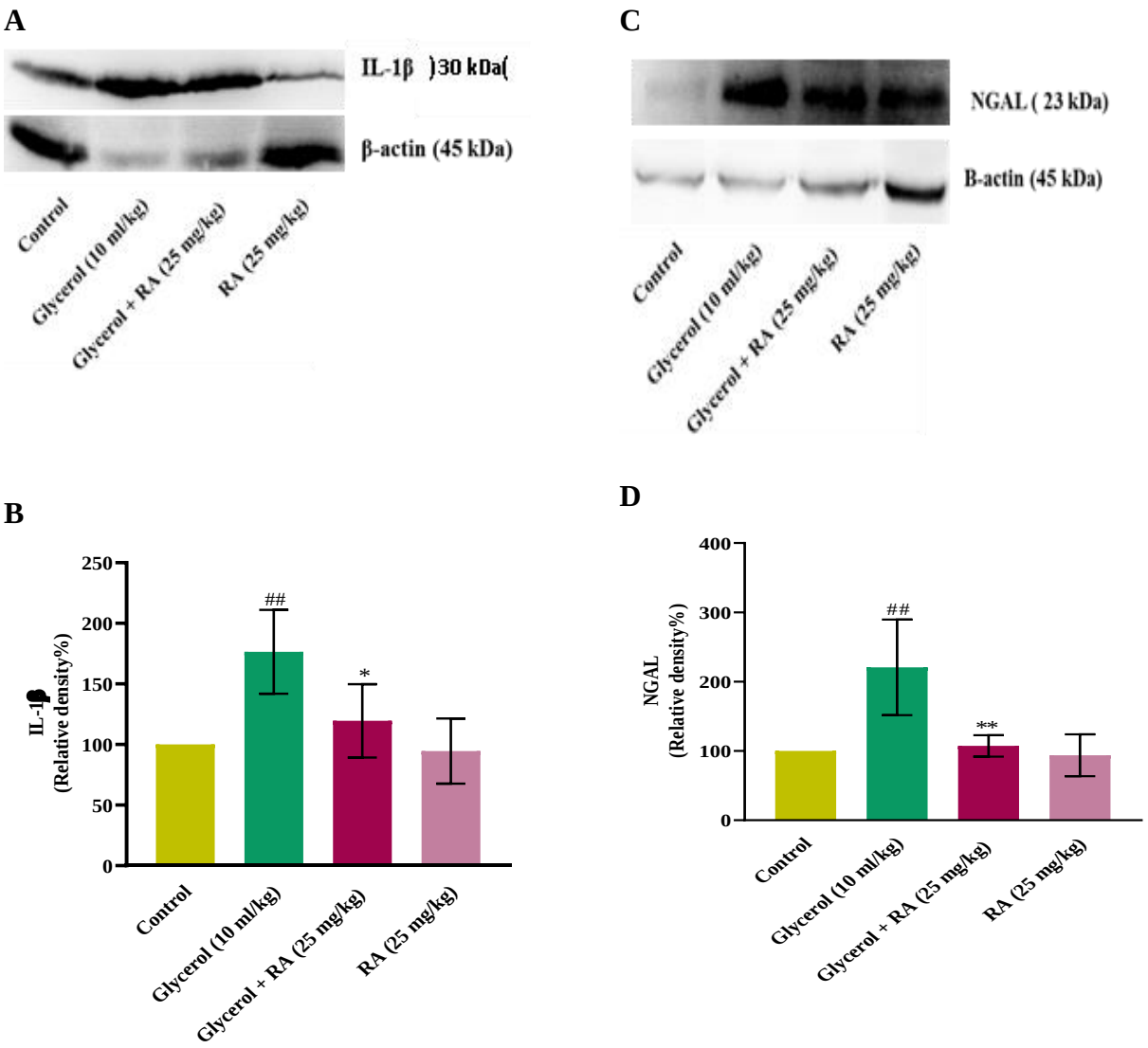

Figure 4. Effect of glycerol 50% (10 ml/kg) and RA (25 mg/kg) on IL-1β and NGAL levels in kidney tissue. (A, C) Immunoblot bands from the western blotting analysis. (B and D) Quantitative analysis of immunoblots is shown. The data is presented as mean ± SD (n=4). One-way ANOVA and Post-test Tukey–Kramer were used to examine statistical differences. ##p<0.01 vs. control group, **p<0.01 and, *p<0.05 vs. glycerol group. RA: Rosmarinic acid, GLY: Glycerol NGAL: neutrophil gelatinase-associated lipocalin.

Discussion

The current investigation assessed the nephroprotective potential of RA against AKI resulting from rhabdomyolysis in a rat model. Intramuscular glycerol injection in rats is a well-known method used for assessing rhabdomyolysis (Abd-Ellatif et al. 2019). In our study, 50% glycerol administered at 10 ml/kg increased biochemical factors, tissue injury, oxidative stress markers, and the amount of proteins, such as IL-1β and NGAL, in rat kidneys. All these changes, except histological alterations, were significantly reversed after intraperitoneal administration of RA.

In assessing nephrotoxicity, it is important to monitor changes in key markers of renal function such as serum Cr and BUN. Reductions in these markers indicate improvements in renal function following drug treatment (Toprak et al. 2008). Experiments have shown that rats that received glycerol have higher serum Cr and BUN levels, indicating potential nephrotoxic effects of glycerol (Nara et al. 2016b; Zhang et al. 2017; Yin et al. 2019). The result of this study indicated that a 10

ml/kg glycerol administration can raise serum BUN and Cr levels in kidney tissue, which is consistent with prior investigations. According to recent research, RA was found to decrease renal injury biomarkers, suggesting its reno-protective features. Zhang et al. also found that RA (20 mg/kg, for 7 days) was effective in reducing Cr and BUN levels after deltamethrin-induced renal injury (Zhang et al. 2015). Our research, consistent with previous research, demonstrated the renal protective effects of RA. Specifically, in this study, a 4-day intraperitoneal treatment with RA (10 and 25 mg/kg) significantly lowered serum BUN and Cr values.

Several studies have reported pathological damage in kidney tissue after exposure to glycerol. In a study by Yin et al., mice that received glycerol showed extensive damage, including tubule dilation, glomerular hypertrophy, and severe interstitial inflammatory infiltration (Yin et al. 2019). The histopathological observations of our research are consistent with previous surveys, demonstrating that administration of 50% glycerol (10 ml/kg, IM) causes pathological lesions in rat kidney tissue, such as tubule necrosis and the formation of myoglobin casts. Prior research has shown a reduction in kidney tissue damage with RA. According to a report, it was found that RA (1, 2, and 5 mg/kg, 2 days, orally) reduced pathological lesions, including myoglobin cast and tubular dilatation in mice suffering from kidney damage caused by cisplatin (Domitrović et al. 2014). In our investigation, although a 4-day intraperitoneal treatment with RA (5, 10, and 25 mg/kg) lowered myoglobin cast formation and tubular necrosis, these changes were not statistically significant. The discrepancy between our findings and earlier investigations could be linked to variations in the period of RA therapy and the severity of renal impairment across experimental models.

It is necessary to investigate the tissue abnormalities following rhabdomyolysis-induced AKI. The elevation of oxidative stress, inflammation, and endothelial dysfunction is notably linked to myoglobin-induced renal injury (Panizo et al. 2015). During AKI, redox imbalance can be exacerbated by glycerol administration, which increases both lipid peroxidation and proinflammatory cytokines (Nara et al. 2016a). This experiment measured the renal GSH and MDA levels in rats to assess the body antioxidant capacity. Tissue levels of MDA indirectly reflect the extent of cell damage caused by free radicals and are known as a marker for lipid peroxidation (Park et al. 2012). Our findings demonstrated that administration of 50% glycerol can elevate the amount of MDA and reduce GSH in kidney tissue. Previous studies have examined the antioxidant effects of RA by measuring MDA and GSH levels. For instance, RA (50, 100, and 200 mg/kg, 12 days, by gavage) significantly reduced MDA and GSH amount in the kidneys of rats suffering from gentamicin-induced nephrotoxicity (Tavafi and Ahmadvand 2011). Moreover, in an investigation by Zhang et al., administration of RA (200 mg/kg, for 30 days) reduced the MDA level in the kidney tissue of aging mice (Zhang et al. 2015). In the study of Tavafi et al., pretreatment with RA (100, 200, and 50 mg/kg, orally) before inducing nephrotoxic injury in rat kidney tissues resulted in a decrease in MDA levels and an elevation in the GSH values (Tavafi et al. 2019).

The pro-inflammatory response and the presence of leukocytes play a role in the pathogenesis of glycerol-induced AKI (Homsi et al. 2006; Liu et al. 2018). Research projects exhibited a substantial elevation in kidney NF-kB levels of rats following glycerol injection (Huang et al. 2017; Amirshahrokhi 2021). The activation of NF-κB is initiated by free radicals released from damaged tissues and inflammatory cells. NF-kB plays a major role in the synthesis of immune signaling

molecules, such as TNF-α, IL-1β, and IL-6, as a strong inflammatory mediator (Amirshahrokhi 2021; Huang et al. 2017; Charan and Kantharia 2013). The activity of these cytokines correlates with kidney injury. Various studies have determined that glycerol injection increases kidney IL-1β levels (Kunak et al. 2016; AlBasher et al. 2020), which is consistent with our findings. On the other hand, the anti-inflammatory effects of RA have been reported by inhibiting the activation of NF-κB and the reduction of inflammatory cytokines (Rahbardar et al. 2022). Moreover, RA (5, 10, and 20 mg/kg, IP, for 5 days) had a downregulatory effect on the expression level of IL-1β mRNA in the tissues of mice exposed to cisplatin (Xiang et al. 2022). Also, RA 100 mg/kg exhibited therapeutic effects against inflammation-induced AKI in mice (Akhter et al. 2022). In line with previous research, RA (25 mg/kg) reduced renal IL-1β expression as a result of its anti-inflammatory profile.

NGAL is classified as a stress protein within the lipocalin family. This protein is known as a valuable marker for AKI because it increases during kidney injury (Zager et al. 2013; Lippi et al. 2012). Additionally, compounds with redox-balancing effects can reduce the NGAL protein (Ozturk et al. 2017). According to published data, NGAL can increase in both renal tissue and blood samples of rats after 50% glycerol administration (Fauzi et al. 2016). In our survey, a single 10 ml/kg IM injection of 50% glycerol elevated the kidney levels of NGAL, confirming AKI. The current study showed that administration of RA (25 mg/kg) modulated the expression of the NGAL level in the kidney tissue. It could be linked to the suppression of NF-κB expression and the antioxidative activity of RA. It is important to explain that the effect of RA on the kidney levels of NGAL has not been evaluated in other studies. In this context, a 2025 study on glycerol-induced AKI reported that NGAL levels strongly correlate with both oxidative stress and inflammation, suggesting its utility as a biomarker for assessing therapeutic efficacy (Aminian et al. 2025). Thus, our finding that RA downregulated NGAL expression may further reinforce its clinical potential as a renoprotective compound.

Although previous studies have demonstrated the renoprotective effects of RA in different models of AKI including gentamicin-induced toxicity, ischemia/reperfusion injury, folic acid-induced renal toxicity and nanoparticle-based delivery systems (Gholampour et al. 2025; Li et al. 2022; Mottaghi et al. 2025; Tavafi and Ahmadvand 2011), our study provides a novel contribution by focusing on glycerol-induced rhabdomyolysis-associated AKI, a less frequently studied model that mimics myoglobin-mediated nephrotoxicity. Furthermore, we employed lower intraperitoneal doses of RA (5, 10, 25 mg/kg) compared with earlier studies using higher oral doses, and we evaluated NGAL protein expression as a valuable detector of renal damage in addition to conventional markers (BUN and Cr) and oxidative stress/inflammatory mediators. Taken together, these aspects highlight the novelty of our work and suggest that RA may exert protective effects through a broader mechanism in rhabdomyolysis-induced AKI compared with previously reported models.

RA applies its nephroprotective effect through several molecular pathways. It can suppress the NF-κB signaling pathway. As a result, upregulation of pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6, which are major contributors to AKI progression, will be inhibited (Rahbardar et al. 2022). Ra activates the Nrf2/HO-1 antioxidant pathway. Consequently, enzymes related to antioxidant defense increase, including GSH peroxidase, SOD, and catalase (Wang et al. 2020). Moreover, RA can inhibit the MAPK signaling cascade, which regulates apoptosis, oxidative stress, and inflammation in renal tissues (Joardar et al. 2019). Additionally, the NLRP3 inflammasome and the

FoxO3/NF-κB axis modulation by RA lead to renal apoptosis and inflammation reduction (Mottaghi et al. 2025). Totally, these molecular pathways suggest that RA may exert its renoprotective effect through redox balance, inflammation, and apoptotic signaling modulation, supporting its therapeutic potential in glycerol-induced AKI.

In conclusion, our research indicates that a 10 ml/kg injection of 50% glycerol leads to rhabdomyolysis and AKI in rats. Increased BUN and Cr levels, tissue damage, and oxidative stress markers support this finding. We also found that RA restores renal activity through rebalancing the amount of BUN and Cr. In addition to its antioxidant properties, RA possesses anti-inflammatory effects through the reduction of IL-1β levels. Therefore, RA appears to be a promising approach to protect against AKI induced by rhabdomyolysis (Figure 5).

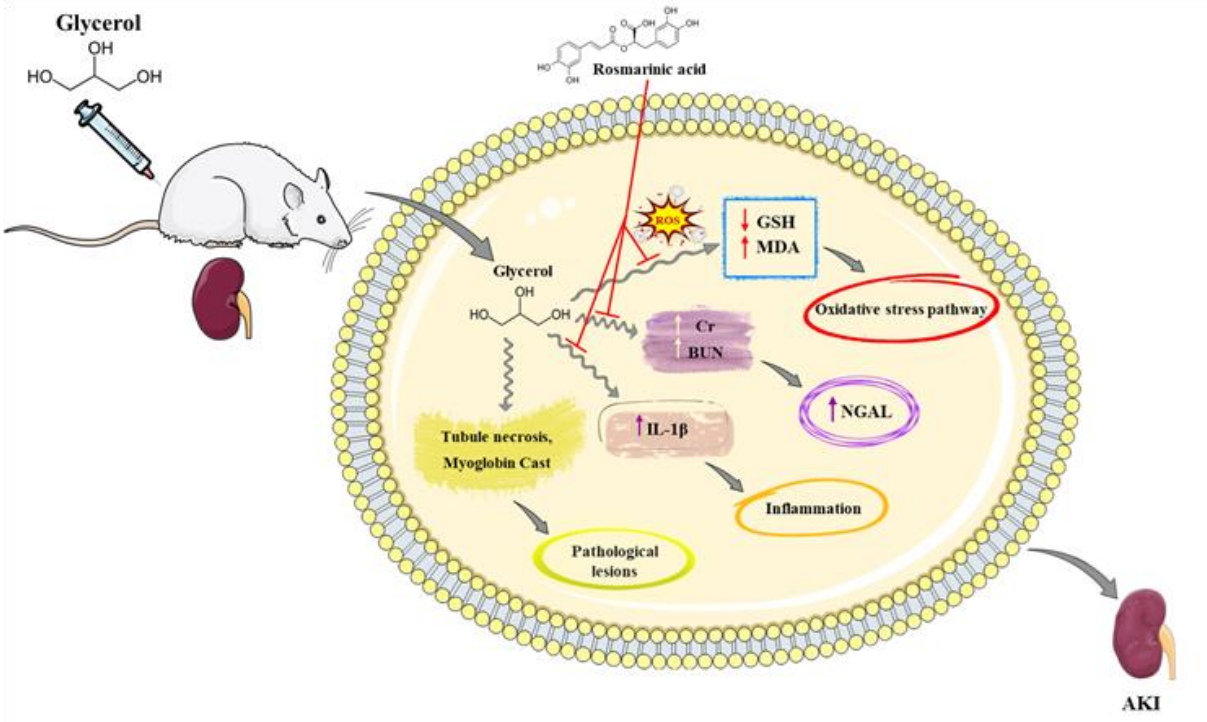

Figure 5. Simplified schematic representation of the mechanism of glycerol-induced AKI in rats and protection by RA. (Images from: smart.servier.com)

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Conflicts of interest

There are no conflicts of interest.

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Ethical Considerations

All examinations were approved by the Animal Care and Use Committee of Mashhad University of Medical Sciences and conducted in accordance with the Internationally Accepted Principles for Animal Use and Care.

Code of Ethics

The ethical number is IR.MUMS.AEC.1401.074.

Authors' Contributions

PFY performed the experiments, analyzed the data and wrote the paper. SM supervised, verified the analytical methods,

and reviewed the entire procedure and paper. TA designed the experiments and supervised. MSS performed the experiments. AJ performed histopathological examination HH conceived the original idea and supervised and reviewed the entire procedure and paper. The authors declare that all data were generated in-house and that no paper mill was used. During the preparation of this work, the authors used AI-assisted technologies to rephrase and reduce plagiarism, improving language and grammar. After using this tool/service, the authors critically reviewed and edited the content to ensure accuracy and accept full responsibility for the publication's content.

Abbreviations

RA: Rosmarinic acid, AKI: acute kidney injury, GSH: reduced glutathione, IM: intramuscular, IL-1β: interleukin-1beta, BUN: blood urea nitrogen, NGAL: neutrophil gelatinase-associated lipocalin, MDA: malondialdehyde, ARF: acute renal failure, ROS: reactive oxygen species, IP: Intraperitoneal.

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