Ameliorative Role of Diallyl Disulfide Against Glycerol-induced Nephrotoxicity in Rats

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INTRODUCTION

Rhabdomyolysis is a clinical syndrome characterized by skeletal muscle injury, which causes release of cell components such as myoglobin, electrolytes, and various sarcoplasmic enzymes into systemic circulation.[1] These released cellular contents result in renal vasoconstriction, tubular cast formation, and apoptosis, which eventually causes nephrotoxicity.[1] Clinical development of rhabdomyolysis is attributed to various reasons such as crush syndrome, exhaustive exercise, medications, infections, and toxins. Rhabdomyolysis is estimated to account for 5%–15% of total cases of acute kidney injury (AKI). In preclinical studies, rhabdomyolysis-induced nephrotoxicity is produced by intramuscular injection of glycerol, which imitates the human condition of rhabdomyolysis-induced AKI.[3]

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors of nuclear receptor superfamily having three subtypes: PPAR-α, PPAR-β/δ, and PPAR-γ. They play a vital role in energy metabolism; however, they differ in the range

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of their activity. More recently, the anti-inflammatory functions of PPAR-γ have received much attention, as its agonists have been shown to exert a broad range of protective effects in several animal models of neurological, cardiovascular, and renal diseases. The PPAR-γ agonists such as rosiglitazone and pioglitazone have protective effects against ischemia reperfusion-induced kidney injury, diabetic-hypertensive-nephropathy, experimental glomerulonephritis, and drug-induced renal injury.

Diallyl disulfide (DADS) is a natural molecule obtained from garlic and possesses anti-inflammatory and antioxidative activities. DADS has been reported as an active ingredient responsible for anti-atherosclerotic effect of garlic oil. Most recently, DADS has been reported to protect against acetaminophen and cisplatin-induced kidney injury in rodents, which primarily owes to its antioxidant and anti-inflammatory properties. Few citations available in literature suggest a crosstalk between DADS and PPAR-γ. However, the relevance of this crosstalk has never been addressed in models of kidney injury. This study investigated the role of DADS against glycerol-induced nephrotoxicity in rats. Moreover, potential involvement of PPAR-γ in DADS-mediated renoprotection is explored.

**Materials and Methods**

The animal experiments were carried out in accordance with the guidelines framed by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forests and Climate Change, Government of India. Institutional Animal Ethics Committee (IAEC) of Guru Nanak Dev University, Amritsar approved the animal protocol (226/CPCSEA/2018/12). Male Wistar albino rats (20–22 weeks) weighing 270–300 g were used in this study. Animals were housed in the Central Animal Facility of Guru Nanak Dev University, Amritsar (Registration No. 226/PO/Re/ S/2000/CPCSEA). Animal had free access to standard chow and water.

**Experimental protocol**

Forty-six animals were randomly divided into six groups. DADS was dissolved in corn oil and pioglitazone was suspended in 0.5% carboxymethyl cellulose. Bisphenol A diglycidyl ether (BADGE) was dissolved in minimum volume of ethanol and final volume was made with distilled water. Group 1 (control, n = 6): no treatment was given to rats. Group 2 (glycerol 50% w/v, 8 mL/kg, n = 8): glycerol was administered intramuscularly in hind limbs of rats to induce nephrotoxicity. After 24 h of glycerol administration, the rats were sacrificed. Group 3 (glycerol + DADS, 50 mg/kg, n = 8): DADS (50 mg/kg, p.o.) was administered for five consecutive days. On fifth day, glycerol was administered after 1 h of DADS treatment and animals were sacrificed after 24 h. Group 4 (glycerol + DADS, 100 mg/kg, n = 8): DADS (100 mg/kg, p.o.) was given for five consecutive days followed by treatment mentioned in group III. Group 5 (glycerol + pioglitazone, n = 8): pioglitazone (10 mg/kg, p.o.) was administered for five consecutive days. On fifth day, glycerol was given after 1 h of pioglitazone treatment and animals were sacrificed after 24 h. Group 6 (glycerol + DADS + BADGE, n = 8): BADGE (30 mg/kg, i.p.) was administered 1 h prior to DADS followed by treatment as mentioned in Group 3.

After respective treatments and glycerol administration, animals were placed in individual metabolic cages for a 24-h urine collection. After 24 h, the rats were anaesthetized. Blood samples were collected using retro-orbital puncture and rats were sacrificed by cervical dislocation. Serum isolated from blood was used for estimation of creatinine, urea, sodium and potassium levels. Moreover, the creatinine, sodium, and protein content in urine were estimated. Rat kidneys were removed and washed with 1.17% potassium chloride (KCl) solution. A part of renal tissue was preserved in neutral buffered formalin (NBF) for histological studies. Small portion of renal tissue was used for estimation of superoxide anion generation (SAG) and the rest of tissue was minced and homogenized in 1.17% KCl solution (10% w/v) using teflon homogenizer. Contents were centrifuged at 800× g for 10 min to remove the cellular debris and re-centrifuged at 11,000× g for 20 min. The clear supernatant was used to estimate lipid peroxides and reduced glutathione (GSH) levels in kidneys. In addition, creatine kinase (CK) was assayed in serum as markers of muscle injury.

**Quantification of kidney injury in rats**

Estimation of creatinine in serum and urine samples was done by using commercially available kits. Creatinine clearance (CrCl) was calculated by using the following formula: (CrCl = urine creatinine × urine volume / serum creatinine × 24 × 60 × animal wt). CrCl was expressed as millilitre per minute per kilogram of rat weight. Estimation of urea in serum samples was done by using commercially available kits and expressed as milligram per milliliter of serum. Potassium level was estimated in the serum sample using kit and expressed as millimole per liter of serum. Sodium level was estimated in the serum and urine samples colorimetrically. Fractional excretion of sodium (FeNa) was calculated by using formula: (FeNa = [serum sodium × urine creatinine/urine sodium × serum creatinine] × 100). Results of FeNa were expressed as percentage change in the values. Microproteins were estimated in urine samples by using colorimetric kit. Microproteinuria was calculated using formula: [microproteinuria = microproteins × urine volume] / (creatinine × 24 × 60 × animal wt). Microproteinuria was expressed as percentage.
collected in 24 h]. Results were expressed as milligram per day.

Quantification of oxidative stress in rat kidneys
Quantitative measurement of TBARS, an index of lipid peroxidation in renal tissues, was performed according to method previously described Brar et al. Results were expressed as reduced NBT picomoles per minute per milligram of tissue. GSH content of renal tissue was estimated and expressed as micromoles of reduced glutathione per milligram of protein Arora et al.

Histological studies
Renal tissues preserved in 10% NBF were dehydrated in graded concentrations of ethanol, immersed in xylene and then embedded in paraffin. Sections of 4 µm thickness were stained with hematoxylin–eosin (H&E) and periodic acid Schiff (PAS) stains using standardized protocols. Sections stained with H&E stain were evaluated under microscope (Nikon Eclipse 80i) at ×100 magnification for gross histopathological changes and neutrophil accumulation, whereas tubular brush border damage was evaluated by PAS staining.

Drugs and chemicals
DADS was purchased from Tokyo Chemical Industry, Chennai, India. BADGE was procured from Sigma Aldrich, Bangalore, India. Pioglitazone was a kind gift from Panacea Biotec, India. Glycerol was purchased from SRL, Mumbai, India. All other reagents used in the study were of analytical grade.

Statistical analysis
Results were expressed as mean ± standard error of mean (SEM). Data obtained from various groups were statistically analyzed using one-way analysis of variance followed by Tukey–Kramer post hoc test. P < 0.05 was considered to be statistically significant.

RESULTS
Mortality of two animals was observed in glycerol-treated rats. One animal died in glycerol + DADS (50 mg/kg) and glycerol + DADS + BADGE group. Data of surviving animals have been presented in the Results section.

Effect of diallyl disulfide on renal parameters
A significant increase in serum creatinine, urea, electrolytes (potassium and FeNa), and microproteinuria was observed in glycerol-treated rats as compared to control group. Glycerol administration resulted in a marked reduction in CrCl in rats. DADS (50 and 100 mg/kg) produced renoprotective effect in rats. Pioglitazone treatment attenuated glycerol-induced changes in renal parameters in rats. Prior administration of BADGE abolished DADS-induced kidney protection in rats [Figure 1].

Figure 1: Effect of DADS on renal parameters in rats. Values are expressed as mean ± SEM. *P < 0.05 vs. Control; †P < 0.05 vs. Gly; ‡P < 0.05 vs. Gly + DADS 50
**Effect of diallyl disulfide on renal oxidative stress in rats**

A significant increase in renal oxidative stress (measured as increased TBARS, SAG, and decreased GSH levels) was observed in glycerol-treated rats as compared to control group DADS significantly attenuated oxidative stress in rats, which was comparable to pioglitazone-mediated antioxidant effect. Pretreatment with BADGE abolished DADS-mediated antioxidant effect of in rats [Figure 2].

**Histological evaluation of the effect of diallyl disulfide in rat kidneys**

H&E staining of renal tissues in control group showed overall integrity of glomerulus and convoluted tubules. Various morphological changes such as mild glomerular expansion, tubular dilation, vacuolization, tubular necrosis, and accumulation of cellular debris in the lumen of tubules were shown in glycerol-treated rat kidneys. PAS staining of renal tissues highlighted the loss of brush border in renal tubules of glycerol-treated rats as compared to control ones with intact brush border. DADS and pioglitazone attenuated glycerol-induced renal histological changes in rats. Prior administration of BADGE attenuated DADS-mediated correction of histological changes of rat kidneys in glycerol-intoxicated rats [Figures 3 and 4].

**DiscuSsion**

Administration of glycerol markedly increased serum and urine biomarkers of kidney injury along with marked oxidative stress and histological changes in glycerol-intoxicated rats. Moreover, CK levels were elevated evidencing muscle injury. Our results revealed that administration of DADS is protective against glycerol-induced rhabdomyolysis in rats. DADS-mediated renoprotection was comparable to the pioglitazone in our model. In addition, prior administration of BADGE attenuated DADS-mediated protection in rats. It highlights the involvement of PPAR-γ in DADS-mediated renoprotection in rats.

Garlic has been used as folk medicine as centuries and contains a wide range of biologically active molecules accounting for its medicinal properties. Garlic possesses sulfur containing constituents such as allicin, DADS, diallyl trisulfide and S-allyl cysteine, which are noted to contribute to its antioxidant properties and anti-aging action.[18] The protective effect of DADS has been shown in various animal models such as gentamicin-induced nephrotoxicity, acetaminophen-induced renal injury, acetaminophen-induced acute hepatotoxicity, and carbon tetrachloride-induced hepatic oxidative damage.[10,11,19,20] DADS has been reported to reduce the expression of iNOS, which inhibits activated NO production in stimulated macrophages.[21] Another study has suggested that the potent antioxidant activity and anti-apoptotic effects of DADS occur via the Nrf-2-antioxidant response element pathway.[22] Glycerol induces intense muscle damage which results in release of myoglobin from skeletal

Figure 2: Effect of DADS on renal oxidative stress and creatine kinase level in rats. Values are expressed as mean ± SEM. *P < 0.05 vs. Control; †P < 0.05 vs. Gly; ‡P < 0.05 vs. Gly + DADS 50
muscles. Myoglobin is one of the key components of rhabdomyolysis-induced renal damage and results in kidney damage through renal vasoconstriction, intra-luminal cast formation, and direct heme protein-induced cytotoxicity.\textsuperscript{[23]} Myoglobin-induced lipid peroxidation leads to the formation of \textit{F}_2-isoprostanes, which further act as potent renal vasoconstrictors.\textsuperscript{[24]} Myoglobin precipitates with Tamm-Horsfall protein in the convoluted tubules thereby forming heme casts, which leads to tubular obstruction.\textsuperscript{[2]} In our study,
H&E staining revealed intense tubular damage and cast formation in glycerol-intoxicated rat kidneys which was marked attenuated by DADS treatment. PAS staining revealed glycerol led loss of brush border which was corrected by DADS in rats.

Free radicals play key role in the pathogenesis of glycerol-induced kidney damage. Glycerol-intoxication led release of myoglobin increases lipid peroxides and F2-isoprostanes, which further augment renal damage.[24] Mitochondria are the target for myoglobin and iron ions, which induce reactive oxygen species.[25] in our study, glycerol administration resulted in marked oxidative stress in renal tissues of rats shown by increase in lipid peroxides, augmented generation of superoxide anion, and decreased glutathione levels in rat kidneys. DADS treatment attenuated oxidative stress and protected the kidneys against oxidative stress. Our results are in accordance in previous findings, where DADS-mediated cyto-protection is attributed to its antioxidant activity.[7,10,11,20]

PPARs belong to the nuclear receptor super family and play a crucial role in regulating lipid metabolism. PPAR-γ agonists are particularly used to treat type 2 diabetes mellitus by restoring insulin sensitivity. Thiazolidinediones, such as pioglitazone and rosiglitazone, are selective activators of PPAR-γ and are widely used for treating diabetes. Anti-inflammatory property of PPAR-γ agonists is well documented as later inhibit production of tumor necrosis factor-α and other pro-inflammatory cytokines.[26] Pioglitazone has noted to attenuate renal damage in diabetic rats through antioxidant activity and reduction in pro-inflammatory cytokines such as nuclear factor kappa B and infiltration of macrophages in kidneys.[27] PPAR-γ mediates the activation of Nrf-2 signaling that plays a vital role in cyto-protection against oxidative stress as well as in suppression of inflammation.[28] In our study, pioglitazone effectively attenuated glycerol-induced oxidative stress and renal damage in rats. Interestingly, relation between DADS-mediated activity and PPAR-γ has been revealed. In 3L3-L1 cells, DADS treatment has been noted to increase the expression of PPAR-γ.[29] Recently, DADS administration has been reported to potentiate the anti-obesity effect of green tea by increasing PPAR-γ activity.[30] To the best of our knowledge, we are the first to report relevance of PPAR-γ in DADS-mediated protection against kidney injury. The major limitation of our study is that we did not quantify the levels of renal PPAR-γ in various groups. Moreover, the CK levels decreased with DADS treatment in rats. Therefore, further investigations are required to prove whether DADS-mediated renoprotection is due to attenuation of muscle injury or its direct renoprotective effect. Further studies are required to confirm renoprotective effect of DADS in acute as well as chronic models of kidney injury.

On the basis of our findings, it is concluded that DADS attenuates glycerol-induced renal damage in rats. In addition, PPAR-γ finds its essential involvement in DADS-mediated renoprotection in rats.

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Conflicts of interest
There are no conflicts of interest.

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