N-(2-hydroxyphenyl)acetamide and its gold nanoparticle conjugation prevent glycerol-induced acute kidney injury by attenuating inflammation and oxidative injury in mice

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Abstract
The protective activity of N-(2-hydroxyphenyl)acetamide (NA-2) and NA-2-coated gold nanoparticles (NA-2-AuNPs) in glycerol-treated model of acute kidney injury (AKI) in mice was investigated. NA-2 (50 mg/kg) and NA-2-AuNPs (30 mg/kg) were given to the animals for four days followed by 24-h water deprivation and injection of 50% glycerol (10 ml/kg im). The animals were sacrificed on the next day. Blood and kidneys were collected for biochemical investigations (urea and creatinine), histological studies (hematoxylin and eosin; and periodic acid-Schiff staining), immunohistochemistry (actin and cyclooxygenase-2, Cox-2), and real-time RT-PCR (inducible nitric oxide synthase, iNOS; nuclear factor-κB p50, NFκB; hemeoxygenase-1, HO-1; and kidney injury molecule-1, Kim-1). NA-2 protected renal tubular necrosis and inflammation, though the result of NA-2-AuNPs was better than compound alone and it also exhibited the activity at far less dose. The test compound and its gold nano-formulation decreased the levels of serum urea and creatinine level in the treated animals. Both NA-2 and NA-2-AuNPs also conserved actin cytoskeleton, and lowered COX-2 protein expression. Moreover, the mRNA expressions of iNOS and NFκB p50 were down-regulated, and HO-1 and Kim-1 genes were up-regulated. We conclude that NA-2 and NA-2-AuNPs ameliorates kidney inflammation and injury in glycerol-induced AKI animal model via anti-oxidant and anti-inflammatory mechanisms which make it a suitable candidate for further studies. We believe that these findings will contribute in the understanding of the mechanism of action of paracetamol-like drugs and can be considered for clinical research for the prevention of AKI.

Keywords Acute kidney injury · N-(2-hydroxyphenyl)acetamide · Glycerol · Rhabdomyolysis · Gold nanoparticles

Introduction
Acute kidney injury (AKI) is a major cause of mortality in the world. It can result from ischemia, drug-induced injury, inflammation, or renal obstruction [1]. Rhabdomyolysis (skeletal muscle damage) is also a leading cause of acute kidney injury which accounts for up to 50% of all cases of AKI and having mortality rate of 5–10% which is constant for several decades [2–5]. In rhabdomyolysis, free myoglobin or hemoglobin is deposited in the kidneys where they cause lipid peroxidation of S1, S2, and S3 segments of renal proximal tubular cells which further leads to the activation of inflammatory cascades [2, 6]. Currently, there is no specific treatment available except supportive treatment or kidney transplant in case of complete loss of kidney functions [4]. However, recent scientific research has found some compounds and extracts such as N-acetylcystiene,
of 7-O-Galloyl-D-sedoheptulose, L-carnitin, ascorbic acid, suramin, and hydroalcoholic extract of flowers of P. graminatum [7–16] may help in the healing of severe kidney injury in future clinical trials. Still there is an immense need to explore new preventive compounds to overcome this problem before the injury occurs due to susceptible cases.

A model of rhabdomyolysis-induced AKI is made by intramuscular injection of glycerol which damages skeletal muscles. The reported mechanism of glycerol-induced damage is that it causes oxidative injury to kidney tubular cells which further leads to tissue necrosis and inflammation [7–9,15,17,18]. NF-κB plays a major role in the activation of inflammatory process by inducing expression of several genes in which COX-2 and iNOS are the key players of inflammation [15]. Moreover, the protection of tubular damage is mediated by HO-1 and Kim-1 and the expression of these proteins may increase in response to injury [18,19].

N-(2-hydroxyphenyl)acetamide (NA-2) also known as 2-acetamidophenol is a synthetic compound and a derivative of salicylic acid. It is reported as a potent anti-inflammatory in arthritis model in vivo [20] and inducer of apoptosis in human glioblastoma cell line in vitro [21]. We selected this compound because of its anti-inflammatory activities. The chemical structure of NA-2 is shown in Fig. 1. We also synthesized the gold nanoparticles conjugated to NA-2 (i.e., NA-2-AuNPs). Property of better drug delivery to the specific site, increased bioavailability, and lower cellular toxicity are added advantages of nano-formulations [22].

Materials and methods

Synthesis of NA-2-coated gold nanoparticles (NA-2-AuNPs)

For the synthesis of NA-2-AuNPs, 1 mM solution of HAuCl₄·3H₂O was mixed with 1 mM solution of NA-2 and kept for stirring. After 20 min, 0.5 mL of NaBH₄ solution having 50 mM concentration was added, resulting in complete reduction of gold ions to form NA-2-coated AuNPs. The NA-2-AuNPs were collected after freeze drying and washed to remove the unreacted ligands and salts. The UV–Visible spectra were recorded on Thermo Scientific Evolution 300 UV–Visible spectrophotometer. The size and shape of the NA-2-AuNPs were also examined by an Atomic Force Microscope (AFM, Agilent 5500).

In vivo model of rhabdomyolysis

International guidelines and Institutional Animal Care and Use Committee (IACUC) approval for the care and use of laboratory animals (Approval Protocol No.: 2014-0002) were followed during the execution of the present study.

Male BALB/c mice were bred and maintained at the animal house facility of International Center for Chemical and Biological Sciences, University of Karachi. Animals were given free access to water and rodent chow in their conventional cages kept at 22–23 °C with 12-h light–dark cycle. Prior to the start of experiment, animals were acclimatized with the experimenter and the environment. Animals were divided into six groups and each group contained twelve (12) animals, i.e., normal control (G1), AKI model group (G2), AKI + NA-2 group (G3), AKI + NA-2-AuNPs group (G4), NA-2 only (G5), and NA-2-AuNPs only (G6) given to normal animals.

Animals in G3 and G4 were pre-treated with NA-2 (50 mg/kg) and NA-2-AuNPs (30 mg/kg) intra-peritoneal, respectively, for 4 days while animals in G1 and G2 received normal saline. The G5 and G6 were set as a control group receiving NA-2 (50 mg/kg) and NA-2-AuNPs (30 mg/kg), respectively. On day 4, all animals were kept on water
deprivation for 24 h followed by glycerol injection at the dose of 10 ml/kg body weight *intra-muscularly* except G1, G5, and G6 which received normal saline. After 24 h of glycerol injection, all animals were sacrificed humanely under anesthesia for further studies.

**Serum urea and creatinine estimation**

At the end of the experiment, blood was collected by cardiac puncture and serum was separated by centrifugation. Serum urea and creatinine were measured using a Reflotron® dry chemistry analyzer (Roche, Switzerland).

**Histology and immunohistochemistry**

Histological and immunohistochemical procedures were carried out following standard protocol. For histology, the tissue sections were stained with hematoxylin and eosin (H&E) and periodic acid Schiff’s (PAS) stain to study the morphological changes to kidney structures and to calculate the proximal tubular damaged area using NIS Element AR 3.2 software. For immunohistochemistry, primary antibodies (Actin 1:100, Santa Cruz, Sc-69879 mouse monoclonal and COX-2 1:100, Santa Cruz, Sc-23983 goat polyclonal) and secondary antibodies (Cy3 Goat anti-Mouse, Millipore, AP-124C and Cy3 Donkey anti-Goat, Jackson ImmunoResearch Laboratories 705-166-147) were used. Nucleus was stained with DAPI (1:10,000 dilutions). The slides were examined by bright-field, fluorescence, and differential interference contrast (DIC) microscope (Nikon 90i, Japan).

**Expression analysis of iNOS, NFκB p50, HO-1 and Kim-1 using real time PCR**

The mRNA was isolated from homogenized mouse kidney tissues using TRIzol reagent (Life Technologies, USA). The mRNA was then reverse transcribed into cDNA by ReverTaid first strand cDNA synthesis kit (Fermentas, USA). The primers sequences used in this study were iNOS (sense, 5′-AGG GAATCTTTGAGCGGTTTT-3′) (antisense, 5′-AGG AATGAGTGAGGCTTGG-3′), NFκB p50 (sense, 5′-TCA TGTCCACAGCCCTTCCT-3′) (antisense, 5′-CCTGCTGTT CTGTCATC-3′), HO-1 (sense, 5′-CAAGGAGTTACA CATCCAAGC-3′) (antisense, 5′-GTACAGGAAGCCCAT CACCG-3′), Kim-1 (sense, 5′-AACATCTCAGCATACATG ACTGACT-3′) (antisense, 5′-TGCTTCAGTGATTACT CCAG-3′), and GAPDH (sense, 5′-GTATGACTCCAC TCACGGCA-3′) (antisense, 5′-TCCACGACATACCTCA GCACC-3′). RT-PCR studies were conducted at 94 °C for 5 min, followed by 40 cycles at 94 °C for 5 s, 60 °C for 30 s, and 94 °C for 15 s. GAPDH was used to normalize mRNA expression, and the expression levels were calculated using the 2−ΔΔCt method.

**Statistical analysis**

Statistical analysis of data was performed using Microsoft Excel. All the values were expressed as mean ± SEM. The data were compared between or within groups by *t* test. *P* values less than 0.05 were considered statistically significant.

**Results**

**Characterization of NA-2-coated gold nanoparticles (NA-2-AuNPs)**

The UV–Visible spectrum (Fig. 1a) of NA-2-AuNPs showed absorption maxima at 544 nm indicating the formation of NA-2-AuNPs. NA-2-AuNPs were spherical in shape and size ranged from 10 to 90 nm (Fig. 1b) as determined by an Atomic Force Microscope (AFM, Agilent 5500) operated in tapping mode.

**NA-2 and NA-2-AuNPs inhibit proximal tubular necrosis and cast deposition**

H&E staining of kidney cortex and medulla is shown in Fig. 2. Normal kidney cortex and medullary structures can be seen in Fig. 2a, e. However, marked proximal tubular necrosis and inflammation were observed in animals treated with glycerol (Fig. 2b). In the same animals, the protein casts were deposited in the loop of Henle (LH) and damage to the LH cells was also observed (Fig. 2f). On the other hand, in NA-2 pre-treated animals (50 mg/kg body weight), notable inhibition of proximal tubular damage (Fig. 2c) and decreased protein casts deposition in tubules are observed (Fig. 2g). The NA-2-AuNPs pre-treated animals (30 mg/kg) showed almost complete protections against kidney injury as shown in Fig. 3d, h. When the tubular damage was calculated, the data revealed about 40% damage occurring in the AKI group as compared to the normal group (*P* < 0.001). Only about 5% and 2% (*P* < 0.001) damages were calculated in NA-2 and NA-2-AuNPs-treated groups, respectively (Fig. 3).

**NA-2 and NA-2-AuNPs decrease serum urea and creatinine levels**

Significant increase (*P* < 0.001) in serum urea and creatinine was observed in the AKI group as compared to the normal group. However, NA-2 and NA-2-AuNPs administration significantly decreased (*P* < 0.001) the level of both parameters compared to the AKI group (Fig. 4a, b).
NA-2 and NA-2-AuNPs preserve brush borders of PCT and LH

PAS staining of kidney cortex and medulla is shown in Fig. 5. The normal brush borders of PCT and LH can be seen in Fig. 5a, e, whereas the brush borders were almost destroyed in the AKI-treated group (Fig. 5b, f). The pre-treatment with NA-2 decreased the damage to brush borders (Fig. 5c, g). However, complete preservation of brush borders was observed in the NA-2-AuNPs-treated group (Fig. 5d, h).
**NA-2 and NA-2-AuNPs preserve the actin cytoskeleton**

Immunohistochemistry of the actin cytoskeleton is shown in Fig. 6a–d. Figure 6a showed normal pattern of actin, whereas the massive destruction can be seen in the AKI group (Fig. 6b). Treatment with NA-2 was observed to decrease actin destruction (Fig. 6c), whereas NA-2-AuNPs completely protected the actin cytoskeleton (Fig. 6d).

**NA-2 and NA-2-AuNPs decrease the COX-2 protein expression**

No remarkable immunostaining of COX-2 in normal kidney sections was observed (Fig. 6e). However, increased expression was seen in the AKI group (Fig. 6f), whereas NA-2- and NA-2-AuNPs-treated groups showed markedly decreased COX-2 protein expression (Fig. 6g, h).

**NA-2 and NA-2-AuNPs down-regulate the mRNA expressions of iNOS and NFκB p50 genes, and upregulate HO-1 and Kim-1**

The real-time RT-PCR data of iNOS, NFκB p50, HO-1, and Kim-1 are shown in Fig. 7. iNOS (Fig. 7a), NFκB, p50 (Fig. 7b) were significantly increased in the AKI group compared to the normal control (P < 0.001) but significantly decreased in the NA-2 and NA-2-AuNPs groups (P < 0.001). There was no significant change in HO-1 in AKI compared to the control group but the levels increased in NA-2- and NA-2-AuNPs-treated groups (P < 0.05) (Fig. 7c). Kim-1 expression was increased in AKI (P < 0.05) compared to normal control, NA-2 (P < 0.01), and NA-2-AuNPs (P < 0.05) (Fig. 7d).

**Discussion**

Most of the animal models studying AKI are based on the administration of nephrotoxic drugs, i.e., gentamycin, cisplatin, etc. [4]. However, none of these models mimic the exact pathology of AKI as that occurs in rhabdomyolysis. Glycerol-induced AKI is the model which is closest to rhabdomyolysis-induced AKI [2]. Basically, glycerol causes damage to muscles as a result of myoglobin release into the blood stream. It also causes hemolysis and hemoglobin is released in the blood. Both myoglobin and hemoglobin contain a catalytic iron metal center. When these hemoglobin and myoglobin go through the kidney, the catalytic iron of both proteins causes lipid peroxidation of the proximal tubular cells of the kidneys. Consequently, oxidative damage occurs followed by activation of inflammatory cascades...
Therefore, we used this model to study the protective effects of NA-2 and NA-2-AuNP on the mouse kidney.

In this study, we elucidated the protective effects of NA-2 and NA-2-AuNPs and found that it nicely prevented the rhabdomyolysis-induced AKI in mice. The tested compounds also protected the renal tubular damage and lowered serum urea and creatinine levels. Drug delivery is one of the important aspects of therapeutics, and adverse effects and bioavailability of drugs are the other major limiting factors. Kidney is the major organ for excretion of drugs and therefore, it is one of the targets of organ toxicity along with the liver. In this study, we wanted to prevent kidney toxicity by incorporating NA-2 gold nanoparticles in our study. Incorporation of these nanoparticles leads to decreased dose of NA-2 required for nephron protection. We also investigated the possible mechanisms underlying
the nephron protection by NA-2 which includes effects on COX-2, iNOS, NF-κB, HO-1, and Kim-1 following NA-2 treatment of animal model of AKI.

The proximal convoluted cells of the kidney have brush borders, which are responsible for increase in the reabsorption of water and other solutes filtered in the kidney glomeruli [23]. Brush borders are mainly composed of filamentous actin (F-actin) which interacts with career molecules involved in the processes of tubular secretion and reabsorption [24]. In rhabdomyolysis-induced kidney injury, the structure and permeability of the cell membrane are altered and enzymes such as endonucleases, phospholipases, and proteases are activated which degrade the actin cytoskeleton in the brush borders of proximal convoluted tubules [25]. In this study, we also elucidated the protection of actin by the tested compound. Our immunohistochemical results...
demonstrate that NA-2 protected the disruption of actin cytoskeleton in the brush borders and that the protection was more enhanced in the NA-2-AuNPs-treated group.

NF-κB is a very complex protein with diverse actions [26]. It plays an important role in the regulation of cytokine production and cell survival [27, 28]. Primarily, the NF-κB controls inflammatory process by several mechanisms to affect the duration and degree of the inflammatory reactions [29, 30]. Our findings demonstrate elevated level of NF-κB in the glycerol treated group in comparison to the normal control group. However, NA-2 and NA-2-AuNPs markedly decreased the expression level of NF-κB. NF-κB induces the expressions of iNOS and COX-2 genes [15, 31] which are key players in inflammation and responsible for initiating the inflammatory processes [15]. We observed a higher protein expression of COX-2 in the glycerol-treated group, whereas NA-2 and NA-2-AuNPs reduced the COX-2 protein expression. Likewise, iNOS is involved in the regulation of many inflammatory cytokines such as nitric oxide (NO) [31]. In the current study, we also noticed a significantly elevated mRNA expression of iNOS in glycerol-treated group. However, in NA-2- and NA-2-AuNPs-treated animals, the expression was down-regulated. This shows that the high oxidative environment in glycerol treated group is negatively modulated by the NA-2 and NA-2-AuNPs treatment.

NO induced by iNOS is responsible for the induction of Heme oxygenase-1 (HO-1) enzyme which is mainly involved in the metabolism of heme, producing iron, biliverdin, and carbon monoxide as products [32]. Stress factors also induce HO-1 where it is known to play a protective role by anti-inflammatory and immunomodulatory functions. It also plays an important role in the regulation of inflammation where it inhibits inflammation by upregulation of interleukin-10 and interleukin-1R antagonist expression [33]. HO-1 also modulates the immune system against inflammatory diseases [19]. In our study, it was found to be significantly increased in NA-2 and NA-2-AuNPs-treated groups compared to normal control and diseases group for AKI animals.

One of the recent molecules identified for the characterization of renal tubular cell injury marker is kidney injury molecule-1 (Kim-1) [34, 35]. In inflammatory conditions, expression of Kim-1 is found to be increased. In our study, expression of Kim-1 in glycerol-treated group is also found to be increased as compared to the normal control which is in accordance to the previous studies.

![Effects of NA-2 and NA-2-AuNPs on iNOS, NF-κB, HO-1, and Kim-1 mRNA expressions. NA-2 and NA-2-AuNPs attenuate mRNA expression of iNOS (a) and NF-κB (b) while both increases the mRNA expression of HO-1 (c) and Kim-1 (d) (*P<0.05, **P<0.01, ***P<0.001)](image-url)
Findings in our study suggest a role of NA-2 in nephron protection in glycerol-induced model of AKI mainly by producing anti-inflammatory and anti-oxidant activities. The compound NA-2 also known as 2-acetamidophenol is structurally similar to the well-known drug paracetamol and to some extent to aspirin as shown in Fig. 1. NA-2 has a hydroxyl (OH) group in the 2 position (ortho), whereas paracetamol has the hydroxyl group in the 4 position (para) of the benzene ring. The para prefix in paracetamol is because of this para position of the OH group. This structural similarity suggests that the mechanism of action of NA-2 might be similar to paracetamol and aspirin. Therefore, we believe that the present study will make valuable contributions in the understanding of the mechanism of action of paracetamol-like drugs.

**Conclusion**

NA-2 protects rhabdomyolysis-induced kidney injury by lowering serum urea and creatinine levels. Its gold nanoparticles conjugation (NA-2-AuNPs) protects kidney structures very well at a low dose as compared to NA-2 alone. The test compound and its nano-formulation nicely preserved kidney tubular brush borders and actin cytoskeleton. The mechanisms by which these compounds protect the kidney appears to be by under-expressing COX-2 genes, and thus protecting from inflammation and oxidant injury. They also down-regulate mRNA expression of NF-κB and iNOS and upregulate HO-1 and Kim-1 expression. We hope that these compounds may be considered for further studies and clinical research.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflicts of interest.

**References**