

Efficacy of Co-administration of Garlic Extract and Metformin for Prevention of Gentamicin–Renal Toxicity in Wistar Rats: A Biochemical Study

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ABSTRACT

Background: Gentamicin (GM) nephrotoxicity has been related to oxidative stress. Garlic and metformin (MF) have anti-oxidant activity and therefore, this study was aimed to evaluate the preventive and curative effects of garlic, MF and their combination on GM induced tubular toxicity in Wistar rats.

Methods: In a pre-clinical study, 70 male Wistar rats were randomly designated into 7 groups of 10 and treated as follows: Group 1: Received saline for 20 days. Group 2: Were injected 100 mg/kg/d of GM intraperitoneally (ip), for 10 days and saline for 10 more days. Group 3: Received GM for 10 days then 20 mg/kg garlic ip for the next 10 days. Group 4: Received GM for 10 days and MF (100 mg/kg) orally for the next 10 days. Group 5: Received GM for 10 days and a combination of MF and garlic for the next 10 days (100 and 20 mg/kg, respectively). Group 6: The same as group 5 but with half-doses of MF and Garlic. Group 7: Received GM for 10 days together with a combination of MF and garlic. On 20th day of the experiment the serum blood urea nitrogen (BUN) and creatinine (Cr) were measured and compared in different groups.

Results: GM injection significantly increased the serum BUN and Cr ($P < 0.05$). Administration of MF, garlic or their combination with or after injection of GM (high doses) could attenuate BUN and Cr.

Conclusions: The results indicate that MF and garlic or their combination have curative and protective activity against GM nephrotoxicity.

Keywords: Garlic, gentamicin, metformin, nephrotoxicity

INTRODUCTION

In contrast to its nephrotoxicity, the aminoglycoside antibiotic gentamicin (GM) is still considered to be an important agent against life-threatening infections.^[1,2] During the last decade, the goal of reducing or protecting against its nephrotoxicity has attracted much effort and attention.^[1-3] The pathogenesis of GM-nephrotoxicity has shown the involvement of oxygen free radicals, and some of free radical scavengers have been shown to ameliorate the nephrotoxicity.^[1-5] Herbal therapies are used by

a substantial proportion of people in the world.^[6] Herbs are often administered in combination with therapeutic drugs, which may raise the potential of herb-drug antioxidant activity. Indeed recent trends in controlling and treating diseases tend to favor natural antioxidant compounds rather than synthetic ones.^[6,7] Garlic, is a commonly worldwide used food, and its medical properties have been well-recognized since the ancient times.^[8] Garlic is known for its properties, as an antioxidant against free radicals.^[8,9] Apart from superiorities of Metformin (MF) to other anti-diabetic drugs,^[10,11] various investigations strongly suggests that this antidiabetic agent prevents oxidative stress-induced death in several cell types through a mechanism dependent on the mitochondrial permeability transition pore (PTP) opening.^[10-13] Thus, MF may afford protection against GM-induced tubular injury by affecting the mitochondria through a mechanism dependent on the mitochondrial PTP opening.^[12-21] Due to the relative safety and effectiveness of antioxidant agents, they seem to be good candidates for testing in human. Furthermore, co-administration of herbal medicines with synthetic drugs is very common, and may potentiate their antioxidant properties. However, their combination effects need testing. Therefore, we investigated the effect of garlic juice and MF co-administration in attenuation of GM induced tubular toxicity in Wistar rats.

METHODS

Drugs and chemicals

MF Hexal, Germany was supplied as white-powder, dissolved in distilled water freshly, as an aqueous solution to be given as a single daily oral dose of 100 mg/kg/day.^[17] GM treatment protocol used in the present study has been reported in a previous study.^[22]

Garlic extract preparation

Fresh garlic was purchased at the peak of their maturity from a local grower in Hamadan, Iran, in May 2011. The garlics were cleaned, separately chopped, crushed and then macerated with 96% ethanol for 48 h. The debris was removed by centrifugation at 200 g for 5 min. The supernatant was then filtered and rotary-evaporated at 40°C. The extract was frozen and stored at -20°C. The

frozen extract was reconstituted with normal saline to prepare final concentration when needed.^[23]

Determination of total flavonoids

The amount of total flavonoids in the garlic extract was determined colorimetrically using the method of Shirzad and coworkers^[23,24] with minor modification. In this method, 0.5 mL of garlic extract or rutin (standard flavonoid compound) was mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. Then it was left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm prepared using rutin solutions at concentrations of 25-500 ppm in methanol. The experiment was repeated in triplicate. Total flavonoids were expressed in terms of rutin equivalents (in mg/g).

Determination of total phenolic compounds

The amount of total phenolic compounds in the garlic extract was determined calorimetrically using the Folin–Ciocalteu reagent described by Bahmani *et al.*,^[25] with minor modification. In brief, 5 mL of garlic extract or gallic acid (standard phenolic compound) was mixed with Folin–Ciocalteu reagent (1:10 diluted with distilled water) and aqueous Na₂CO₃ (4 mL, 1 M). The mixture was allowed to stand for 15 min, and the total phenols were determined by colorimetry at 765 nm. A standard curve was prepared using 0, 50, 100, 150, 200, and 250 mg/L solutions of gallic acid in methanol: water (50:50, vol/vol). Total phenol values were expressed in terms of gallic acid equivalent (in mg/g). The experiment was repeated in triplicate.

Determination of antioxidant activity

The ferric thiocyanate method was employed to evaluate antioxidant activity of the extract.^[26] In a suitable vial, 500 µg of the extract was dissolved in ethanol and added to a reaction mixture containing 2.88 mL of 2.5% linoleic acid and 9 mL of 40 mM phosphate buffer. The vial was incubated at 40°C for 96 h. Every 12 h (during incubation), 0.1 mL of the vial content was diluted with 9.7 mL of 75% ethanol, 0.1 mL of ammonium thiocyanate, and 0.1 mL of FeCl₂. The absorbance of sample was measured at 500 nm, and the percentage inhibition (the capacity to inhibit the peroxide

formation in linoleic acid) was determined using the following equation;

Percentage of inhibition = $(1 - [\text{absorbance of sample} / \text{absorbance of control}]) \times 100$

A high inhibition percentage indicates a high antioxidant activity. Ethanol within the sample and without reagents was used as the negative control.

Allicin determination

Allicin content was measured in garlic extract using the method of Miron *et al.*^[25] In brief, 200 mg of extract was added to 1.0 mL (final volume) of 2-nitro-5-thiobenzoate (1.2×10^{-4} M) in 50 mM sodium phosphate and 1 mM ethylenediamine tetraacetic acid (pH 7.2). The decrease in optical density at 412 nm was determined after a 30 min incubation at room temperature.^[23] The concentration (C) of allicin was calculated according to the following equation:

$$C_{\text{allicin}} \text{ (mg/mL)} = \frac{\Delta A^{412} \times 162}{28,300} \\ = \Delta A^{412} \times 5.72 \times 10^{-3}$$

ΔA_{412} in the above formula is the decrease in optical density compared with the initial absorption at 412 nm.

Animals

Study samples included 70 male Wistar rats with a weight range of 200-250 g. The rats were purchased from Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. All animals were similarly handled in the animal house of the research center, had free access to food and water. They were housed at a controlled temperature ($25^\circ\text{C} \pm 3^\circ\text{C}$) and humidity (50-60%) environment with a 12 h dark-light cycle (lights on at 7 AM) and allowed free access to pelleted diet and tap water. Their general health state and activity were monitored closely during the experiment. The animal experimentation was conducted in accordance with the National Institute of Health guide for the careful use of laboratory animals.

Experimental design

The animals were divided into seven groups (10 rats each) as follows:

Group 1: Received saline for 20 days. Group 2: Were injected 100 mg/kg/d of GM intraperitoneally (ip), for 10 days and saline for 10 more days. Group 3: Received

GM for 10 days then 20 mg/kg garlic ip for the next 10 days. Group 4: Received GM for 10 days and MF (100 mg/kg) orally for the next 10 days. Group 5: Received GM for 10 days and a combination of MF and garlic for the next 10 days (100 and 20 mg/kg, respectively). Group 6: The same as group 5 but with half-doses of MF and Garlic. Group 7: Received GM for 10 days together with a combination of MF and garlic.

In the first day (before experiment) and on the final day (20th day), serum samples were obtained to measure blood urea nitrogen (BUN), serum creatinine (Cr) for all rats. Rats were sacrificed by injecting ketamine (ip) under general anesthesia.

Determination of serum blood urea nitrogen and Cr levels

BUN and Cr levels were measured using colorimetric method employing commercial kits by an auto analyzer (BT 300, Japan).

Statistical analysis

Data were expressed as mean \pm SEM. One way ANOVA was applied to compare the serum BUN and Cr levels between the groups. Values of $P < 0.05$ were considered statistically significant.

RESULTS

The effect of metformin on BUN and Cr levels

The data for serum levels of BUN and Cr are demonstrated in Figure 1. No significant differences were observed before the experiment.

The levels of BUN and Cr in group II which received GM were significantly higher than the ones in group 1, after the experiment ($P < 0.05$). The results indicate that GM injection induced nephrotoxicity in animals. The levels of BUN and Cr. in groups which received garlic or MF were significantly lesser than group 2 which receive GM. Co-administration of high-doses of MF and garlic vanished the induced nephrotoxicity. However, their low-doses had no effect on GM nephrotoxicity.

DISCUSSION

The main objective of this research was to determine the protective and curative role of garlic, MF and combination of both on GM induced

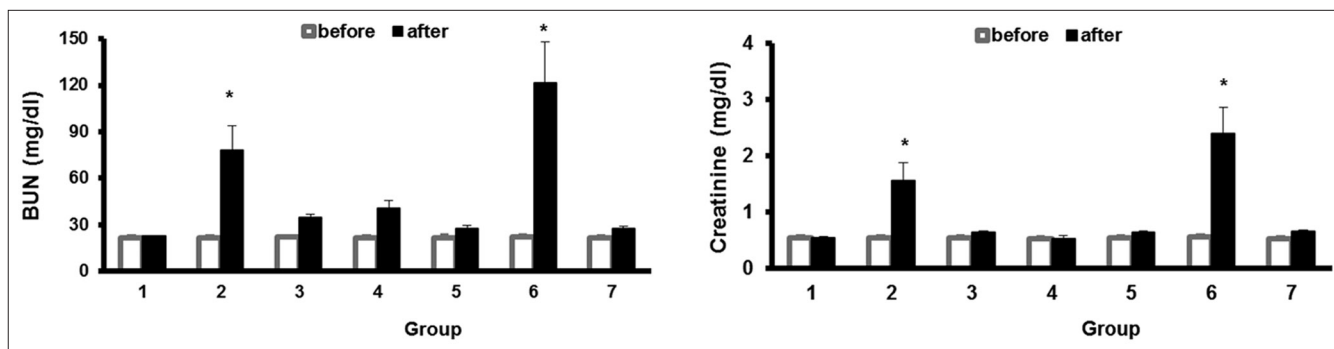


Figure 1: The serum levels of BUN and creatinine before and after the experiment in seven groups of animals. Group 1, sham group; group 2, positive control group treated with gentamicin (GM); group 3 treated with GM for 10 days and post-treatment with garlic extract intraperitoneally (ip) for next 10 days; group 4 treated with GM for 10 days and post-treatment with MF (oral) for next 10 days; group 5, treated with GM for 10 days and post-treatment with combination of MF (oral) and garlic extract (ip) for the next 10 day (100 and 20 mg/kg, respectively); group 6, treated with GM and combination of low dose (50 and 10 mg/kg, respectively); of MF (oral) and garlic extract (ip) for 10 days, and group 7, treated with GM and combination of high dose of MF (oral) and garlic extract (ip) for 10 days. The symbol (*) stands for significant difference from other groups ($P < 0.05$)

nephrotoxicity. The findings indicate that 10 days post-treatment with garlic, MF, or combination of both after 10 days of GM administration, the serum levels of BUN and Cr reduced significantly ($P < 0.05$), when compared with GM treated group. Similar result was observed when co-administration of GM, MF and garlic was applied for 10 days. In this group of animals, the serum levels of BUN and Cr were also reduced statistically ($P < 0.05$) when compared with the positive control group. When we administered concurrently MF and garlic juice with GM, they could prevent GM induced nephrotoxicity. These results indicate that MF and garlic have curative effect other than protective activity. The aminoglycoside antibiotic GM is still widely used against infections by Gram-positive and Gram-negative aerobic bacteria. However, its use has been limited due to renal impairment that occurs in up to 30% of treated patients.^[1,2] Moreover, GM has been widely used as a model to study renal failure in experimental animals.^[3] The drug may accumulate in epithelial tubular cells causing a range of effects starting with loss of the brush border in epithelial cells and ending in overt tubular necrosis, activation of apoptosis, and massive proteolysis. GM also causes cell death by generation of free radicals, phospholipidosis, extracellular calcium-sensing receptor stimulation and energetic catastrophe, reduced renal blood flow, and inflammation.^[1-5] Various drugs or antioxidants have been shown to ameliorate GM-nephrotoxicity. Because of their relative safety and effectiveness, antioxidant agents seem to be good candidates for

testing in human. To the best of our knowledge, this is the first study in which the combination of MF and Garlic extract was applied for the treatment of GM-tubular toxicity. Garlic is known for its antioxidant properties against free radicals.^[27] The protective effect of the garlic-derived antioxidant S-allylcysteine on renal injury and oxidative stress induced by ischemia and reperfusion was shown by Segoviano-Murillo *et al.*^[28] The results of this study showed that garlic possess high level of antioxidant activity. In another study, Pedraza-Chaverrí *et al.* showed that S-allylmercaptocysteine (one of the water soluble organo-sulfur compounds found in garlic extract scavenges hydroxyl radical *in vitro* and attenuates GM-induced oxidative and nitrosative stress and renal damage *in vivo*.^[29] MF is used for the treatment of diabetes as a sugar-lowering agent.^[18,21] MF exerts its metabolic activity through the induction of the adenosine monophosphate-activated protein kinase (AMPK) pathway, which acts as a sensor detecting variations of intracellular energy levels.^[21] Alterations in epithelial cell polarity and in the subcellular distributions of epithelial ion transport proteins are key molecular consequences of acute kidney injury and intracellular energy depletion.^[30] AMPK, a cellular energy sensor, is rapidly activated in response to renal ischemia, and AMPK activity may influence the maintenance or recovery of epithelial cell organization in mammalian renal epithelial cells subjected to energy depletion.^[31-34] At a molecular level, energy deprivation causes key energy-dependent membrane proteins to become

displaced and dysfunctional.^[30,31] Specially, in the proximal tubule, the Na, K, ATPase (Adenosin triphosphatase) is internalized from the basolateral membrane, disrupting the cell's capacity to maintain normal transepithelial sodium transport.^[12,18,34-36] Preservation of a polarized plasma membrane distribution of Na, K, ATPase in renal epithelia is essential for the maintenance of both solute reabsorption and volume homeostasis. It was shown that Na, K, ATPase becomes mislocalized after energy deprivation.^[18,37-43] ATP depletion also perturbs the distribution of tight junction proteins, further disrupting epithelial cell polarity and organization^[12,18,36] and leading to back leak of extracellular fluid into the urinary space. Such molecular insults result in accumulation of potentially harmful toxins.^[1] MF activates AMPK in rat kidney lysates.^[41-43] MF treatment increases detectable *p*-AMPK in a dose-dependent manner and MF-induced AMPK activation occurs in proximal tubules as well as in distal segments.^[41-43] Mitochondria represents one of the major cellular sources of reactive oxygen species (ROS) generation,^[10,43-50] and mitochondrial toxicity can also be mediated by ROS. ROS are normally produced at low levels by mitochondria themselves. However, under pathological conditions, the intracellular and intramitochondrial ROS content may be amplified.^[10,43-50] In accordance with our laboratory results, prevention of histologic changes due to GM toxicity by MF was shown by Morales *et al.*^[18] They found that control and MF-treated rats showed no structural alterations in renal tissues, while massive and diffuse cell necrosis was observed in the proximal tubules of kidneys from rats injected with GM.^[18] Similar results were obtained in our study.

CONCLUSIONS

The results of our study showed that Garlic extract could safely be used together with MF to increase the antioxidant potency to ameliorate GM-tubular toxicity.

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