Lycopene Attenuates Colistin-Induced Nephrotoxicity in Mice via Activation of the Nrf2/HO-1 Pathway

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Nephrotoxicity is the major dose-limiting factor for the clinical use of colistin against multidrug-resistant (MDR) Gram-negative bacteria. This study aimed to investigate the protective effect of lycopene on colistin-induced nephrotoxicity in a mouse model. Fifty mice were randomly divided into 5 groups: the control group (saline solution), the lycopene group (20 mg/kg of body weight/day administered orally), the colistin group (15 mg/kg/day administered intravenously), the colistin (15 mg/kg/day) plus lycopene (5 mg/kg/day) group, and the colistin (15 mg/kg/day) plus lycopene (20 mg/kg/day) group; all mice were treated for 7 days. At 12 h after the last dose, blood was collected for measurements of blood urea nitrogen (BUN) and serum creatinine levels. The kidney tissue samples were obtained for examination of biomarkers of oxidative stress and apoptosis, histopathological assessment, and quantitative reverse transcription-PCR (qRT-PCR) analysis. Colistin treatment significantly increased concentrations of BUN and serum creatinine, tubular apoptosis/necrosis, lipid peroxidation, and heme oxygenase 1 (HO-1) activity, while the treatment decreased the levels of endogenous antioxidant biomarkers glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD). Notably, the changes in the levels of all biomarkers were attenuated in the kidneys of mice treated with colistin by lycopene (5 or 20 mg/kg). Lycopene treatment, especially in the colistin plus lycopene (20 mg/kg) group, significantly downregulated the expression of NF-κB mRNA (P < 0.01) but upregulated the expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and HO-1 mRNA (both P < 0.01) in the kidney compared with the results seen with the colistin group. Our data demonstrated that coadministration of 20 mg/kg/day lycopene can protect against colistin-induced nephrotoxicity in mice. This effect may be attributed to the antioxidative property of lycopene and its ability to activate the Nrf2/HO-1 pathway.

Over the past 2 decades, there has been a pronounced increase in the emergence of Gram-negative “superbugs” (1). This has led to serious infections that are resistant to almost all clinically available antibiotics. Such a dire situation is perpetuated by a lack of novel antibiotics in the drug developmental pipeline, leaving the world in a vulnerable state with respect to these life-threatening infections (1). The dry antibacterial drug development pipeline has led to the revival of the polymyxin class of antibiotics, colistin (i.e., polymyxin E) and polymyxin B, as a last-line defense for treatment of infections caused by multidrug-resistant (MDR) Gram-negative pathogens (2, 3). However, the use of polymyxins has largely been limited by nephrotoxicity, the major dose-limiting factor occurring in up to 60% of patients (4–6). As a result, development of nephroprotective agents is very important for optimizing clinical use of polymyxins.

Polymyxin-induced nephrotoxicity potentially arises from the manner in which the antibiotics are handled by the renal system (7–9). For both colistin and polymyxin B, only a very minor proportion of the dose is renally eliminated (8, 10); the majority appears to undergo extensive reabsorption after being filtered by glomeruli (8), leading to accumulation in renal tubular cells and subsequently to apoptosis and tubular damage (6, 7, 10–12). Recent studies from our laboratory have demonstrated that polymyxin-induced apoptosis in renal tubular cells appears to be mediated by oxidative stress (11, 13). Reactive oxygen species (ROS) molecules are mainly generated by mitochondria and are among the major mediators of oxidative stress; they initiate renal cell apoptosis, which finally leads to renal dysfunction (14, 15). ROS-mediated oxidative stress plays a key role in colistin-induced nephrotoxicity (12, 16–18).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a critical transcription factor that regulates antioxidant genes by binding to antioxidant response elements (AREs) (19–22). Nrf2 activation promotes the expression of several phase II and antioxidative enzymes such as the heme oxygenase 1 (HO-1), which protects the cell against apoptosis. The cytoprotection of the HO-1 isozyme depends on its ability to catabolize free heme, thereby preventing it from sensitizing cells to undergo apoptosis (23). Notably, Nrf2 is particularly active in tissues (e.g., kidney tissue) that are susceptible to oxidative stress from exposure to xenobiotics (24, 25). Under nominal cellular conditions, the transcriptional activity of Nrf2 is suppressed, as it remains bound by Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm (26). However, when the cell experiences oxidative stress, Nrf2 becomes dissociated from Keap1 and translocates to the nucleus, where it induces expression of ARE-dependent target genes. The Nrf2/HO-1 pathway plays an important role in the nephrotoxicity of antibiotics and toxins, including gentamicin, cisplatin, Adriamycin, and manganese (22, 26–30).
Lycopene is a carotenoid compound (Fig. 1) and acts as a highly efficient antioxidant in scavenging singlet-oxygen and free radicals (31–35). Lycopene has been shown to activate the Nrf2/HO-1 pathway and can protect against nephrotoxicity induced by gentamicin, Adriamycin, and cisplatin (28, 31, 36–39). Lycopene displays antioxidant and anti-inflammatory effects; in one clinical trial, the data suggested that it may play an important role in protection against various chronic diseases (e.g., cancer) (40). In the present study, we investigated the nephroprotective effect of lycopene on polymyxin-induced nephrotoxicity and the involvement of the Nrf2/HO-1 pathway in a mouse model.

MATERIALS AND METHODS

Chemicals. Colistin (sulfate) was purchased from Zhejiang Shenghua Biotechnology Co., Ltd. (Zhengjiang, China) (20,400 units/mg). Lycopene was obtained from the Pure Biochemical Technology Co., Ltd. (Shanghai, China). Hemin, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were from Sigma-Aldrich (St. Louis, MO, USA).

Animal experiments. This animal study was approved by the Institutional Animal Care and Use Committee at the China Agricultural University. Adult Kunming mice (female, 6 to 8 weeks of age, 18 to 22 g) were obtained from Vital River Animal Technology Co., Ltd. (Beijing, China). The animal laboratory was maintained at approximately 22°C and 50% relative humidity with a 12-h light-dark cycle. An acclimation period of 1 week was employed prior to the experiments. Mice had free access to food and water during the experiments. Fifty mice were randomly divided into five groups (n = 10 in each group): saline solution (control group), lycopene administered at 20 mg/kg of body weight/day (lycopene group), colistin at 15 mg/kg/day (colistin group), colistin at 15 mg/kg/day plus lycopene at 5 mg/kg/day (colistin/lycopene 5 group), and colistin at 15 mg/kg/day plus lycopene at 20 mg/kg/day (colistin/lycopene 20 group). For colistin (sulfate) administration, mice were intravenously injected at 15 mg/kg/day in two doses via a 3-min infusion. In the colistin plus lycopene group at 5 mg/kg or 20 mg/kg, groups, mice were orally administered 5 or 20 mg/kg/day lycopene, respectively, at 2 h before administration of intravenous colistin (15 mg/kg/day). Mice in the control group were given an equal volume of saline solution. All mice were treated for 7 days, and blood samples and kidneys were collected at 12 h after the last dose for biochemical, histopathological, and gene expression studies described below.

Biochemical analyses. Blood samples were centrifuged at 3,000 × g for 10 min for biochemical analysis. Blood urea nitrogen (BUN) and creatinine levels were analyzed using commercial kits according to the manufacturer’s instructions (Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China).

Part of kidney tissue was homogenized in 9 volumes of cold Tris buffer (0.01 M Tris-HCl, 0.1 mM EDTA-Na2, 0.01 M sucrose, 0.9% saline solution, pH 7.4) to prepare a 10% tissue homogenate and then centrifuged at 3,000 × g (4°C) for 15 min. The supernatant was collected for measuring the concentrations of malondialdehyde (MDA), nitric oxide (NO), catalase (CAT), inducible nitric oxide synthase (iNOS), glutathione (GSH), and superoxide dismutase (SOD) using commercial kits (Nanjing Jiancheng Institute of Biological Engineering). Protein concentrations in the supernatant were measured using the bicinchoninic acid (BCA) protein assay (Wuhan Boster Bio-engineering Limited Co., Wuhan, China).

Histopathological examination. For the light microscopy histological examination, the right kidneys were randomly selected from 4 mice in each group and fixed in 10% neutral buffered formalin. The formalin-fixed tissue was embedded in paraffin, divided into 4-μm sections, and stained with hematoxylin-eosin (H&E). A semiquantitative evaluation of kidney injury was conducted, and a semiquantitative score (SQS) was employed to grade the lesion severity for each kidney sample. Scores of 0, +1, +2, +3, +4, and +5 corresponded to no change, mild change, mild to moderate change, moderate change, moderate to severe change, and severe change, respectively (12).

Activities of caspase-3, caspase-9, and HO-1. The activities of caspase-3 and caspase-9 in the kidney were determined using commercial kits (Beyotime Bioengineering Institute Beijing, China). Total HO-1 activity was measured as described previously (41). In brief, reactions were performed in a 1.2-ml reaction mixture consisting of 0.5 mg protein from tissue homogenate, 2 mmol/liter glucose-6-phosphate, 0.2 U glucose-6-phosphate dehydrogenase, 0.8 mmol/liter NADP, and 20.0 mmol/liter hemin. Incubation was allowed to proceed for 1 h at 37°C. The results were determined spectrophotometrically by measuring the optical density at 464 nm against a baseline absorbance at 530 nm. Activity was normalized to the value for the control.

Quantitative RT-PCR (qRT-PCR) examination. Total RNA was isolated using the TRIzol extraction method according to the manufacturer’s instructions (Invitrogen Inc., Carlsbad, CA). The quality of RNA was verified by evaluating the optical density (OD) at 260 nm and 280 nm. One microgram of total RNA was processed to produce cDNA by the use of reverse transcription and a PrimeScript reverse transcription-PCR (RT-PCR) kit (TaKaRa, Dalian, China). The PCR conditions and primers used were as follows: Nrf2 forward, 5′-CAT CTC ACC AAA CAA GAT GC-3′; Nrf2 reverse, 5′-TCT TTT TCC AGC GAG GAT AT-3′; HO-1 forward, 5′-GCT CGA ATG AAC ACT CT-3′; HO-1 reverse, 5′-GGA AGC TGA GAG TGA GGA CC-3′; NF-kB forward, 5′-CAG TCT CCT CTC TGG TCT-3′; NF-kB reverse, 5′-AAG GAT GTC TCC ACA CCA CTG-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward, 5′-AGA GTC CAT GGC ATC ACT GCC-3′; GAPDH reverse, 5′-GCC TGC TTT ACC ACC TTT GTT-3′. Standard cycling conditions were used, including a preamplification step of 95°C for 10 min, followed by amplification for 40 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 20 s. All reactions were conducted in three replicates. GAPDH was used as an internal control, and fold change in gene expression was calculated using the threshold cycle method (2−ΔΔCt).

Statistical analyses. Results are presented as means ± standard deviations (SD). A one-way analysis of variance, followed by a Fisher’s least significant difference (LSD) test, was employed to compare any two means when the variance was homogeneous; otherwise, Dunnett’s T3 test was used (SPSS Inc., Chicago, IL, USA). A P value of <0.05 represented significant difference.

RESULTS

Effect of lycopene on the levels of BUN, serum creatinine, and oxidative stress biomarkers in the kidney. Compared to the control level, BUN and serum creatinine levels did not change in the lycopene treatment group but significantly increased in the colistin-treated mice, indicating impaired kidney function (Table 1). The BUN and creatinine levels were normal after coadministra-
tion of lycopene at 20 mg/kg of body weight/day with colistin (Table 1). Colistin treatment also significantly increased the concentrations of MDA and NO as well as iNOS activity (all \( P < 0.01 \)) in the kidney tissue, while the GSH level and activities of SOD and CAT substantially decreased (all \( P < 0.01 \); Table 2). The levels of MDA, iNOS, CAT, and GSH in the colistin/lycopene 20 mg/kg group were comparable to those in the untreated control group. These results show the attenuation of colistin-induced nephrotoxicity by coadministration of 20 mg/kg/day lycopene.

**Histopathological assessment.** The representative histopathological results are shown in Fig. 2. In the kidneys from the control group and the group administered lycopene alone, no marked pathological changes were observed (Fig. 2A and B). However, in the kidneys from the colistin group, there was severe tubular damage with focal necrosis of tubular epithelial cells and numerous casts (Fig. 2C); the corresponding SQS increased to 2.75 ± 0.50 (\( P < 0.01 \)). These pathological changes were significantly attenuated in the colistin/lycopene 5 mg/kg and colistin/lycopene 20 mg/kg groups (Fig. 2D, E, and F). The corresponding SQSs decreased to 1.50 ± 0.58 (\( P < 0.05 \)) and 0.75 ± 0.50 (\( P < 0.01 \)), respectively.

**Activity of caspase-3, caspase-9, and HO-1 in the kidney.** There was a significant increase in the activities of both caspase-3 and caspase-9 in the colistin group, while the GSH level and activities of SOD and CAT markedly decreased (all \( P < 0.01 \)) in the kidney tissue, while the GSH level and activities of SOD and CAT substantially decreased (all \( P < 0.01 \); Table 2). The levels of MDA, iNOS, CAT, and GSH in the colistin/lycopene 20 mg/kg group were comparable to those in the untreated control group. These results show the attenuation of colistin-induced nephrotoxicity by coadministration of 20 mg/kg/day lycopene.

**Expression of Nrf2 and OH-1 mRNAs.** Significant global medical challenge. Colistin is increasingly used as the only therapeutic option. However, recent pharmacological data indicate that plasma concentrations achieved with the currently recommended dosage regimens are suboptimal in many patients (42), potentially leading to poor clinical outcome and emergence of resistance. Simply increasing daily doses of colistin is not an option because nephrotoxicity can occur in a large proportion of patients (4, 43). In the present study, we investigated the potential nephroprotective effect of lycopene, a red-colored carotene pigment found in red fruits and vegetables, such as tomatoes, watermelons, *Momordica cochinchinensis* Spreng fruit, and papayas (31). Lycopene is 10-, 47-, and 100-fold more effective in quenching singlet oxygen than \( \alpha \)-tocopherol, \( \beta \)-carotene, and vitamin E, respectively (32, 34). Clinical studies also demonstrated that dietary supplementation of lycopene or tomato products reduced levels of biomarkers of oxidative stress (e.g., cellular DNA damage and lipid oxidation) in healthy subjects, smokers, and type-2 diabetics (44–49). Another study showed that daily intake of 150 ml tomato juice (equal to administration of lycopene at approximately 15 mg/day) for 5 weeks can significantly reduce the serum concentration of 8-OHdG (8-hydroxy-2'-deox-yguanosine, a critical biomarker of oxidative stress) after extensive physical exercise (50). In addition, lycopene is the most effective quencher of oxygen free radicals across all of the naturally occurring carotenoids and demonstrates potential antioxidant properties (35, 51); the latter has been evaluated in clinical trials in several countries (52–54).

In a mouse model, we demonstrated that coadministration of lycopene attenuated colistin-induced nephrotoxicity (Fig. 2) as shown by the decrease of BUN, serum creatinine, and caspase-dependent apoptosis levels (Table 1). These data are in line with previous studies that showed that coadministration of antioxidants can protect against colistin-induced nephrotoxicity (12,

### TABLE 2 Effect of lycopene on the biomarkers of renal oxidative stress induced by colistin

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Conc (mean ± SD; ( n = 10 )) for indicated group*</th>
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<tr>
<td>MDA (mmol/mg pro)</td>
<td>1.48 ± 0.41</td>
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<tr>
<td>NO (( \mu )mol/g pro)</td>
<td>4.56 ± 0.72</td>
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<tr>
<td>iNOS (U/mg pro)</td>
<td>0.45 ± 0.21</td>
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<tr>
<td>CAT (U/mg pro)</td>
<td>96.5 ± 8.42</td>
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<tr>
<td>GSH (mmol/mg pro)</td>
<td>55.1 ± 6.18</td>
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<tr>
<td>SOD (U/mg pro)</td>
<td>68.7 ± 6.23</td>
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*MDA, malondialdehyde; NO, nitric oxide; iNOS, inducible nitric oxide synthase; CAT, catalase; GSH, glutathione; SOD, superoxide dismutase; pro, protein. Values are presented as means ± SD (\( n = 10 \)). *, \( P < 0.05 \); **, \( P < 0.01 \) (compared to the control group); #, \( P < 0.05 \); ##, \( P < 0.01 \) (compared to the colistin group).
Increased production of ROS is an important mechanism in colistin-induced nephrotoxicity and neurotoxicity (17, 56–58). Excessive ROS levels cause damage to DNA, lipids, and proteins, eventually leading to apoptosis (14, 59). Lycopene has been shown to interact with ROS and thereby to prevent ROS-induced tissue and cellular damage (35). Consistent with previous studies, we revealed that colistin-induced nephrotoxicity is associated with significantly increased MDA levels and a concomitant decrease in the levels of antioxidant enzymes CAT, SOD, and GSH (Table 2) (16, 18, 60, 61). In the present study, lycopene treatment restored the levels of all of these biomarkers to the normal range observed in the untreated control group (Table 2), confirming its nephroprotective effect. This protective property was also confirmed by histopathological examination of the mouse kidneys, which revealed a marked amelioration in tubular necrosis and decrease of SQSs in the lycopene-cotreated groups compared to the colistin group (Fig. 2).

Caspase-3 is a key biomarker of apoptosis (18), which can be activated by both intrinsic and extrinsic apoptotic pathways and consequently lead to DNA breakdown (14, 57, 62). Several studies showed that activated caspase-3 played a key role in colistin-induced apoptosis in rat kidney tissue (12, 17, 18). The mitochondrion is a major producer of ROS and plays a central role in the process of oxidative stress (58, 63). Release of cytochrome c into

**FIG 2** Representative histopathological results and the semiquantitative scores. (A) Control group: no marked injury. (B) Lycopene group: no marked injury. (C) Colistin (15 mg/kg/day) group: marked tubular damage with necrosis and exfoliation of epithelial cells (arrows), cast formation (arrowheads), and dilation. (D) Colistin (15 mg/kg/day) plus lycopene (5 mg/kg/day) (Colistin/lycopene 5) group: mild tubular damage with necrosis (arrows) of epithelial cells and cast formations (arrowheads). (E) Colistin (15 mg/kg/day) plus lycopene (20 mg/kg/day) (Colistin/lycopene 20) group: minor tubular damage with tubular dilatation (arrows) and cast formations (arrowheads). (F) SQS values are presented as means ± SD (n = 4). **, P < 0.01 (compared to the control group); #, P < 0.05; ##, P < 0.01 (compared to the colistin group). Data represent the results of H&E staining. Bars, 100 µm.

**FIG 3** Activities of caspase-3 (A), caspase-9 (B), and HO-1 (C). Values are presented as means ± SD (n = 10). **, P < 0.01 (compared to the control group); #, P < 0.05; ##, P < 0.01 (compared to the colistin group).
the cytosol is associated with the mitochondrial apoptosis pathway which is mediated by the activation of mitochondrial permeability transition pores. The cytochrome c in the cytosol also leads to apoptosisosome formation, which activates caspase-9 and caspase-3-mediated apoptosis (62). Caspase 9 is an important bio-marker in the mitochondrial apoptosis pathway (11, 62, 64). Our previous study demonstrated that mitochondrial dysfunction and the mitochondrial apoptosis pathway play important roles in colistin-induced nephrotoxicity (11). Previous studies identified that lycopene can protect against cell or tissue injury induced by toxins (e.g., 3-nitropropionic acid, trimethyltin, and HgCl2) via inhibiting the mitochondrial apoptosis pathway (34, 64, 65). Our findings here demonstrate that lycopene treatment markedly decreased the activities of both caspase-9 and caspase-3 (Fig. 3A and B). It is very likely that lycopene treatment minimized ROS production and the subsequent mitochondrial dysfunction (32).

The Nrf2 pathway is important in cytoprotective adaptive responses to xenobiotic insults (19). Nrf2 regulates the expression of cytoprotective genes encoding antioxidants and phase II detoxifying enzymes such as CAT, SOD, and HO-1 (22). It has been demonstrated that activation of Nrf2 is involved in drug-induced nephrotoxicity by cisplatin, gentamicin, and aristolochic acid (28, 66–70). Similarly, the mRNA levels of Nrf2 and its corresponding downstream HO-1 gene significantly increased with colistin or lycopene treatment (Fig. 4). Decreases in the GSH level in damaged cells can lead to adaptive increases in the intracellular antioxidant defense; an important event of this process is the increase of the nuclear translocation of Nrf2 and its DNA binding capacity (71). The activated Nrf2 can lead to increased GSH levels through inducing the expression of the cysteine-glutamic acid exchange transporter, which protects cells from oxidative stress (71). This may explain the moderate increase of Nrf2 mRNA expression after colistin treatment. Dezoti Fonseca and colleagues showed that hemin, an inducer of HO-1, can significantly attenuate polymyxin B-induced nephrotoxicity in rats (61), which also suggested that HO-1 is involved in colistin-induced nephrotoxicity.

NF-κB plays a critical role in the regulation of many important genes involved in cellular homeostasis and cell death (26). It acts as a sensor of ROS-mediated oxidative stress (11). NF-κB activation can induce iNOS expression and then the production of NO and exacerbate oxidative stress injury (33, 51). Ozkan et al. reported that caspase-1, an activator of NF-κB, and p38 mitogen-activated protein kinase (MAPK) (72), played an important role in colistin-induced nephrotoxicity in rats (18). It has been demonstrated that lycopene can suppress oxidative stress-induced NF-κB DNA binding, NF-κB/p65 nuclear translocation, and phosphorylation of IκB kinase (IKK) and IκBα, which in turn reduces oxidative stress response (33, 73). In line with these findings, our results revealed that colistin-induced nephrotoxicity is associated with a marked increase in the level of NF-κB expression (Fig. 4), iNOS activity, and NO concentrations (Table 2). Importantly, the expression of iNOS, NO, and NF-κB in the groups treated with colistin was significantly attenuated by lycopene treatment (Table 2 and Fig. 3). This is consistent with previous findings that lycopene suppresses the production of iNOS and NO by blocking the phosphorylation of p38 MAPK and translation of NF-κB (33). In addition, cross talk between Nrf2 and NF-κB systems has been identified. Nrf2 activation caused by increased HO-1 activity can lead to the inhibition of NF-κB nuclear translocation via HO-1 end products, i.e., bilirubin and CO (71). In the present study, results indicated that the Nrf2 activation may be partially responsible for the downregulated expression of NF-κB mRNA by lycopene, which can directly scavenge singlet-oxygen and free radicals. The detailed mechanism warrants further investigation.

In conclusion, this report is the first to reveal that the Nrf2/ HO-1 pathway plays a protective role in colistin-induced nephrotoxicity in mice. Importantly, our data indicate that lycopene may be able to attenuate colistin-induced nephrotoxicity via activation of the Nrf2/HO-1 pathway.

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We declare that we have no conflicts of interest with regard to this study.

REFERENCES


