Accepted Manuscript

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PII: S0278-6915(17)30584-7

DOI: [10.1016/j.fct.2017.10.006](https://doi.org/10.1016/j.fct.2017.10.006)

Reference: FCT 9331

To appear in: *Food and Chemical Toxicology*

Received Date: 5 August 2017

Revised Date: 21 September 2017

Accepted Date: 7 October 2017

Please cite this article as: Ma, Z.-n., Liu, Z., Wang, Z., Ren, S., Tang, S., Wang, Y.-p., Xiao, S.-y., Chen, C., Li, W., Supplementation of American ginseng berry extract mitigated cisplatin-evoked nephrotoxicity by suppressing ROS-mediated activation of MAPK and NF- κ B signaling pathways, *Food and Chemical Toxicology* (2017), doi: 10.1016/j.fct.2017.10.006.

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Supplementation of American ginseng berry extract mitigated cisplatin-evoked nephrotoxicity by suppressing ROS-mediated activation of MAPK and NF- κ B signaling pathways

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Abbreviations

The following abbreviations are used in this manuscript:

GSH	Glutathione	MDA	Malondialdehyde
SOD	Superoxide dismutase	TNF- α	Tumor necrosis factor- α
BUN	Blood urea nitrogen	IL-1 β	Interleukin-1 β
CRE	Creatinine	iNOS	Inducible nitric oxide synthase
COX-2	Cyclooxygenase-2	CYP2E1	Cytochrome P450 E1
4-HNE	4-hydroxynonenal	NF- κ B	Nuclear factor-kappa B
MAPK	Mitogen activated protein kinase	JNK	c-Jun N-terminal kinase
ERK	Extracellular signal-regulated kinase	AGB	American ginseng berry
ROS	Reactive oxygen species		

Running title: AGBE ameliorates cisplatin-induced nephrotoxicity

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Abstract

Nephrotoxicity induced by cisplatin in 30% of all cisplatin treated patients seriously limits its clinical implication as a widely used anticancer agent, and may even cause patients to alter or give up cisplatin therapy. The purpose of this study is to test a protective effect of American ginseng berry extract (AGBE) on cisplatin-induced nephrotoxicity in mice. In this study, the histopathological changes and elevated levels of serum creatinine (CRE) and urea nitrogen (BUN) caused by cisplatin were significantly diminished by AGBE treatment. Oxidative stress caused by cisplatin, evidenced by increases in kidney tissues malondialdehyde (MDA) content, cytochrome P450 E1 (CYP2E1), renal 4-hydroxynonenal (4-HNE) levels and decreases of glutathione (GSH) and superoxide dismutase (SOD) contents, was significantly ameliorated by AGBE pretreatment. The expression levels of tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) were inhibited by AGBE treatment, suggesting a suppression of inflammatory response. Additionally, AGBE clearly inhibited cisplatin-induced activations of nuclear factor-kappa B (NF- κ B) and mitogen activated protein kinase (MAPK) signal pathways. Supplementation of cisplatin-intoxicated mice with AGBE also significantly reduced apoptotic protein levels of Bax, cleaved caspase-3, cytochrome c and increased anti-apoptotic protein Bcl-2. These findings highlight nephroprotective effect of AGBE against cisplatin-evoked nephrotoxicity through ROS-mediated MAPK and NF- κ B signaling pathways.

Keywords: American ginseng berry extract; Cisplatin-induced nephrotoxicity; MAPK; NF- κ B, Anti-apoptosis; Anti-inflammatory

1. Introduction

Cancer treatment using chemotherapeutic drugs has unfolded new prospect to improve the quality of life of cancer patients. Unfortunately, numerous anticancer agents have been considered to be carcinogenic in clinical experimental application systems (Cherry et al., 2004). Cisplatin (cis-diamminedichloroplatinum), a typical platinum-based agent during “the war on cancer”, is widely used to treat many types of cancers within the effective dose range, including cervical, ovarian, head, neck and non-small cell lung carcinoma. It is particularly effective in testicular cancer, with a cure rate of more than 90% (Karakoc et al., 2015; Miyagi et al., 2014). Nevertheless, the high dose cisplatin is primarily restricted by accumulative nephrotoxicity and neurotoxicity, in which renal toxicity and kidney dysfunction are common (Kim et al., 2014b; Pabla and Dong, 2008). Studies revealed that accumulation of cisplatin in renal tubular cells was five times more in comparison to other tissues, suggesting the principal renal toxicity (van Angelen et al., 2013).

Renal oxidative stress, apoptosis and inflammation have been considered as potential mechanisms underlying nephrotoxicity caused after cisplatin exposure (Ma et al., 2017). In most cases, cisplatin appears in the plasma and passes through renal tubular epithelial cells, to activate numerous pro-inflammatory cytokines, such as $\text{TNF-}\alpha$, $\text{IL-1}\beta$, and to induce the generation of the inflammatory mediators iNOS and COX-2 (Honma et al., 2013; Ma et al., 2015). Oxidative stress is an imbalance in the redox reaction, leading to the generation of reactive oxygen species (ROS) in the pathophysiology of cisplatin-evoked nephrotoxicity (Santos et al., 2008). Excess ROS production by cisplatin may result in lipid peroxidation (LPO) and delayed-onset renal damage. Likewise, ROS-mediated oxidative stress triggers the activation of a series of signaling proteins, including MAPK (Malik et al., 2015) and $\text{NF-}\kappa\text{B}$ (Sahu et al., 2014). Simultaneously, cisplatin may induce DNA damage in the kidney, which is related with ROS formation leading to caspase-3 dependent apoptosis (Liu et al.,

²⁰¹⁰). Overall, presently available data revealed that the oxidative stress, inflammation, apoptosis exert major roles in the pathogenesis of cisplatin-evoked renal toxicity.

Recently, various reports have concentrated on traditional Chinese herbal medicines to assess novel therapeutic agents for treatment of nephrotoxicity. Root of Red ginseng (Araliaceae) and leaves of *Panax quinquefolius* (Araliaceae) have been displayed with nephroprotective effect against cisplatin-evoked nephrotoxicity through *in vivo* experiments (Cayir et al., 2011; Gutierrez et al., 2010; Sultana et al., 2012). American ginseng, aka *Panax quinquefolius*, has a long history worldwide as a medicinal herb and is viewed as one of the most well-known perennial herbal plants of genus *Panax* (family Araliaceae) in China (Szeto et al., 2015). American ginseng berry (AGB) is the ripe fruit of *Panax quinquefolius*. A lot of reports have proved that ABE exerts similar or same physiological activities as *P. ginseng* or *P. quinquefolius*. So far, researchers have focused on the chemical constituents and biological activities of roots and leaves of American ginseng, while the study on its berry is rare (Shao et al., 2004). In order to fully utilize the resources and to enhance the economic value of American ginseng, in-depth studies on the saponins and their biological activities of AGB have been carried out for several decades. Previous studies showed that AGB contained ginsenosides, polysaccharides, volatile oil, amino acids, fatty acids, flavonoids, inorganic elements and sterols; of which ginsenosides were the main active ingredients (Wang et al., 2006). The American ginseng berry extract (AGBE) was similar to AGB showing a variety of pharmacological activities in clinical therapies. Shao *et al.*, reported that antioxidant effects of AGBE in cardiomyocytes exposed to acute oxidant stress (Shao et al., 2004). The polysaccharide fractions of AGBE showed anti-hyperglycemic effect in *ob/ob* mice (Xie et al., 2004). Anticancer effects of AGBE were demonstrated *in vitro* and *in vivo* (Xie et al., 2009). Although many pharmacological effects by AGBE have been discovered, nephron-protective effect of AGBE against cisplatin-evoked nephrotoxicity has not been reported so far.

In this study, potential mechanisms of AGBE in protecting acute renal injury by cisplatin were investigated in mice. The underlying molecular mechanisms of AGBE were mainly involving ROS-mediated activation of MAPK and NF- κ B signaling pathways.

2. Materials and Methods

2.1 Preparation of samples

AGBE extracted from the berry of *P. quinquefolium* (American ginseng) was prepared and quantified in our laboratory as described previously (Ma et al., 2017). First of all, AGBE was refluxed with 70% ethanol for three times. The crude extracts (ginsenosides, sugars, starch etc.) were separated by column chromatography with AB-8 resin. Later the saponins analysis was built on a Hypersil ODS column and detected by high performance liquid chromatography (HPLC) at 203 nm. Seven ginsenosides (Rg1, Re, Rb1, Rc, Rb2, Rb3, and Rd) were determined by comparing their retention times with corresponding standard compounds, which contents of the saponins in the AGBE as follows: 0.336% Rg1, 9.107% Re, 0.504% Rb1, 8.805% Rb2, 29.523% Rb3, 3.171% Rc, 6.022% Rd.

2.2 Reagents

Cisplatin was obtained from Sigma-Aldrich (St. Louis, MO, USA). The commercial assay kits of CRE, BUN, GSH, SOD, MDA and dye kits hematoxylin and eosin (H&E) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The mitochondria isolation kit was obtained by (Beyotime Biotechnology, Shanghai, China). Antibodies directed against GAPDH, Bax, Bcl-2, COX-2, iNOS, 4-HNE, CYP2E1, cytochrome c, caspase-3, and cleaved caspase-3, IKK α , IKK β , I κ B α , NF- κ B, phospho-IKK α , phospho-IKK β , phospho-I κ B α , phospho-NF- κ B, JNK MAPK, p38 MAPK, ERK MAPK, phospho-JNK MAPK, phospho-p38 MAPK, phospho-ERK MAPK. The secondary antibodies for western blot were received from Cell Signaling Technology (Danvers, MA,

USA). The enzyme-linked immunosorbent assay (ELISA) kit of mouse TNF- α and IL-1 β were obtained from R&D systems (Minneapolis, MN, USA). The dye kits for Hoechst 33258 were purchased from Beyotime Co. Ltd (Shanghai, China) and TUNEL apoptosis detection kits were bought from Roche Applied Science (Shanghai, China). The secondary antibodies of DyLight 488-labeled were obtained by BOSTER Bio-Engineer Co. Ltd. (Wuhan, China). All of the other reagents were of analytical grade.

2.3. *Experimental animals and treatment protocol*

This study was carried out with 48 male ICR mice (22-25g), offered by YISI Experimental Animals Co. Ltd (Changchun, Jilin province, China). Mice were housed at controlled temperature ($25 \pm 2^\circ\text{C}$) and humidity ($60 \pm 10\%$) with 12 h light/dark cycle with food and water *ad libitum*. Animal experiment processing procedures were executed strictly in line with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006). All animal experiment was authorized by Ethical Committee of Jilin Agricultural University (Permit Number: ECLA-JLAU-16033).

The mice were divided into five groups with 8 animals each and treated as follows: untreated control mice group, AGBE alone mice group (300 mg/kg), cisplatin-injected mice group (20 mg/kg) and cisplatin + AGBE mice groups (150 mg/kg and 300 mg/kg). Mice were administered intragastrically with AGBE daily for ten continuous days and received a single intraperitoneal injection of cisplatin on the 7th day after 1 h from the last administration of AGBE in cisplatin group and AGBE + cisplatin groups. The experiment was terminated at the end of 10 days and all the animals were anesthetized and were sacrificed. Kidneys were gathered and weighted, blood samples were collected immediately, allowed to put an hour or so, then centrifuged at 3500 rpm for 15min at 4°C to separate serum, which was stored at -80°C for different biochemical index

detection. Subsequently, right kidney was fixed in 10% neutral formaldehyde for histological examination, left kidney was frozen in liquid nitrogen and stored at -80°C till analysis. Kidney weight/ body weight was calculated as the kidney index ($\text{mg/g} \times 100\%$).

2.4 Estimation of kidney functions tests

Serum BUN and CRE were determined by commercially available diagnostic kits according to manufacturer's programs (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China).

2.5 Estimation of antioxidant and mitochondrial lipid peroxidation parameters

The GSH and SOD contents were analysed according to the kits protocol as mentioned earlier. Lipid peroxidation was measured through determination of mitochondrial MDA level. Mitochondrial MDA level was measured using a thiobarbituric acid reactive substances (TBARS) method as described by our previous method ^(Qi et al., 2017). Briefly, the mitochondria in kidney tissues were isolated with tissue mitochondria isolation kit (Beyotime Biotechnology, Shanghai, China) and the detection of MDA levels judging by manufacturer's instructions. Then, the protein concentrations were measured by Bradford protein assay with bovine serum albumin as the standard (Beyotime Biotechnology, Shanghai, China).

2.6. Estimation of inflammatory cytokines

The contents of $\text{TNF-}\alpha$, $\text{IL-1}\beta$ in the serum were examined with commercially available enzyme-linked immunosorbent assay (ELISA) kits (Minneapolis, MN, USA).

2.7. Estimation of histopathological examination

The tissue of fixed in 4% neutral formaldehyde was cut into $5\text{ }\mu\text{m}$ -thick sections, which were dehydrated and fixed in paraffin. Later, the slices were stained with hematoxylin and eosin for histopathological analysis according to the procedure earlier in our laboratory (Hu et al., 2017). Tubular damage was assessed by scoring inflammatory infiltration and tubular necrosis in 10

different fields in the corticomedullary junction (microscopy, Leica DM750, Germany). Histopathological changes were blindly scored by a pathologist on a 5-point scale: 0 = no damage, 1 = 10% of the corticomedullary junction injured, 2 = 10-25%, 3 = 25-50%, 4 = 50-75%, 5 = more than 75%.

2.8. Estimation of Hoechst 33258 staining analysis

Hoechst 33258 staining analysis was performed as mentioned earlier with minor modifications. The 5 μ m-thickness kidney sections were stained by Hoechst 33258 dyeing kits and then were washed 3 times each time 10 min in PBS, stained nuclei were visible below UV excitation. These actions were photographed used for a fluorescent microscope (Leica TCS SP8, Germany).

2.9. Estimation of TUNEL staining analysis

In order to evaluate the in situ apoptosis in kidney tubular cells, the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) method was performed. Briefly, TUNEL staining was performed with TUNEL apoptosis detection kits from Roche Applied Science (Shanghai, China). TUNEL positive staining patterns were photographed with microscope (Leica TCS SP8, Germany).

2.10. Estimation of immunohistochemistry (IHC) and immunofluorescence analysis

Paraffin slices were used to deparaffinizing and rehydrating with xylene and graded ethanol series. The sections were incubated in 3% hydrogen peroxide to quench the activity of endogenous peroxidase. Sequentially, these slides were placed in citrate buffer and heated at 100°C to retrieve antigens and then rinsed 3 times with Tris-buffered saline (TBS 0.01 M, pH 7.4) for 10 min. Later, these sections incubated with 1% bovine serum albumin for 60 min and incubated overnight at 4°C with primary antibodies against Bax (1:200), COX-2 (1:200), iNOS (1:200) and Bcl-2 (1:200). The sections followed by incubated with horseradish peroxidase conjugated secondary antibody.

Sections were then washed with distilled water, incubated with diaminobenzidine hydrogen peroxide, and counterstained with hematoxylin. Immunoreactivity was identified as brown nuclear in kidney sections counterstained with hematoxylin. Immunostaining intensity was analyzed by light microscopy (Leica DM750, Germany).

The immunofluorescence staining steps are the same as described above. After all slices were incubated with primary antibodies 4-HNE (1:200) and CYP2E1 (1:200) at 4 °C overnight, then were incubated by DyLight 488-labeled secondary antibodies. Nuclear was stained with 4-6 diamidino-2-phenylindole (DAPI). Immunofluorescence staining was noticed with microscope (Leica TCS SP8, Solms, Germany).

2.11. Estimation of western blot analysis

The total proteins were abstracted with radio immunoprecipitation assay (RIPA) buffer, and the protein concentrations were then determined with a BCA protein assay kit according to previous method. Then these proteins were segregated used for 10% SDS polyacrylamide gels and distracted to polyvinylidene difluoride (PVDF) membranes. Next, these membranes were blocked with 3% BSA for 1.5 h at room temperature and incubated with primary antibodies at 4 °C overnight. Subsequently, membranes were rinsed three times used for TBST for 5 min each time with gentle agitation and incubated with secondary antibodies. Finally, the membranes were visualized with the emitter coupled logic (ECL) plus western blot detection system (Media Cybernetics, State of California, USA).

2.12. Statistical analysis

The data are denoted as mean $\pm$ standard deviation (SD). All statistical analysis was carried out with SPSS software (SPSS Science, Chicago, Illinois, USA). Differences between the groups were

evaluated with one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. *The effect of AGBE on cisplatin-evoked renal dysfunction*

To research the effect of AGBE against cisplatin-evoked renal dysfunction, the serum contents in CRE and BUN were determined at 72 h following cisplatin single injection (20 mg/kg). Severe renal injury as manifested via obvious elevation in serum CRE and BUN levels after cisplatin exposure, which were ameliorated by AGBE administration. Cisplatin management also caused apparent weight loss and raised relative kidney index. Interestingly, these relative kidney index changes were evidently dose-dependently mitigated by AGBE. The results of AGBE alone mice group showed that no obvious effects on CRE, BUN, body weight and relative kidney index when mice were orally given with AGBE for 10 consecutive days. ($p < 0.05$ or $p < 0.01$) (Figure 1).

3.2. *The effect of AGBE against cisplatin-evoked renal oxidative stress damage*

The GSH level and SOD contents reduced clearly in cisplatin injection group, while MDA, a maker of lipid peroxidation, was elevated in comparison to control group. As indicated in Figure 2, supplementation with AGBE decreased the MDA level and restored antioxidant capacity as elaborated by the elevation of GSH and SOD levels. These results clarified that AGBE extenuated oxidative stress damage via up-regulating anti-oxidant enzyme activity ($p < 0.05$ or $p < 0.01$).

4-HNE is the final products in lipid peroxidation, which is considered as indicator in free radical generation. The normal group displayed low expression of the kidney tissues in mice, while following cisplatin induced evident 4-HNE expression in kidney tissues, which was extenuated by AGBE pretreatment. Concomitantly, these results revealed the expression level of CYP2E1 metabolizing enzyme was low in normal and AGBE-treated mice group (300mg/kg), obviously raised by cisplatin

management. Above results illustrated that AGBE administration ameliorated kidneys on cisplatin-evoked oxidative stress in mice (Figure 3).

3.3. The effect of AGBE against cisplatin-evoked histopathological changes

The inflammatory infiltration and tubular necrosis were clearly observed in cisplatin control group in comparison to normal and AGBE groups (Figure 4). Nevertheless, AGBE effectively ameliorated cisplatin-induced pathological changes in this group served with high dose of AGBE (300 mg/kg) and regular renal tubules, no inflammatory infiltrate cells and necrosis appeared in kidney tissues.

3.4. The effect of AGBE against cisplatin-evoked apoptosis

Hoechst 33258 staining was applied to detect the apoptosis level in renal tubular cells. The results of analysis showed that the nuclear fragmentations and condensations in the cisplatin injection group were markedly higher (Figure 5). A mass of cell nucleus emerged circular nucleus with uniform fluorescence intensity and conventional contours in AGBE pre-administration group when compared with cisplatin control group. Additionally, apoptosis level were detected and quantified with TUNEL staining assay, which showed renal tubular epithelial cell apoptosis by cisplatin. After 10 days' treatment, AGBE with low dose reduced the number in TUNEL-positive cells, and more apparently reduction by it at high dose is almost similar to the normal group ($p < 0.05$ or $p < 0.01$) (Figure 6).

To further verify the renoprotective of AGBE against cisplatin-evoked renal cell apoptosis, immunohistochemical and western blot were used to explore the protein expression levels of Bax, Bcl-2, cytochrome c and cleaved caspase-3 in kidney tissues. These results displayed that cisplatin management evidently elevated the expression levels in Bax, cleaved caspase-3, cytochrome c and reduced the expression level of Bcl-2 when in comparison to normal group. Fortunately, these

changes were powerfully ameliorated with AGBE administration. All above results suggested that AGBE exerted repressive effects on cisplatin-evoked apoptosis of kidney tissues in mice ($p < 0.05$ or $p < 0.01$) (Figure 7 and Figure 8).

3.5 The effect of AGBE on cisplatin-evoked activation of MAPK signaling pathways

The MAPK signaling pathway plays a vital role in mediating cisplatin-evoked renal toxicity^(Jo et al., 2005). In researching the role of MAPK activation, JNK MAPK, p38 MAPK and ERK MAPK in renal tubular cell death evoked after cisplatin management, the induction of phosphorylated forms of JNK and p38 were noticed, while no phosphorylated ERK was noted. Treating kidney tissues with cisplatin strongly increased p-JNK and p-p38 expressions in cisplatin control group when in comparison to normal group. Interestingly, the p-JNK and p-p38 expression levels by AGBE pretreatment for 10 days exerted dose-dependent decrease ($p < 0.01$, $p < 0.05$) (Figure 8).

3.6 The effect of AGBE on cisplatin-evoked activation of NF- κ B signaling

Taking into account that NF- κ B could regulate transcription of pro-inflammatory cytokines, we analyzed the protein levels. Western blot analysis was executed to assess the level of phosphorylation of IKK α , IKK β , I κ B α and NF- κ B, which was markedly elevated following cisplatin exposure. Interestingly, these expression levels were negligible in AGBE group (300mg/kg) of the kidney tissues, reflecting almost no inflammatory response (Figure 8). To inspect whether the prohibitive effect of AGBE on the expression level of inflammatory cytokines and mediators was related with its effect on the NF- κ B signaling pathway in our experiment, the pro-inflammatory cytokine levels TNF- α , IL-1 β in the serum and renal protein expression level of iNOS and COX-2 in kidney tissues were determined. Following cisplatin resulted in evidently higher these levels of TNF- α and IL-1 β in comparison to normal group (Figure 9), but the elevations were ameliorated by AGBE effectively ($p < 0.01$, $p < 0.05$). Likewise, immunohistochemical analysis was applied to

assess the expression levels of COX-2 and iNOS in all groups. The results revealed that the positive expression area of iNOS and COX-2 of cisplatin control group was obviously observed in kidney tissues compared with normal group, whereas AGBE pretreatment for 10 days exerted dose-dependent decrease ($p < 0.05$ or $p < 0.01$).

4. Discussion

Nephrotoxicity is one of the most limiting adverse effects of cisplatin, and it is necessary to manage cisplatin-induced nephrotoxicity to enhance the safety and efficacy of cisplatin-containing chemotherapy regimens. In addition, it is important to develop new prophylactic strategies for preventing and treating cisplatin-induced nephrotoxicity, and to determine their mechanisms of action. Though the same experimental methods and thinking were applied with previous reports in our laboratory^{(Ma et al., 2017) (Li et al., 2016)}, the broader molecular mechanisms and deeper signaling pathways were presented in our study. As a non-traditional medicinal part of *P. quinquefolium*, the extracts from its berries are relative with no difficulty and cost less. In order to improve the utilization of the ground part of *P. quinquefolium* and solve the puzzle of treatment costs of patients with kidney disease in clinical practice. We evaluated the nephroprotective effect of AGBE administration on cisplatin-evoked nephrotoxicity. Ginsenoside is the main active ingredient in American ginseng berry, which has indicated multiple pharmacological activities previously. Wang Y *et al.* showed that ginsenoside Rb3 attenuated oxidative stress by suppressing over-production of ROS *in vivo*^(Wang et al., 2014b). The underlying anti-apoptotic mechanisms of ginsenoside Rd was achieved through the inhibition of ASK1-JNK pathway^(Wang et al., 2014a). Likewise, ginsenoside Re ameliorates inflammation by inhibiting IKK- β phosphorylation and NF- κ B activation in mice^(Lee et al., 2012). It is speculated that American ginseng berry saponins may provide a novel therapeutic approach for treating cisplatin-induced renal injury in mice. However, it is not clear whether other

small molecule components have exerted effect. Our work suggested that AGBE has a dramatically alleviative effect in mice mainly focused on ROS-mediated activation of MAPK and NF- κ B signaling pathways.

Current study, the results displayed that cisplatin challenge resulted in typical clinical symptoms and pathological changes of cisplatin-injected mice, such as necrosis, inflammatory infiltration, relative kidney index increase and body weight loss. Moreover, CRE and BUN levels were evidently elevated following cisplatin in comparison to control group, which demonstrated the decrease of glomerular filtration rate. But these symptoms and pathological changes in kidney damage were clearly improved with AGBE pre-administration. All the above consequences indicated that the model in cisplatin kidney injury mice was successfully established, AGBE executed an ameliorate effect in cisplatin-evoked renal toxicity.

Oxidative stress plays a central role in the pathophysiology of cisplatin-evoked renal toxicity (Santos et al., 2008). Previous reports have displayed that cisplatin injection lead to overproduction of free radicals, involving hydroxyl radical and superoxide anion, and further cause oxidative injure and lipid peroxidation (LPO) in kidneys (Wei et al., 2015). Additionally, it illustrated that cisplatin damage anti-oxidant defense mechanisms followed an apparent reduction in the levels of SOD, GSH and elevation in MDA content (Fernandez-Rojas et al., 2014). In present reports, supplementation with AGBE for 10 days evidently repress the elevation of MDA content and decrease of GSH and SOD contents, respectively. 4-HNE, the end production of LPO in response to oxidative stress, is a highly reactive aldehyde that can modify cellular components and induce cytotoxicity. Concomitantly, CYP2E1 mediated biotransformation of cisplatin generates ROS, including hydrogen peroxide and hydroxyl radical (Liu et al., 2002), a inducer of LPO (Valko et al., 2007). Interestingly, these findings were coincident with previous report, which has verified a major role of drug-metabolizing enzyme CYP2E1 on

cisplatin-evoked renal damage^(Liu and Baliga, 2003). The results of immunofluorescence revealed that the expression levels of 4-HNE and CYP2E1 were low in control and AGBE-treated mice significantly increased following cisplatin management, which was decreased with AGBE in a dose-dependent manner. In short, our findings evidenced that AGBE administration recovered antioxidant ability by inhibiting oxidative stress damage.

There is growing evidence that the pathological process of cisplatin-evoked renal cell apoptosis is related with the release of many inflammatory cytokines and mediators, such as TNF- α and IL-1 β ^(Ma et al., 2017). ROS has given an account of playing a leading part in renal tubule interstitial inflammation and activating NF- κ B and MAPK^(Li et al., 2014). NF- κ B is related with the course of cisplatin-evoked renal inflammation via regulating the contents of pro-inflammatory cytokines and inflammatory mediators. Additionally, NF- κ B activity is repressed by designated inhibitor I κ B in cytoplasm. Upon activation, I κ B is rapidly removed by IKK α , IKK β , and activated NF- κ B is released and translocated to nucleus, where it activates the transcription of target genes. These results of western blot showed that AGBE inhibited NF- κ B activation by restraining the activation of IKK α , IKK β and I κ B α . Moreover, NF- κ B-dependent elevation in the contents of inflammatory cytokine mediators was evidently repressed. AGBE decreased the contents of iNOS and other inducible genes, like COX-2, which an inducible form of COX, can emerge in tissue damage. It has been affirmed that COX-2 acts a vital role on cisplatin-evoked nephrotoxicity. Powerful evidence demonstrated that COX-2 and iNOS are notably expressed at positions of inflammation and iNOS shows synergy with COX-2 to quicken the inflammatory response^(Honma et al., 2013; Itzkowitz, 2006). The results of this study, expression levels of phosphorylation IKK α , IKK β , I κ B α , NF- κ B and COX-2, iNOS, TNF- α , IL-1 β induced by various stimuli, including growth factors and cytokines, is consistently absent in normal tissue, while increased in inflammatory condition of cisplatin

induction. Pre-administration with AGBE, the expressions of these protein levels were evidently decreased. In summary, these results declared that AGBE may as an anti-inflammatory drug on cisplatin-evoked renal damage.

Mitochondrial pathway of apoptosis induced following cisplatin can be regulated with pro-apoptotic protein Bax and anti-apoptosis protein Bcl-2^(Jiang et al., 2009). Investigations found that Bax genes were activated to elevate the expression of Bax protein when cells were stimulated after cisplatin exposure^(Wei et al., 2007). Moreover, Bax protein binds to mitochondria membrane after conformational change, and then arouses the emancipation of cytochrome c from mitochondria^(Tayem et al., 2006), eventually accelerates apoptosis. The intrinsic signals of apoptosis generally center on mitochondria and particularly cytochrome c, is identified as a susceptible apoptotic indicator of a crucial importance. Pore formation in the outer mitochondrial membrane and opening of permeability transition pore are the mechanisms that regulate discharge of cytochrome c^(Ying and Sanders, 2001). Which has until recently been considered only as a participant in oxidative phosphorylation in mitochondria^(Green and Reed, 1998). Correspondingly, the anti-apoptosis protein Bcl-2 could stabilize mitochondrial membrane potential through a series of ways to restrain the release of cytochrome c^(Breckenridge et al., 2003; Cummings and Schnellmann, 2002), and thus prohibits mitochondrial pathway of cell apoptosis.

Another central mechanism underlying renal toxicity of cisplatin is pro-apoptotic effects created via inducing the activation of caspases. As we all know, caspases, a family in intracellular cysteine proteases, are indispensable for the performance of apoptosis by activating of caspase-3^(Checinska et al., 2007). Notably, caspase-3 is a basic apoptotic regulatory factor causing cytoskeleton decomposition, nuclear demise, and other cell changes related with apoptosis^(Arjumand et al., 2011). In current research, we detected caspase-3, cytochrome c, Bax, Bcl-2 protein expression levels by

immunohistochemistry and western blot analysis. These results displayed that cleaved caspase-3, cytochrome c, Bax protein expression levels in cisplatin group obviously elevated in comparison to that of the control group, further implying the characteristic of renal tubular cell apoptosis. The above results cleared that proximal renal tubular apoptosis was major pathological changes following cisplatin. Interestingly, the elevations in these protein levels were evidently decreased by AGBE pretreatment. Simultaneously, the TUNEL staining and Hoechst 33258 staining assay emerged that apoptosis rate were markedly decreased by AGBE when in comparison to cisplatin control group. In short, these results illustrated that AGBE mitigated apoptosis of kidney tissues and appeared alleviative effects against acute nephrotoxicity against cisplatin-evoked mice.

The ROS produced after cisplatin exposure also activate series of downstream proteins that mediate necrosis and apoptosis, especially MAPK family proteins. Briefly, MAPK family consist of three main serine/threonine kinase proteins, containing JNK, ERK and p38, which are related to cell proliferation and differentiation and widely linked to inflammation, apoptosis and cell death (Malik et al., 2015). Past studies have shown that the members of mitogen activated protein kinase (MAPK) family, like c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 are activated following cisplatin exposure, further results in renal cell death (Pabla and Dong, 2008). Kim et al explored that activation of JNK (phospho-JNK) exacerbated renal function, caused apoptosis and tubular inflammation following cisplatin treatment, showing a mechanistic part of JNK in renal toxicity (Kim et al., 2014a). The blockade of p38 MAPK activation extenuated cisplatin mediated oxidative stress, inflammation and apoptosis in kidneys (Francescato et al., 2009). Additionally, a work illustrated that cisplatin injection evoked phos-phorylation and accumulation of ERK MAPK in the mitochondria of renal proximal tubule epithelial cell (Nowak, 2002). Thence, the MAPK pathway could act as a potential target to probe new therapeutic interventions to extenuate cisplatin-evoked kidney

damage in mice. Administration of cisplatin to mice, increased expression of phosphorylation JNK and p38 in kidneys demonstrating elevated oxidative stress, inflammation and apoptosis. Supplementation with AGBE decreased phosphorylation formation and extenuated activation of apoptotic pathways in our study. Although many works reflected cisplatin induced ERK activation in several acute kidney injury^(Potocnjak and Domitrovic, 2016), no ERK induction after cisplatin exposure of kidney tissues. The reason may be interpreted with different experimental programs, like a relatively higher concentration of cisplatin was utilized in present research. Furthermore, continuous activation of p-JNK and p-p38 with high concentration of cisplatin may suppress p-ERK activation^(Shen et al., 2003; Zhong et al., 2006).

In summary, significant prophylactic effects of AGBE on cisplatin-evoked renal toxicity is achieved, via inhibiting oxidative stress, inflammation and apoptosis. However, the exhaustive mechanism underlying this effect remains ambiguous, further researches to evaluate the effects of AGBE administration against cisplatin-evoked nephrotoxicity are warranted.

Acknowledgements:

This work was supported by the grants of Scientific Research Foundation for the Returned Overseas Chinese Scholars (Jilin Province, 2015), Jilin Science & Technology Development Plan (No. 20160209008YY), and the Program for the Young Top-notch and Innovative Talents of Jilin Agricultural University (2016-2018).

Conflicts of Interest:

The authors declare no conflict of interest

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Figure legends

Figure 1. Effects of AGBE on body weight change, kidney index, the levels of CRE and BUN. All data were expressed as mean $\pm$ S.D., $n = 8$. * $p < 0.05$, ** $p < 0.01$ vs. normal group; # $p < 0.05$, ## $p < 0.01$ vs. cisplatin control group.

Figure 2. Effects of AGBE on contents of: GSH, SOD and MDA in cisplatin-evoked renal toxicity. All data were expressed as mean $\pm$ S.D., $n = 8$. * $p < 0.05$, ** $p < 0.01$ vs. normal group; # $p < 0.05$, ## $p < 0.01$ vs. cisplatin group.

Figure 3. Effects of AGBE on expression contents of: 4-HNE and CYP2E1. The expression level of 4-HNE and CYP2E1 (Green) in tissue section isolated from different groups was evaluated with immunofluorescence. The column chart showed relative fluorescence intensity. Representative immunofluorescence images were taken at 400 $\times$, 6-Diamidino-2-phenylindole (DAPI) (Blue) acted as a nuclear counterstain. All data were expressed as mean $\pm$ S.D., $n = 8$. ** $p < 0.01$ vs. normal group; # $p < 0.05$, ## $p < 0.01$ vs cisplatin group.

Figure 4. Histological analysis of morphological changes in kidney tissues, were stained with H&E (100 $\times$, 400 $\times$) Arrows show necrotic cell and inflammatory infiltrate cells. Column chart showed renal tubular damage score. All data were expressed as mean $\pm$ S.D., $n = 8$. * $p < 0.05$ vs. normal group; # $p < 0.05$, ## $p < 0.01$ vs cisplatin group.

Figure 5. Renal tissues stained with: Hoechst 33258 (200 $\times$, 400 $\times$). Column chart showed renal tubular cell apoptosis. All data were expressed as mean $\pm$ S.D., $n = 8$. * $p < 0.05$ vs. normal group; # $p < 0.05$, ## $p < 0.01$ vs cisplatin group.

Figure 6. Renal tissues stained with TUNEL staining (400 $\times$). The presence of TUNEL positive cells were evaluated by image analyzer. All data were expressed as mean $\pm$ S.D., $n = 8$. ** $p < 0.01$ vs. normal group; # $p < 0.05$, ## $p < 0.01$ vs. cisplatin group.

Figure 7. Effects of AGBE on the expression levels of: Bax, Bcl-2, and iNOS, COX-2. The column chart show antibodies stained area. The protein expression was examined by immunohistochemistry in kidney tissues from normal, cisplatin, cisplatin + AGBE (150 mg/kg), and cisplatin +AGBE (300 mg/kg). ** $p < 0.01$ vs. normal group; # $p < 0.05$, ## $p < 0.01$ vs. cisplatin control group.

Figure 8. Effects of AGBE on the protein expression levels of: Bax, Bcl-2, cytochrome c, cleaved-caspase-3; p-ERK, p-JNK, p-p38; and p-IKK α , p-IKK β , p-IkBa, p-NF- κ B. Column chart show antibodies relative expression. The protein expression was examined by western blott analysis in kidney tissues from normal, cisplatin, cisplatin

+ AGBE (150 mg/kg), and cisplatin + AGBE (300 mg/kg). All data were expressed as mean $\pm$ S.D., $n = 8$. ** $p < 0.01$ vs. normal group; # $p < 0.05$, ## $p < 0.01$ vs. cisplatin group.

Figure 9. Effects of AGBE on the levels of: TNF- α and IL-1 β in cisplatin-evoked renal toxicity. All data were expressed as mean $\pm$ S.D, $n = 8$. * $p < 0.05$, ** $p < 0.01$ vs. normal group; # $p < 0.05$, ## $p < 0.01$ vs. cisplatin control group.

Figure 1

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Figure 8

Figure 9

1. Cisplatin's toxicity limits the use of it in clinical cancer treatment.
2. As purified parts of *P. quinquefolium* are not available, berry extracts are easy and cost less to prepare.
3. ABGE eases cisplatin-induced nephrotoxicity by suppressing ROS-mediated activation of MAPK and NF- κ B signaling pathways.