ejpmr, 2016,3(12), 548-553

SJIF Impact Factor 3.628

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

EFFECTS OF AMLODIPINE AGAINST CISPLATIN-INDUCED NEPHROTOXICITY IN RABBITS

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Article Received on 20/10/2016 **Article Revised on 10/11/2016** **Article Accepted on 30/11/2016**

ABSTRACT

Nephroprotective effect of amlodipine was investigated in rabbits with acute renal injury induced by a single i.p. injection of cisplatin (6.5 mg/kg). Amlodipine treatment (5mg/ kg/ day, orally) was applied for 7 consecutive days, starting 4 days before cisplatin administration. Amlodipine had highly significantly restored all of the studied parameters to near normal values with histopathology sections showing normal looking tubules with minimal necrosis. Amlodipine significantly compensated deficits in kidney tissue glutathione level, suppressed lipid peroxidation and decreased the elevations of serum tumor necrosis factor-alpha resulted from cisplatin administration. Also, histopathological renal tissue damage mediated by cisplatin as well as inflammatory cells infiltration was greatly ameliorated by amlodipine treatment. It was concluded that amlodipine represents a potential therapeutic option to protect against acute cisplatin nephrotoxicity commonly encountered in clinical practice.

KEYWORDS: Amlodipine, Cisplatin, Nephrotoxicity.

INTRODUCTION

Nephrotoxicity is one of the leading causes of morbidity and mortality and its prevalence is continuously increasing worldwide. Among the various factors which lead to nephrotoxicity, several drugs such as antibiotics, non steroidal anti-inflammatory agents, immunosuppressive agents and chemotherapeutic agents are known to cause nephrotoxicity and it is one of the serious disadvantages with these agents.[1]

Although cisplatin has been a mainstay for cancer therapy, its use is mainly limited by two factors: acquired resistance to cisplatin and severe side effects in normal tissues especially renal tissues. Cisplatin-induced nephrotoxicity is a major complication in the cancer therapy and had a dose limiting toxicity.[2]

Nephrotoxicity was reported in the initial clinical trials of cisplatìn chemotherapy. Now, it is recognized that the prevalence of cisplatìn nephrotoxicity is high, occurring in about one-third of patient undergoing cisplatin treatment.[3]

Several interrelated mechanisms of action have been hypothesized to induce the nephrotoxicity. These include apoptosis and inflammatory mechanism, productìon of tumor necrosis factor (TNF)-α by renal parenchymal cells and oxidative stress.[4]

Cisplatin may induce injury in renal vasculature and result in decreased blood flow and ischemic injury of the kidneys, contributing to a decline in glomerular filtration rate. These events, together, culminate in the loss of renal function during cisplatìn nephrotoxicity.[5]

Calcium-channel blockers represent a group of organic chemical structures that share the ability to inhibit Ca2+ entry into excitable cells. Calcium channel blockers may help in renal vessel dilatation since there is a key role of voltage-dependent Ca2+ channels and intracellular Ca2+ stores in the alpha1A-adrenoceptor induced contraction of the renal artery.[6,7]

There is now increasing evidence that calcium channel blockers, including amlodipine, have a beneficial nephroprotective effect beyond their blood pressure lowering effect in terms of maintaining glomerular filtration rate, reducing proteinuria, decreasing production of lymphokines and decreasing oxidative stress.[8,9,10]

Aim of the Study

The aim of the present study was to investigate the probable nephroprotectìve effects of Amlodipine Against Cisplatin-induced nephrotoxicity in rabbits.

MATERIALS AND METHODS

Animals

Male New Zealand white rabbits, weighing 1.0 to 1.5 kg were obtained from local market in Baghdad, Iraq. The animals were housed in the animal house of the college of medicine Al-Nahrain University. The animals were fed on standard rodent pellet diet and water ad libitum. Before starting the study, the animals were left for 48 hours to acclimatize to the animal room conditions which were maintained on an environment of controlled temperature, a 12 hours light-dark cycle and proper ventilation. The study protocol was approved by the ethical committee of College of medicine, Al-Nahrain University.

Drugs

Cisplatin solution was obtained from ebewe pharma, Austria. Amlodipine obtained from Pfizer, USA. The required doses were taken and reconstituted in 5 ml of distilled water just before oral administration.

Experimental Design

The rabbits were randomly divided into three equal groups (n= 8, each). The first group received a single i.p. injection of normal saline (vehicle of cisplatin) and served as negative control. Nephrotoxicity was induced in animals of the second and third groups by a single i.p. injection of cisplatin at a dose of 6.5 mg/kg. The second group received distilled water orally for 7 days and served as positive control. The third group was treated with treated with Amlodipine 5mg/ kg/ day for 7 consecutive days starting 4 day before cisplatin administration.

Sample preparation and biochemical analysis

Following 72 hours of cisplatin administration the animals were sacrificed. The blood aspirated from rabbits' heart was immediately transferred into plastic test tubes without anticoagulant and allowed to clot overnight at 4°C before centrifugation for 15 minutes at 1000 rpm.

The supernatant (serum) was carefully aspirated and transferred into another clean plastic test tube, then stored in refrigerator at -20°C for subsequent measurement of serum urea, serum creatinine and serum albumin using colorimetric assay kits according to the instructions of the manufacturer (BioMérieux, France). Serum TNF-α level was measured using ELISA kit according to the instructions of the manufacturer (Cusabio, China). Kidneys were extracted free of their peritoneal attachments. Right kidneys were placed in 10% neutral buffered formalin for histopathological examination while left kidneys were homogenized in Phosphated buffered saline (PBS) and stored overnight in the refrigerator at -20°C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 rpm at 2 - 8°C. The supernatant was collected and stored at -20°C to be used for the estimation of tissue glutathione (GSH) and tissue malondialdehyde (MDA) by ELISA according to the instructions of the kit's manufacturer (Cusabio, China).

Histopathological examination of renal tissue

The right kidneys were fixed in10% formalin solution and then dehydrated in ascending grades of alcohol and embedded in paraffin. Sections at 5µm-thickness were taken, stained with hematoxylin and eosin (H&E) and examined under light microscope by a pathologist unaware of the treatment conditions.

Scores were assigned according to the percentage of cortical tubules having epithelial necrosis Scores were assigned according to the percentage of cortical tubules having epithelial necrosis by examining 10 high power fields per kidney under higher power magnification (x200) (Table 1).

Table 1: Cortical tubular necrosis scores

Score	Score Percentage of tubules with necrosis
0	0%
1	<10%
2	10%-25%
3	26%-75%
4	>75%

Statistical analysis

Statistical analysis was performed using IBM SPSS version 21 statistical software and Microsoft Excel 2010. Descriptive statistics for the numerical data were formulated as mean and standard error. Parametric independent samples t-tests were carried out for comparison between two groups whenever data were normally distributed, while non-parametric Mann-Whitney U tests were carried out whenever the data were not normally distributed. The significant difference level (p-value) is below 0.05.

RESULTS

Comparison between negative control group (normal group) and Positive control group (cisplatin group)

Serum Urea Level

Serum urea analysis done on blood collected on day 8 of experiment (72 hours after nephrotoxicity induction by cisplatin) showed that cisplatin causes elevation of serum urea level. Independent samples *t*-test showed highly statistically significant elevation of blood urea in the group received cisplatin (positive controls) compared to the negative control, $p = 0.000$ (Table 2).

Serum Creatinine Level

Independent samples *t*-test revealed a highly statistically significant difference (higher serum creatinine levels) between the negative and positive controls, $p = 0.000$(Table 2).

Serum Albumin Level
Independent samples *t*-test showed a highly statistically significant decrease in serum albumin levels between the negative and positive controls, $p = 0.000$ (Table 2).

Serum Tumor Necrosis Factor-alpha Level
Highly statistically significant difference (higher TNF-α levels) between the negative and positive controls was revealed via Independent samples *t*-test, $p = 0.000$(Table 2).

Kidney Tissue Malondialdehyde Level
Independent samples *t*-test revealed a highly statistically significant difference (higher kidney tissue MDA levels) between the negative and positive controls, $p = 0.000$(Table 2).

Kidney Tissue Glutathione Level
Highly statistically significant difference (higherkidney tissue GSH levels) between the negative and positive controls was revealed via Independent samples *t*-test, $p = 0.000$(Table 2).

Histopathological Examination
In the negative control group (normal group), normal histological picture was seen in rabbit kidneys (Figure 1). While kidney sections of the positive control group (cisplatin group) obtained on day 8 of experiment (72 hours after nephrotoxicity induction by cisplatin) shows renal damage mostly in form of glomerular and tubular injury. The tubular injury is seen mostly in the proximal convoluted tubules in which the basement membranesof the affected tubules appeared to be irregular and lost at many sites with necrosis and sloughing of their epithelial cells lining. Some of the renal tubules are distended and dilated with proteinaceous casts especially in severe nephrotoxicity. Glomerular damage including glomerular capillary necrosis with obliteration of capillary lumens and increase of cellularity as a result ofacute inflammatory cellsinfiltration (neutrophils) and mesangial cells hyperplasia resulting in the reduction of the glomerular filteration rate and protein urea with cast formation in renal tubules (Figure 2).

Cortical Tubular Necrosis Scores
Scores of cortical tubular necrosis were calculated on kidney tissue sections on day 8 of experiment (72 hours after nephrotoxicity induction); it showed that cisplatin causes cortical tubular necrosis. Independent samples *t*-test showed a highly statistically significant difference (higher cortical tubular necrosis score) in the positive control compared to the negative control, $p = 0.000$ (Table 2).

Neutrophil Count
Average of neutrophil count showed that cisplatin causes marked elevation of neutrophil count. Independent samples *t*-test showed a highly statistically significant difference (higher neutrophil count) in the positive control group compared to the negative control, $p = 0.001$ (Table 2).

Comparison between positive control group (cisplatin group) and treatment group (amlodipine)
Serum Urea Level
Serum urea analysis done on blood collected on day 8 of experiment (72 hours after nephrotoxicity induction by cisplatin) showed that Amlodipine effectively reduced the serum urea level in the present study to near normal levels. Independent samples *t*-test showed highly statistically significant lower levels of serum urea in the group treated with amlodipine compared to the positive control, $p = 0.000$(Table 2).

Serum Creatinine Level
Independent samples *t*-test showed a highly statistically significant difference (lower serum creatinine levels) in the amlodipine treated group and had effectively reduced the serum creatinine level in the present study to near normal levels. Independent samples *t*-test showed a highly statistically significant difference (lower serum creatinine levels) in the amlodipine group in comparison to the positive controls, $p = 0.000$ (Table 2).

Serum Albumin Level
In the present study, amlodipine had effectively increased the serum albumin level to near normal levels. Independent samples *t*-test showed a highly statistically significant difference (higher serum albumin levels) in the amlodipine group in comparison to the positive controls, $p = 0.000$ (Table 2).

Serum Tumor Necrosis Factor-alpha Level
Independent samples *t*-test showed a highly statistically significant difference (lower serum TNF-α levels) in the amlodipine group in comparison to the positive control, $p = 0.000$ yet the levels are still higher than the normal levels (Table 2).

Kidney Tissue Malondialdehyde Level
Amlodipine very effectively reduced the kidney tissue MDA level in the present study. Independent samples *t*-test revealed a highly statistically significant difference (lower kidney tissue MDA levels) in the amlodipine group compared to the positive control group, $p = 0.000$ (*T*able 2).

Kidney Tissue Glutathione Level
amlodipine seem to increase the tissue glutathione level very effectively in this study. Independent samples *t*-test showed a highly statistically significant difference in the amlodipine group compared to the positive control group, $p = 0.000$ (Table 2).

Histopathological Examination
Kidney sections of Amlodipine group and Thyme group showed nearly normal histological picture with minimal pathological changes (Figure 3).

Cortical Tubular Necrosis Scores

Amlodipine and thyme extract effectively reduced the cortical tubular necrosis scorein this study. Independent samples *t*-test showed a highly statistically significant difference (lower cortical tubular necrosis score) compared to the positive control group, $p = 0.000$ (Table 2).

Neutrophil Count

Amlodipine very effectively reduced the neutrophil count in the present study. Mann-Whitney U test showed a highly statistically significant difference (lower neutrophil count) in the amlodipine treatment group compared to the positive controls, $p = 0.000$. (Table 2).

Table 2: Comparison between negative control group (normal group), positive control group (cisplatin group) and treatment group (amlodipine group).

Groups / Parameters	Negative control group (normal group)	Positive control group (cisplatin group)	Amlodipine group
Serum urea	35.34±1.40[a]	100.23±0.86[b]	35.43±1.70[c]
S. creatinine	0.42±0.09[a]	4.84±0.08[b]	0.36±0.04[c]
Serum albumin	2.92±0.03[a]	0.92±0.02[b]	2.09±0.08[c]
Serum TNF-α	42.91±1.84[a]	322.55±5.33[b]	136.88±6.67[c]
Tissue MDA	180.50±0.64[a]	505.39±1.70[b]	181.68±0.58[c]
Tissue GSH	62.41±0.60[a]	11.40±0.19[b]	60.05±0.54[c]
Tubular necrosis score	00.00±0.00[a]	3.63±0.18[b]	1.00±0.00[c]
Neutrophil count	00.00±0.00[a]	5.03±0.92[b]	0.58±0.14[c]

Results represent mean±SD, different superscript (a,b,c) identify highly significant change ($p \leq 0.001$) between different groups.

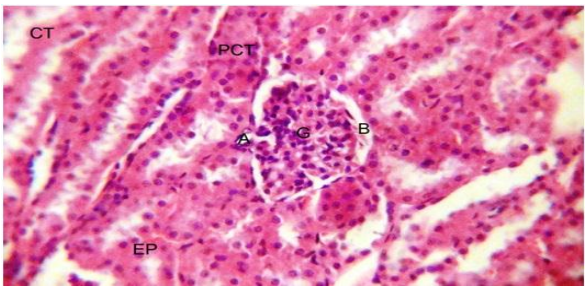

Figure 1: Section of right kidney of a White New Zealand rabbit of negative control group (normal group) showing normal renal tubules with viable epithelial cells.40X, H&E. A: Afferent glomerular arteriole, B: Bowman's capsule, CT: Collecting tubule, EP: Epithaelial cells, G: Glomerulus, PCT: Proximal convoluted tubule.

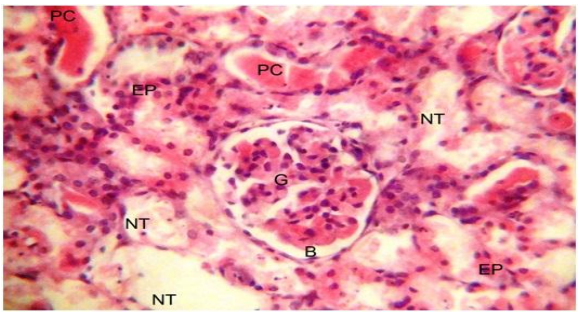

Figure 2: Section of right kidney of a White New Zealand rabbit of positive control group (cisplatin group) 72 hours after administration of 6.5mg/ kg cisplatin I.P. showing tubular necrosis with loss of nuclei, fragmentation of cells and leakage of content in 79% of tubules. Some tubules have proteinaceuos casts. (A) 20X, H&E. (B) 40X, H&E. B: Bowman's capsule, EP: Epithelial cells, G: Glomerulus, NT: Necrotic tubule, PC: proteinaceous cast.

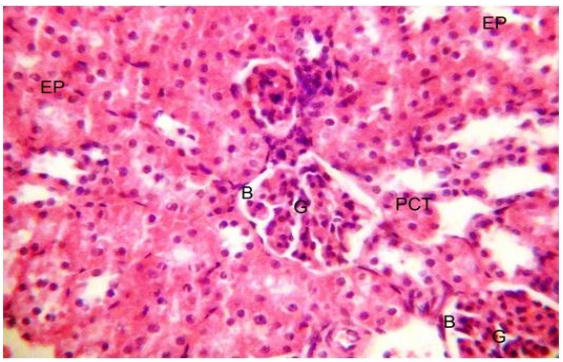

Figure 3: Section of right kidney of a White New Zealand rabbit of amlodipine group treated with amlodipine 5mg/ kg/ day, 72 hours after administration of cisplatin 6.5mg /kg I.P. showing normal looking renal tubules with viable epithelial cells.40X, H&E.B: Bowman's capsule, EP: Epithaelial cells, G: Glomerulus, PCT: Proximal convoluted tubule.

DISCUSSION

Cisplatin Induced Nephrotoxicity

Cisplatin is one of the potent anticancer agents that is not only cause nephrotoxicity but also induces vascular endothelial dysfunction mediated by inflammation and oxidative stress. Cisplatin-induced nephrotoxicity is a major complication in the cancer therapy and had a dose limiting toxicity.[2] The important parameters that should be taken into account in the evaluation of kidney damage caused by cisplatin are urea and creatinine. These markers are the end products of various metabolic pathways that are excreted in the urine via glomerular filtration whose serum levels are an indicator of renal functions. In cisplatin induced nephrotoxicity, a significant elevation in the levels of urea and creatinine

were observed which serves as an indicator of impaired renal functions.[11]

In the present study, using an experimental model of cisplatin induced nephrotoxicity in rabbits (single dose of 6.5 mg/ kg I.P.) characterized by alterations in renal function as a significant increase in serum creatinine, urea, albumin and TNF-α as well as increased MDA and decreased GSH levels compared to normal group and this results are compatible with those observed by many other studies.[12,13] Oxidative stress injury is actively involved in the pathogenesis of cisplatin induced nephrotoxicity. Reactive Oxygen Species (ROS) directly act on cell components, including lipids. Free radicals damage the lipid components of the cell membrane by peroxidation and denaturation of proteins, which lead to enzymatic inactivation. The production of MDA is an index of lipid peroxidation.[14] It has been reported that cisplatin induced nephrotoxicity is closely associated with an increase in lipid peroxidation in the kidney manifested by increased MDA as well as a decrease in anti oxidant activity with depletion of GSH which is in agreement with the results obtained in the present study.[15] Glutathione (GSH) is a major non protein thiol in living organisms that plays an important role in protection against free radicals, peroxides, and other toxic the compounds.[16] Platinum sulhydryl group complexes are taken up by renal cells and stabilized by intracellular GSH for several hours. In case of intracellular GSH depletion, the complexes undergo rapid transformation to reactive metabolites. Thus GSH depletion results in increased toxicity of cisplatin.[17]

The present study along with other studies have shown increased tissue content of inflammatory mediators together with inflammatory cell infiltration, suggesting that inflammation plays an important role in cisplatin induced renal injury.[18] Although the precise inflammatory mechanisms are unknown, marked attenuation of cisplatin induced renal damage by inhibition of tumor necrosis factor alpha (TNF-α) indicates that TNF-α has a central role of mediation cisplatin induced renal injury.[13]

Histopathological results of this study showed severe kidney damage characterized by severe necrosis of tubular cells and inflammatory cell infiltration that could be correlated with the harmful effects of cisplatin parallel to high MDA and low GSH levels. These results are in agreement with the results in other studies.[19,12] The increase in thickness of the glomeruli basement membrane could be a result of membrane disturbance due to cisplatin administration. Arda-Pirincci *et al*., 2009 mentioned that lipid peroxidation mediated by oxygen free radicals causes destruction and damage to cell membranes resulting in necrosis.[20]

Amlodipine Effects on Cisplatin-induced Nephrotoxicity

Cisplatin may induce injury in renal vasculature and result in decreased blood flow and ischemic injury of the kidneys, contributing to a decline in glomerular filtrationrate.[5] Nifedipine and amlodipine effectively reversed the effect of gentamicin-induced renal tubular toxicity in rats. Amlodipine has nephroprotective effect against high- and low-osmolar contrast media and doxorubicin induced nephrotoxicity.[10]

Effect on Kidney Functions

Results of the present study showed that pretreatment with amlodipine (5mg /kg /day) for 4 days before cisplatin administration followed by 3 more days had effectively maintained normal kidney functions. Amlodipine reduced serum urea and serum creatinine and increased serum albumin levels in statistically highly significant difference almost to the normal levels. The present study is in agreement with other previous studies.[10] Calcium-channel blockers may help in renal vessel dilatation since there is a key role of voltage-dependent Ca2+ channels and intracellular Ca2+ stores in the alpha1A-adrenoceptor induced contraction of the renal artery. Cisplatin significantly increased the intracellular calcium level of renal epithelial cells in a dose-dependent manner. Increased intracellular calcium levels result in oxidative damage and necrosis associated with up regulation of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and Inducible Nitric-oxide Synthase.[21]

Effect on Inflammation

Results of this study revealed that amlodipine alleviates inflammation. Amlodipine highly significantly reduced serum TNF-α level. The present study is in agreement with other previous studies.[22]

Effect on Oxidative Stress

Results of the present study showed that amlodipine had effectively maintained normal levels of oxidative stress parameters. Amlodipine reduced tissue MDA and increased tissue GSH levels in statistically highly significant difference almost to the normal levels. The present study is in agreement with other previous studies.[8,10]

Effect on Renal Histopatology

Histopathological examination of kidney tissues in the present study showed that amlodipine has marked beneficial effect. Amlodipine greatly ameliorated the histopathological changes; histological examination showed very mild inflammatory cells infiltration, no atrophy, minimal necrosis and tubules almost back to normal. The present study is in agreement with other previous studies.[10]

In Conclusion, results of this study showed that treatment with amlodipine (5 mg/ kg/ day, orally), had protective effects against cisplatin induced nephrotoxicity in rabbits. Amlodipine found to induce significant effects in

alleviating inflammation & oxidative stress, maintaining normal kidney functions and preventing histopathological changes.

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