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Role of Autophagy Activation in Prevention of Cisplatin Induced Acute Kidney Injury in Rats

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ABSTRACT

Acute kidney injury (AKI) is a public health problem associated with significant morbidity and mortality, and is still a major challenge for nephrologists. This work aimed to evaluate the role of autophagy activation in the prevention of cisplatin-induced acute kidney injury. This experimental work included 50 male Sprague–Dawley rats that were 60–80 days old. The rats were divided into five groups randomly. We used rapamycin, a well-known activator of autophagy, and chloroquine, a known agent that inhibits autophagy flux. By quantifying serum creatinine, we assessed the impact of rapamycin on kidney function after exposure to the nephrotoxic agent cisplatin. To assess autophagy induction, we used the LC3-II antibody. As regards the use of rapamycin, rats received cisplatin plus rapamycin at 2 mg/kg daily intraperitoneal. The change in serum creatinine was statistically significantly higher than that in rats that received cisplatin only (P = 0.028). With the use of chloroquine, we found that rats received cisplatin plus rapamycin plus chloroquine, serum creatinine was statistically significantly lower than that in rats that received cisplatin plus rapamycin (P = 0.001) and also lower than that in rats that received cisplatin only (P = 0.015). Also, histopathological data in rats that received cisplatin plus rapamycin plus chloroquine revealed a lower ATN score than cisplatin alone (P = 0.001). We can conclude that use of rapamycin didn't offer nephroprotection while chloroquine use attenuated the level of acute kidney injury.

1. INTRODUCTION

Acute kidney injury (AKI) is one of the most common diseases among critically ill adults and children admitted to the intensive care unit (ICU), and it is linked to serious short-and long-term outcomes, including increased mortality [1]. Nephrotoxicity can be caused by a variety of cytotoxic drugs. While many medications cause a dose-related and predictable decrease in glomerular filtration rate (GFR), others cause a more prolonged and, in many cases, permanent deterioration in renal function [2]. Cisplatin is an inorganic

platinum derivative used to treat different types of malignancies, including the bladder, ovary, lung, and testis [3]. Cisplatin nephrotoxicity is a common side effect that affects thirty percent of patients [4], limiting the dose of cisplatin that can be safely administered to cancer patients, which is also a clinical issue in cancer patients receiving cisplatin-based therapy, on the other hand, this is one of the most commonly prescribed anticancer medications [5]. Cisplatin predominantly damages the S3 section of the proximal tubule, resulting in a reduction in GFR, it causes necrosis and apoptosis, as well as reduced renal blood flow owing to vasoconstriction, which is noticed shortly after cisplatin injection [6]. Also, tumour necrosis factor, interleukin-6 (IL-6) and interferon-gamma (IFN-gamma) production, as well as caspases, are all increased. [7]. There is no particular treatment approach available for treating patients with cisplatin-induced AKI, except in the case of supportive treatments such as fluid resuscitation and renal replacement therapy [8]. As a result, developing new drugs to cure or prevent cisplatin-induced acute kidney injury might benefit patients receiving cisplatin-based chemotherapy [9]. Targeting the autophagy pathway is one of the novel strategies that has been demonstrated in different models of AKI in vivo and in vitro, but there is no consensus on whether autophagy is protective or deleterious in AKI.

Autophagy is a cellular "autodigestive" process that allows intracellular components to be transferred from the cytoplasm to lysosomes or vacuoles for destruction and recycling, it involves the removal and recycling of bulk cytoplasmic components, misfolded proteins, and damaged intracellular organelles to maintain cellular homeostasis [10]. It works as a dynamic recycling system, producing new components and energy for cellular regeneration and homeostasis while also removing waste [12]. Because mTORC1 inhibition increases autophagy, mTORC1 inactivation activates the ULK1 complex of the autophagy pathway, allowing ULK1 to phosphorylate its related components Atg13 and FIP200, resulting in ULK1 activation [14]. Rapamycin and its analogues have been used as intervention techniques to promote autophagy in several experimental models of acute kidney injury [15].

Therefore, the aim of the study is to explore the potential effects of rapamycin on cisplatin induced acute kidney injury in rats.

2. Materials and Methods

Research ethical approval was obtained from the research ethical committee (REC), Mansoura University, Faculty of medicine, Mansoura, Egypt, as established by the institutional animal care and use committee (IACUC). Furthermore, all methods were completed in accordance with arrive guideline. This study was conducted on 50 male Sprague–Dawley rats who were 60–80 days old and weighed 150–200 grams each at the beginning of the study.

To generate a cisplatin induced AKI model, rats were injected with a single dose of intraperitoneal cisplatin 7 mg/kg (16). To inhibit autophagy flux, rats were treated with chloroquine (60 mg/kg intraperitoneal) 1 hour before cisplatin injection, followed by an additional chloroquine injection at 24 hours after the cisplatin injection.

Experimental male Sprague-Dawely rats were randomly divided into 5 groups, each group contained 10 rats as follows:

Group (1): Rats received intraperitoneal normal saline.

Group (2): Rats received a single dose of cisplatin 7 mg/kg intraperitoneal.

Group (3): Rats received intraperitoneal dimethyl sulfoxide (DMSO) equal to rapamycin volume plus intraperitoneal cisplatin.

Group (4): Rats received rapamycin dissolved in dimethyl sulfoxide (DMSO), rapamycin dose was 2 mg/kg

daily (2 days before the cisplatin injection and continued after, till rats' sacrification).
Group (5): Rats received rapamycin dissolved in dimethyl sulfoxide (DMSO) with the same previous regimen plus chloroquine.

At the end of the experimental period, rats were sacrificed 72 hours after the cisplatin injection.

2.1 Renal function

Renal function was monitored by serum creatinine. Two blood samples were collected. The first assay was just before the cisplatin injection, and the second assay was just before sacrificing rats.

2.2 Renal histopathology

Kidney samples were embedded in paraffin after being fixed in 10% neutral formalin. H & E staining was done on 3 mm thick sections. A semi-quantitative scoring approach was employed to assess kidney injury, as indicated by tubular necrosis, cellular casts, and tubular injury. Score 0 reflects an injury area of less than 10%, whereas scores 1, 2, 3, or 4 represent an injury involving 10–25%, 25–50%, 50–75%, or > 75% of the field, respectively. For each rat, at least ten randomly selected fields under the microscope (×400) were examined, and an average score was obtained (9).

2.3 Immunohistochemistry analysis

Antibodies directed against LC3-II have been used for Immunohistochemical staining (dilution 1:50). Serial sections (4 m thick) were obtained, deparaffinized in xylene, and hydrated in a graded series of alcohols. Microwave pretreatment in citric acid buffer (10 mM, pH 6.0) for 10 minutes was used to perform heat induced antigen retrieval. Endogenous peroxidase was inhibited, and detection was performed using the DAKO EnvisionTM system (K4011; DAKO Corp, Carpinteria, CA). Blinded grading of the slides was used to assess immunohistochemical staining, and four intensity categories were recorded: 0 (no staining), 1+ (weak), 2+ (moderate), and 3+ (strong) (17).

2.4 Electron microscope

To identify autophagosomes in renal tissues. The cortical kidney tissue samples were cut into small portions using a scalpel and then fixed with 2.5% glutaraldehyde at room temperature. After that, the sections were post-fixed in 1% osmium tetroxide for 2 hours in a dark room. The specimens were dehydrated in graded ethanol washes and infiltrated with propylene oxide for 3 h at room temperature. Following that, the sections were re-infiltrated with pure epoxy resin overnight at 4 °C before being implanted and polymerized for 48 hours at 60 °C. The ultrathin sections (60–100 nm) were stained with 1% uranyl acetate for 10 min, followed by 2% Reynolds lead citrate buffer for 2 min (18).

2.5 Statistical Analyses

IBM-SPSS software (Version 26.0, 2019) was used to enter and analyse data. Quantitative data was first assessed for normality using Shapiro-Wilk's test with $p > 0.050$ indicating that the data was normally distributed. Boxplots were examined for the existence of significant outliers (extreme values). The mean, standard deviation (SD), or median were used to express quantitative data. To compare normal distributed data amongst the five groups, one way ANOVA was performed. Welch ANOVA was used, followed by the Games Howell post hoc test, because homogenicity of variance was violated. To compare non-normally distributed data amongst different groups, the Kruskal-Wallis H-test was used. If the p value for any of the tests employed was less than 0.050, the findings were considered statistically significant. Appropriate charts were used to graphically present the results whenever needed.

3. Results

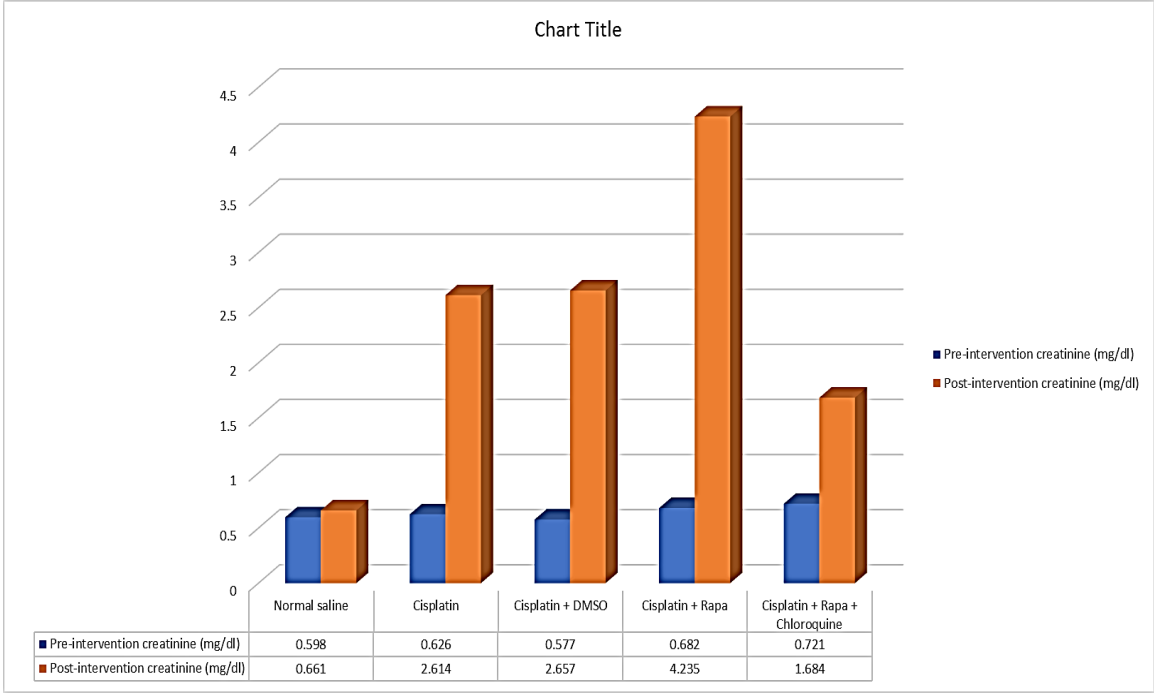

Figure (1): Bar chart for pre- and post-intervention serum creatinine in the 5 groups

Table (1): Serum creatinine change in the study groups

Group	Change
NS	0.06 ± 0.09
Cis	1.99 ± 0.7
Cis+ DMSO	2.08 ± 1.1
Cis+ Rapa	3.55 ±1.25
Cis+Rapa+ Chloro	0.96 ± 0.46

Notes: Data are mean ± SD. NS = Normal saline. Cis = Cisplatin. Cis+ DMSO = Cisplatin + DMSO. Cis+ Rapa = Cisplatin + Rapamycin. Cis+Rapa+ Chloro = Cisplatin + Rapamycin + Chloroquine. The Homogeneity of variances was violated (Levene test, P<0.001).

Accordingly, Welch ANOVA was reported, and it shows a statistically significant difference in serum creatinine change between the 5 groups (P<0.001). So, Games-Howell Post-Hoc tests were reported.

Games-Howell post hoc analysis revealed the following:

1. In normal saline group (NS): Serum creatinine was statistically significantly lower than that in Cis group, Cis+DMSO group, Cis+Rapa group, and Cis+Rapa+Chloro group.
2. In Cisplatin group (Cis): Serum creatinine was not statistically significantly different from Cis+DMSO group. It was statistically significantly lower than that in Cis+Rapa group, and, statistically significantly higher than that in the normal saline group (NS) and Cis+Rapa+Chloro group.
3. In Cisplatin/DMSO group (Cis+DMSO): Serum creatinine was not statistically significantly than that in Cis group, Cis+Rapa group and Cis+Rapa+Chloro group. It was statistically significantly higher than that in the normal saline group (NS).
4. In Cisplatin/Rapamycin group (Cis+Rapa): Serum creatinine was statistically significantly higher

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than that in all groups except Cis+DMSO group.

5. In Cisplatin/Rapamycin/Chloroquine group (Cis+Rapa+Chloro): Serum creatinine was statistically significantly lower than that in Cis group and Cis+Rapa group. It was statistically significantly higher than that in the normal saline group (NS).

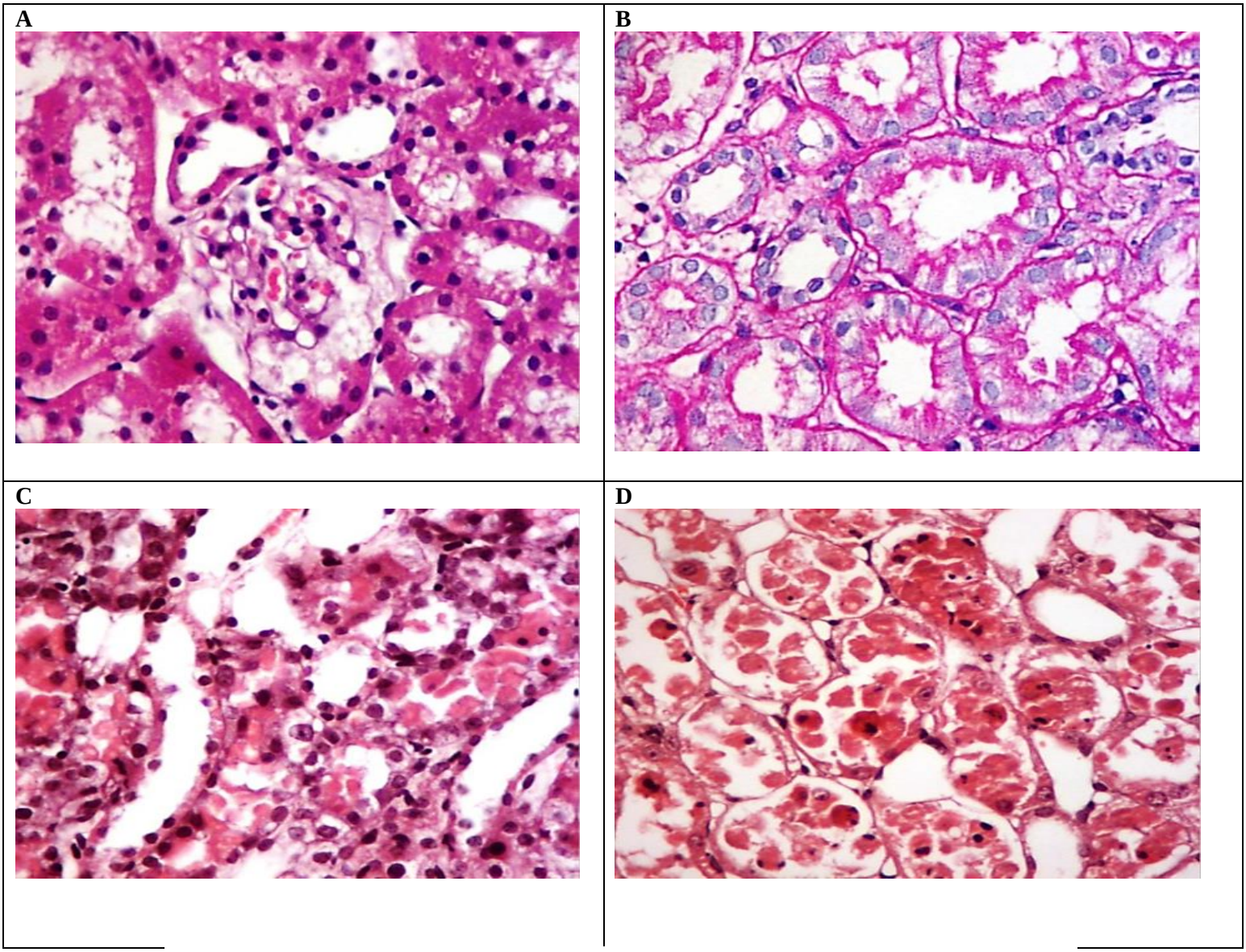

Figure (2): Histopathological results: Light microscopical examination of H&E and PAS stains. **(A)** Cortex with normal glomerulus and intact tubules with no necrotic changes or regenerative changes (H&E X 400) in normal saline group **(B)** Tubules with intact architecture & intact brush border (PAS X 400) in normal saline group **(C)** Necrosis involving 10–25% of tubules score (1) (H&E X400) in Cisplatin +Rapamycin+ Chloroquine group **(D)** Necrosis involving >75of tubules score (4) (H&E X400) in Cisplatin group.

Table (2): ATN score in the 5 groups

Group	Median	Minimum – Maximum	H [4]	P value
NS	0	0 – 0	33.77	**<0.001**
Cis	4	3 – 4		
Cis+ DMSO	3	2 – 4		

Cis+ Rapa	3	2 – 4		
Cis+Rapa+ Chloro	1.5	0 – 4		

Notes: Data are median (minimum – maximum). Test of significance is Kruskal-Wallis H-test. NS = Normal saline. Cis = Cisplatin. Cis+ DMSO = Cisplatin + DMSO. Cis+ Rapa = Cisplatin + Rapamycin. Cis+Rapa+ Chloro = Cisplatin + Rapamycin + Chloroquine.

This table shows that the ATN score was statistically significantly different among the 5 study groups. Pairwise comparisons revealed a statistically significantly lower ATN score in NS group vs Cis group, Cis+ DMSO group, and Cis+ Rapa group, a statistically significantly lower ATN score in Cis+Rapa+ Chloro group vs Cis group. In all other pairwise comparisons, the difference was not statistically significant.

Table (3): LC3-II score in the 5 groups

Group	Median	Minimum – Maximum	H [4]	P value
NS	0	0 – 1	35.67	**<0.001**
Cis	1	0 – 2		
Cis+ DMSO	1	0 – 2		
Cis+ Rapa	3	2 – 3		
Cis+Rapa+ Chloro	3	1 – 3		

Notes: Data are median (minimum – maximum). Test of significance is Kruskal-Wallis H-test. NS = Normal saline. Cis = Cisplatin. Cis+ DMSO = Cisplatin + DMSO. Cis+ Rapa = Cisplatin + Rapamycin. Cis+Rapa+ Chloro = Cisplatin + Rapamycin + Chloroquine.

This table shows that LC3 scores were statistically significantly different among the 5 study groups. Pairwise comparisons revealed a statistically significantly lower LC3 score in NS group vs. Cis+ Rapa group, and Cis+Rapa+ Chloro group, a statistically significantly lower LC3 score in Cis group vs. Cis+ Rap group a, and Cis+Rapa+ Chloro group, and a statistically significantly lower LC3 score in Cis+ DMSO group vs Cis+ Rapa group, and Cis+Rapa+ Chloro group. In all other pairwise comparisons, the difference was not statistically significant.

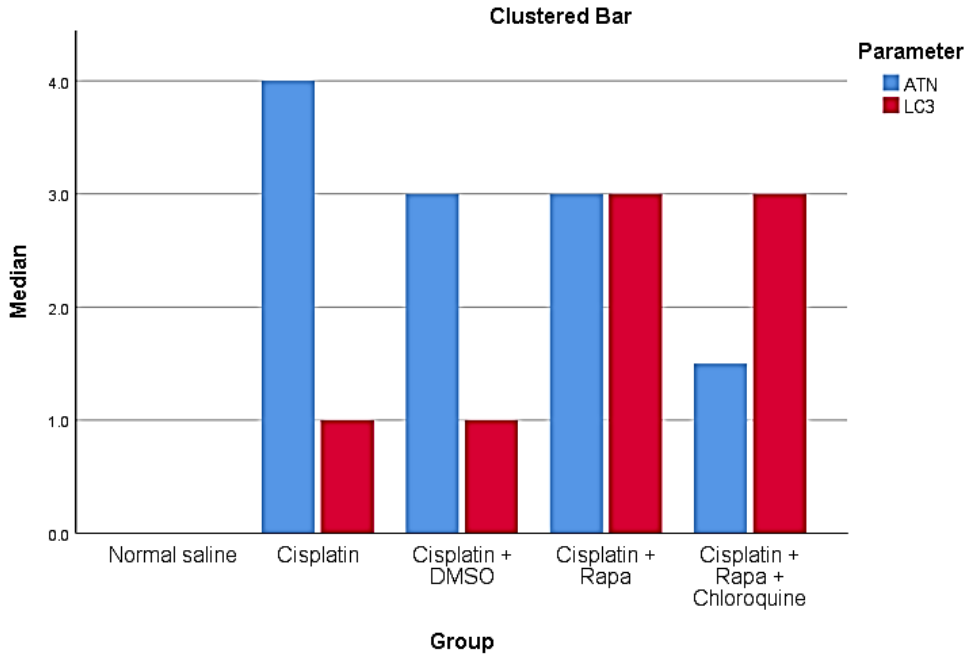

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Figure (3): Bar chart for Median ATN Score and LC3-II Score in the 5 groups

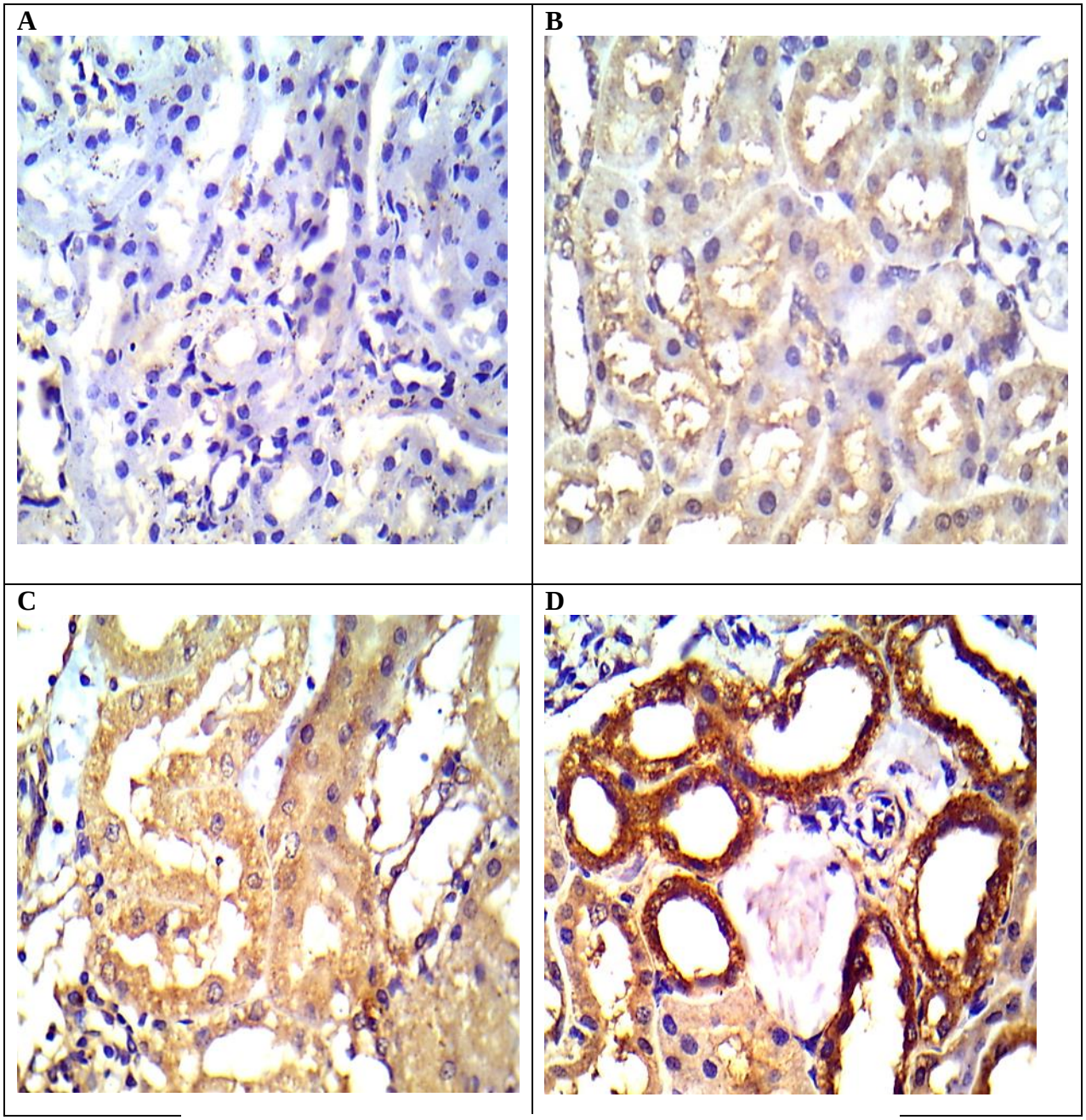

Figure (4): Immnuo-staining images for LC3 II among different groups
(A) Tubules with no staining for LC3 (Score 0) in normal saline group **(B)** Tubules with weak staining for LC3 (Score 1) in Cisplatin only group **(C)** Tubules with moderate staining for LC3 (Score 2) in Cisplatin +Rapamycin group **(D)** Tubules with strong staining for LC3 (Score 4) in Cisplatin +Rapamycin+ Chloroquine group.

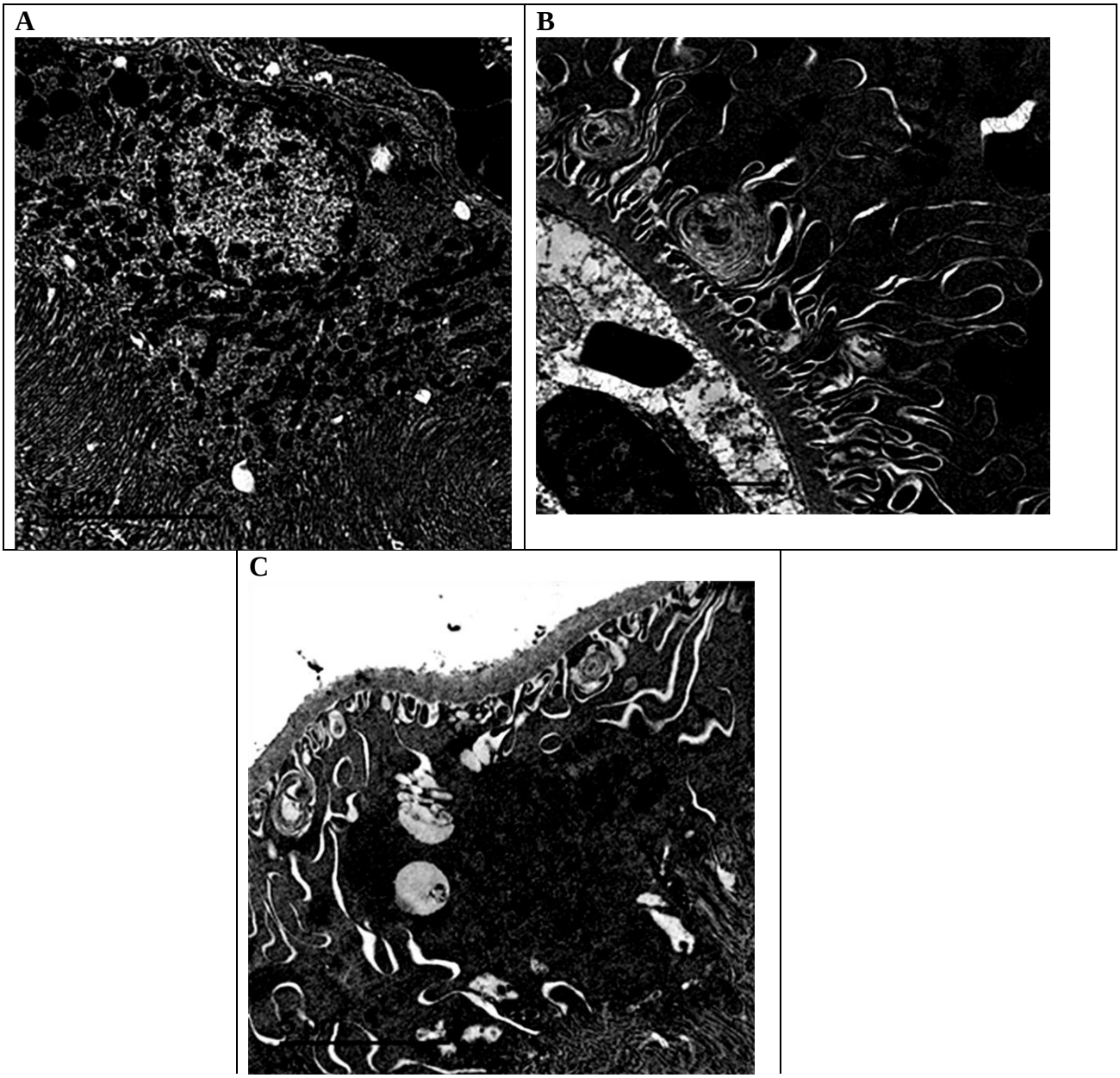

Figure (5): Electron microscope images among different groups
(A) Tubules with low number of autophagosomes in Cisplatin only group **(B)** Tubules with mild increase in number of autophagosomes in Cisplatin +Rapamycin group **(C)** Tubules with marked increase number of autophagosomes in Cisplatin +Rapamycin+ Chloroquine group.

4. Discussion

Acute kidney injury (AKI) is a major global health problem, and even though the International Society of Nephrology's goal of eliminating preventable deaths due to AKI by 2025, AKI has harmful short-term consequences, such as longer hospital stays, greater disability after discharge, and a higher risk of in-hospital mortality, as well as long-term consequences, such as progression to chronic kidney disease, development of cardiovascular disease, and an increased risk of long-term mortality [19]. According to previous research, autophagy is rapidly triggered during AKI to protect tubular cells from damage and death [20]. Autophagy has been shown to have a protective function in the pathogenesis of AKI, but other studies have found that autophagy increases the development of cellular senescence, which adds to renal ageing and accelerates the progression from AKI to CKD, so autophagy acts as a double-edged sword [21]. Autophagy reduces the

activation of apoptosis-associated caspase and limits the induction of apoptosis, potentially reducing cellular damage [22]. On the other hand, autophagy or autophagy-related proteins may assist in the induction of apoptosis in some cases, thereby aggravating cell damage [23].

We investigate the role of autophagy in prevention of cisplatin induced acute kidney injury. In our study we used single toxic dose of cisplatin 7mg/kg injected intra-peritoneal to generate cisplatin induced acute kidney injury rat model. We confirmed the nephrotoxicity of cisplatin by finding that, Serum creatinine and ATN score were statistically significantly higher in rats received intraperitoneal cisplatin only than those received normal saline. This is in agreement with Abd El-Rhman and his group who gave the same dose of cisplatin and noted the same nephrotoxicity in rats received cisplatin only [24].

We selected rapamycin as it is a potent, specific inhibitor of mTOR and an activator of autophagy. Based on previous reports, there is conflicting data on whether rapamycin is protective in the prevention of cisplatin induced acute kidney injury or not.

In our study, rats received cisplatin plus rapamycin 2 mg/kg daily intraperitoneal. The change in serum creatinine was statistically significantly higher than that in rats that received cisplatin only. Also, in rats that received cisplatin plus rapamycin, there was increased expression of LC3 antibody by immunohistochemical analysis, confirming the ability of rapamycin to induce autophagy.

Regarding biochemical and histopathological data, we didn't notice any nephro-protection with the use of rapamycin. This is consistent with the findings of who discovered that Rapamycin at 1 mg per kg intraperitoneal dose is not protective against cisplatin-induced kidney injury [25]. Furthermore, discovered that Rapamycin at a dose of 2 mg per kg I.P failed to improve renal function following ischaemia–reperfusion-induced kidney injury [26].

In LC3-GFP stably transfected proximal tubular cells, Inoue and colleagues used rapamycin to trigger autophagy and autophagosome production in vitro, they found that taking rapamycin increased cisplatin-induced apoptosis [27]. Observed that rapamycin impaired recovery from acute renal failure by inhibiting proliferation and inducing cell cycle arrest and apoptosis in tubular cells [28]. Found that rapamycin significantly decreased GFR, rapamycin elicited a 25% reduction in creatinine clearance in Sprague-Dawley rats [29].

In contrast to our finding, found that rapamycin-induced autophagy augmentation may protect against cisplatin-induced AKI, providing evidence for autophagy's renoprotective role in the kidneys. [30]. Discovered that rapamycin-induced increased autophagy protects against renal I/R injury by suppressing tubular cell death [31].

According to, rapamycin autophagy activation via the mTORC1/ATG13/ULK1 signalling pathway alleviates acute kidney damage following cerebral ischemia and reperfusion [32]. Wang and his colleagues used rapamycin in Cisplatin-treated mice but noticed a decrease in serum creatinine, histological findings also suggested that rapamycin therapy reduced renal tubular damage during Cisplatin treatment [33]. This controversy can be explained by the fact that Rapamycin nephrotoxicity is strain and species-dependent [29].

We used chloroquine, which inhibits autophagy by preventing the fusion of the autophagosomes with lysosomes [34]. We found that adding chloroquine reduced the severity of acute renal injury. In rats that received cisplatin plus rapamycin plus chloroquine, serum creatinine was statistically significantly lower than

that in rats that received cisplatin plus rapamycin, and in rats that received cisplatin only. Also, histopathological data in rats that received cisplatin plus rapamycin plus chloroquine revealed a lower ATN score than cisplatin only. These findings raise questions about the potential role and mechanisms of chloroquine in attenuating cisplatin induced acute kidney injury.

This is in line with, who concluded that acute pretreatment with chloroquine attenuates renal I/R injury in rats [35]. Found that hydroxychloroquine, which is a chloroquine analog, attenuates renal ischemia-reperfusion injury by blocking cathepsin-mediated NLRP3 inflammasome activation [36]. Found that cisplatin-induced apoptosis is suppressed by inhibition of macroautophagy in proximal tubular cells, but in this study, another autophagy inhibitor was used, 3-methyladenine (3-MA), which is widely used as an autophagy inhibitor [27]. In their research, Zhang and colleagues found that chloroquine reduced acute kidney injury caused by lipopolysaccharide therapy, as demonstrated by a reduction in KIM-1 and HMGB-1 expression [37]. Mei and his colleagues found that pharmacological suppression of autophagy in animal model of endotoxic acute kidney damage with chloroquine improved LPS-induced AKI [38].

On the other hand, Periyasamy and colleagues found that cisplatin activated autophagy in cultured renal proximal tubular cells within hours, before apoptosis. Importantly, pharmacological inhibitors of autophagy and beclin-1 knockdown increased apoptosis during cisplatin therapy, suggesting that autophagy may have a protective role in cisplatin-induced kidney damage and nephrotoxicity [39]. Found that cisplatin-induced tissue damage in the renal cortex and outer medulla, as well as chloroquine-induced autophagy inhibition, worsens AKI [30]. Wei and his colleagues discovered that administering chloroquine to block autophagy was ineffective in reversing cisplatin-induced kidney damage [40].

Many of the same stimuli that induce autophagy also activate cell apoptosis, which occurs before autophagy [20]. This clear disparity exists among research examining the involvement of autophagy in acute renal injury, is unknown as stated by Livingston MJ and his group, also assuming that those discrepancies depending on experimental settings, autophagy can be either beneficial or harmful [20].

5. Conclusion

Rapamycin activation of autophagy didn't offer nephron-protection; on the contrary, it aggravates acute kidney injury. Chloroquine attenuates the level of acute kidney injury. This might be owing to chloroquine's ability to suppress autophagy flux, its anti-inflammatory properties, or other pathways that chloroquine can interact with. This is an interesting point that needs thorough research.

Conflict of interest statement:
No conflict of interest

Authorship:
Study designed by Ahmed Samir Megahed, Khaled Farouk Eldahshan and Tarek Medhat Abbas
Data collected by Ahmed Samir Megahed, Rasha Mahmoud Abd Elfattah Attia and Samar Ahmed
Data analyzed by Ahmed Samir Megahed, Dina Abdallah Ibrahim Atwa and Ghada Mohamed Hassan El Kanishy
The paper wrote by Ahmed Samir Megahed

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