

# Renoprotective Effect of Atorvastatin Against Contrast-induced Acute Kidney Injury in Male Rats

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## ABSTRACT

Contrast-induced nephropathy defined as an increase in serum creatinine  $\geq 25\%$  from baseline at 48–72 hours after exposure to contrast media in the absence of another cause for acute kidney injury. Atorvastatin, a lipid-lowering agent, may protect the renal functions from contrast-induced acute kidney injury by other effects not related to lipid levels termed “pleiotropic effects” such as decreasing endothelin synthesis, angiotensin receptors, expression of endothelial adhesion molecules, inflammation, and reactive oxygen species production. This study was designed to evaluate the protective effect of atorvastatin against nephropathy induced by iopromide, as a contrast media. Forty adults’ male Wistar rats were used in this experiment, each group contains ten rats, as follow: first group (control) received normal saline; second group (contrast-induced nephropathy) received iopromide as contrast media; the third group received atorvastatin (20mg/kg) and contrast media, and the fourth group received atorvastatin (40mg/kg) and contrast media. Renal function tests (creatinine clearance, serum urea, microalbuminuria, and lipocalin-2) and vascular endothelial function tests (nitric oxide and prostaglandin) were measured. Histopathological study and immunohistochemical staining were achieved to evaluate caspase-3 and vascular endothelial growth factor expression. The renal functions and endothelial functions notably deteriorated in contrast-induced nephropathy group; while treatment with low and high dose atorvastatin significantly improved these functions, compared with contrast-induced nephropathy group. Histopathological study showed severe tissue damage (medullary congestion and tubular necrosis) in contrast-induced nephropathy group, while treatment with low and high atorvastatin dose significantly protected the renal tissue. Immunohistochemical staining for caspase-3 and vascular endothelial growth factor significantly overexpressed in contrast-induced nephropathy group, while treatment with low and high dose atorvastatin reverse this upregulation in a dose-dependent manner. In conclusion, atorvastatin may act as a prophylactic agent to prevent contrast-induced nephropathy in a dose-dependent manner. Proposed mechanisms of this nephroprotective activity may involve improving vascular endothelial function, anti-apoptotic, and angiogenic effects.

**Keywords:** Atorvastatin, Contrast-induced nephropathy; Contrast media, Iopromide.

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## INTRODUCTION

Contrast medium (CM) is a substance used to improve the visibility of tissue and structure of the body, most frequently used for blood vessels and gastrointestinal tract. There are two types of contrast media, negative contrast media (more radiolucent than neighboring tissues) and positive contrast media (more radiopaque).<sup>1</sup> Barium and iodine are examples of positive contrast agents.<sup>2</sup> Iodinated contrast media classified via water solubility as ionic or non-ionic. Ionic agents break up into negative and positive ions and hence have higher

toxicity, also the ionic contrast agents more toxic because they are highly osmolar compared with non-ionic contrast agents. Therefore, nonionic agents, like iopromide, are used frequently now but still having an incidence of nephrotoxicity, and often involving additional polar-OH groups that make them more water-soluble. There are two main other types of water-soluble iodinated contrast agent, the first contained single benzene ring and three iodine atoms attached and are known as monomeric agents, the second has two tri-iodinated benzene rings and known as dimeric agents.<sup>3,4</sup>

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Iodinated contrast agent has a toxic effect on the kidneys called contrast-induced nephropathy (CIN) which can be defined as an increase in serum creatinine of  $\geq 0.5$  mg/dL or  $\geq 25\%$  from baseline at 48–72 hours after exposure to contrast media in the absence of other causes for acute kidney injury.<sup>5,6</sup> The excellent mechanism of CIN not fully understood until now. Many factors may play a central role in the pathogenesis of this type of nephropathy. These factors include indirect toxicity; like increase vasoconstriction of blood vessels, decrease prostaglandin synthesis and decline local nitric oxide, also involve the direct toxic effect on tubules by reactive oxygen species, high oxygen consumption, increase urine viscosity, and elevated internal pressure due to tubular obstruction. All these conditions lead to renal medullary ischemia.<sup>7</sup>

Different risk factors have role in development of CIN, these factors may be modifiable or nonmodifiable, procedure or patient-related such as age, class IV congestive heart failure, diabetes mellitus with renal impairment, hypoalbuminemia, hypotension, low cardiac output, renal insufficiency, renal transplant, taking other nephrotoxic drugs, and depletion of volume.<sup>8</sup> There are two types of prevention strategies, the first were proved effectiveness such as intravenous extracellular fluid volume expansion with saline or sodium bicarbonate, decreasing the dose of CM, exchange ionic high osmolar CM by iso or low osmolar non-ionic CM, avoiding short intervals between two measures required CM, and stop intake of nephrotoxic drugs, all these shown useful in prevention of CIN. The second prevention measurements are less efficient or with conflicting or limited evidence in the prevention of CIN such as using acetylcysteine, fenoldopam, calcium channel blockers, nebivolol, theophylline and pentoxifylline.<sup>9</sup>

Atorvastatin is a lipid-lowering drug; it is a member of a family called 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors, also called statin family, that inhibit the synthesis of cholesterol in the liver.<sup>10</sup> Atorvastatin has other effects not related to lipid levels and termed "pleiotropic effects" such as improving the stability of atherosclerotic plaques, enhancing endothelial function, reduce inflammation, decreasing oxidative stress, enhance insulin resistance, impede tumor cells and inhibit thrombosis in the vascular wall.<sup>11</sup> According to these pleiotropic activities, this study was designed to evaluate the proposed protective effect of atorvastatin against nephropathy induced by iopromide, as a contrast media.

## MATERIALS AND METHODS

### Animal's preparation

Forty adults' male Wistar rats (weighing 200–300mg) were used in this experiment, getting from the animal house in the College of Pharmacy/Baghdad University. They were placed in the animal house of Pharmacy College/Mustansiriyah University. Animal foods were ordinary pellets and freely reached to water. All animals kept in plastic cages (20 × 25 × 35 cm) each cage contains three animals. Before starting the experiment, animals left for one week under controlled environmental conditions for acclimatization, temperature (23 ± 3°C) and 12/12 hours light/dark cycle, where animal houses contain air vacuum apparatus to maintain the environmental conditions (temperature and humidity). The research study begins after approved by the ethics committee of the Pharmacy

College/Mustansiriyah University.

### Experimental Design

Animals were divided randomly into four groups, each contains ten rats, as follow: Control group: received normal saline by oral gavage tube for six days; CIN group: dehydration for three days, in the fourth day, received furosemide (10 mg/kg) by intramuscular route, after 20 minutes iopromide 370 mg I/mL administered intravenously by tail vein in a dose of 10 ml/kg;<sup>12</sup> low-dose atorvastatin group: received atorvastatin (20mg/kg)<sup>13</sup> for six consecutive days (day 1–6). Dehydration began from day 2 to 4, in the fifth day furosemide (10mg/kg) given by intramuscular route, after 20 minutes iopromide 370 mg I/mL administered intravenously by tail vein in a dose of 10 ml/kg; High-dose atorvastatin group: received atorvastatin (40mg/kg)(13) for six consecutive days (day 1–6). Dehydration began from day 2 to 4, in the fifth-day furosemide (10mg/kg) given by intramuscular route, after 20 minutes iopromide 370 mg I/mL administered intravenously by tail vein in a dose of 10 mL/kg. All animals were allowed to recover for 48 hours in adapted metabolic cages (for urine collection) before being sacrificed on the 7th day. Under anesthesia (ketamine and xylazine 90 mg/kg and 10 mg/kg body weight, respectively)<sup>14</sup> blood collected by cardiac puncture and animals euthanized by cervical decapitation and the kidney was harvested for histopathological study.

Considering drugs administration, 40 mg of atorvastatin calcium salt (as a white crystalline powder, Tabuk-KSA) was dissolved in 5 mL of distilled water to get the oral suspension of 8 mg/mL. Contrast media (iopromide 10 mL/kg, Bayer-Germany) was administrated intravenously during 5–7 minutes after cannula insertion to tail vein, while furosemide (20 mg/2 mL, Sanofi-France) was given via IM route.

### Samples Collection

The blood sample placed in gel serum separatory tube; then left for one hour at room temperature to clot, after that put in a centrifuge for 15 minutes at around 3000 rpm. Serum samples placed in Eppendorf tubes and stored in deep freeze at (–40 to –80) for the detection of serum urea, creatinine, neutrophil gelatinase-associated lipocalin (lipocalin-2), and nitric oxide (NO).

On the sixth day of the study, each rat placed in an adapted metabolic cage, urine collected for 24 hours (between days 6 and 7). After that, the samples centrifuged for 10 minutes at 3000 pm. The supernatant was taken and stored in deep freeze (–40 to –80). Then creatinine, microalbuminuria (MAU), and prostaglandin E2 (PGE2) urinary levels were measured.

Under anesthesia, an abdominothoracic cavity of animals was opened. The kidney was located to the back of the abdominal cavity on both sides of the spinal cord. It's separated by cutting longitudinally and after that, washing with distilled water then transferred to 10% buffered formalin for histopathological study.

### Determination of Creatinine and Creatinine Clearance.

Creatinine level was measured by using modified kinetic Jaffe technique.<sup>15</sup> Creatinine clearance (CrCl) was calculated as follow and expressed as mL/min of body weight:  $CrCl = U \times V / P$ , where  $U$  = urine creatinine (mg/dL),  $V$  = urine volume (mL/min), and

P = serum creatinine (mg/dL).

### Determination of other Biomarkers

Other biomarkers (urea, lipocalin-2, NO within serum) and (MAU, PGE2 within urine) were measured by sandwich ELISA technique. All reagents, samples, and standards were prepared as directed by the manufacturer's instructions (Mybiosource-USA).<sup>16</sup>

### Tissue Preparation for Histopathological Examination

After organ fixation with 10% buffer formalin, tissues were processed as described by Bancroft and Stevens.<sup>17</sup> Blocks were cut by microtome (KARL KOLB, Germany) into 5  $\mu$ m thickness for hematoxylin and eosin staining (H&E) and 4  $\mu$ m thickness for immunohistochemistry (IHC). Histopathologic changes were assessed using an arbitrary scale, where medullary congestion and tubular necrosis were staged from grade 0 (normal tissue) to grade 4 (very severe changes).<sup>18</sup>

### Immunohistochemical Staining (IHC)

After processing of renal tissue, 4  $\mu$ m sections were fixed on positively charged slides and supplemented with caspase-3 and vascular endothelial growth factor (VEGF) (mybiosource, USA) for IHC staining, using ImmunoCruz® ABC Kit (Santa-Cruz Biotechnology, USA) according to the manufacturer datasheet, then digital images of the slides were captured using Leica® DM4000B LED Microscope folding. The immunohistochemical staining score system was used for measuring caspase-3, and VEGF expression depends on the

intensity of stain uptake by tissues per field in the blind fashion and the scores were calculated as the following<sup>19</sup>: score 0 = no staining (no expression), score +1 = weak staining, score +2 = moderate staining, and score +3 = strong staining.

### Statistical Analysis

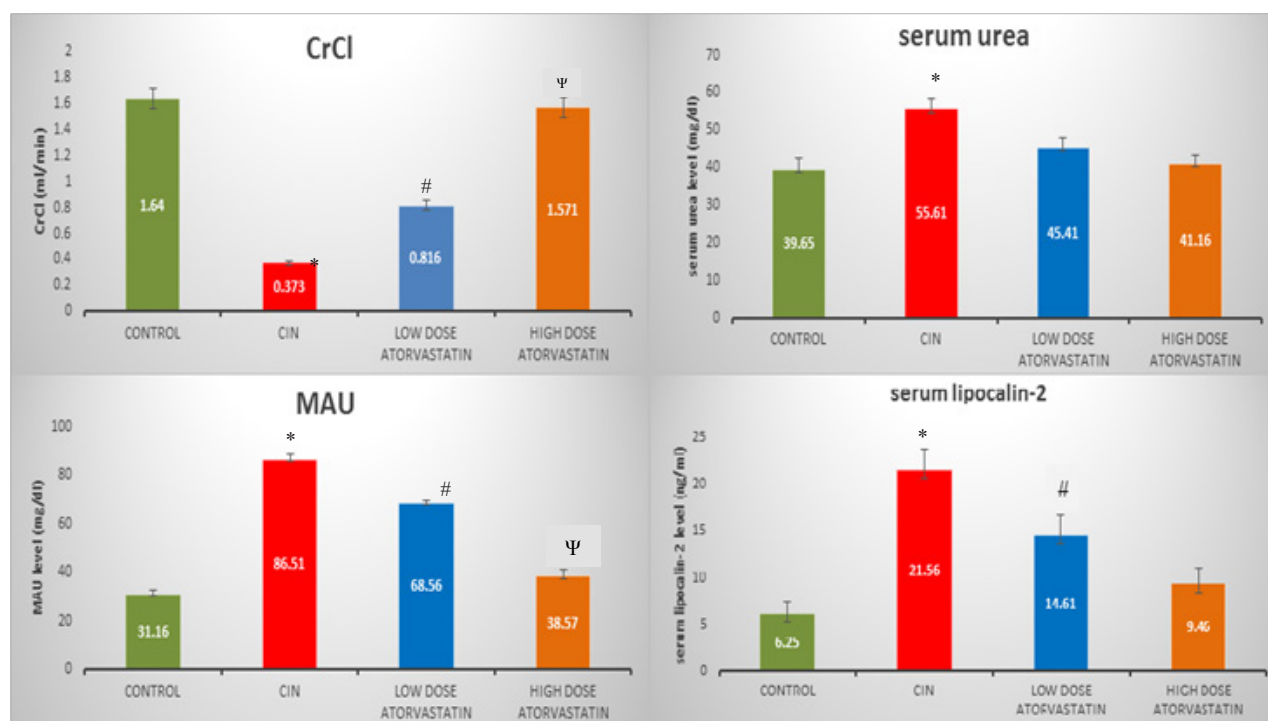
The data were presented as mean  $\pm$  standard error of the mean (M  $\pm$  SEM). Analysis of data was done by using Statistical Packages for Social Sciences-version 16 (SPSS-16). The significance of different means was established by using a one-way analysis of variance (ANOVA) test followed by least significant difference (LSD) for comparison among the four groups. Statistical significance difference was considered when  $p < 0.05$ .

## RESULTS

### Effect of Atorvastatin on Renal function tests after CIN

Regarding renal function parameters, creatinine clearance (CrCl) reduced significantly in the CIN group compared with other groups ( $p < 0.05$ ). Low dose atorvastatin group elevate CrCl significantly compared with CIN group, but still significantly lower than control and high dose atorvastatin groups ( $p < 0.05$ ). High dose atorvastatin significantly elevates CrCl compared with low dose atorvastatin and the CIN group ( $p < 0.05$ ) and approach to control group ( $p = 0.746$ ), as shown in Figure 1.

Considering serum urea levels, CIN significantly elevated values of this marker compared with a control group and high



**Figure 1:** Effect of atorvastatin on renal function tests after CIN. Data were expressed as M  $\pm$  SEM.

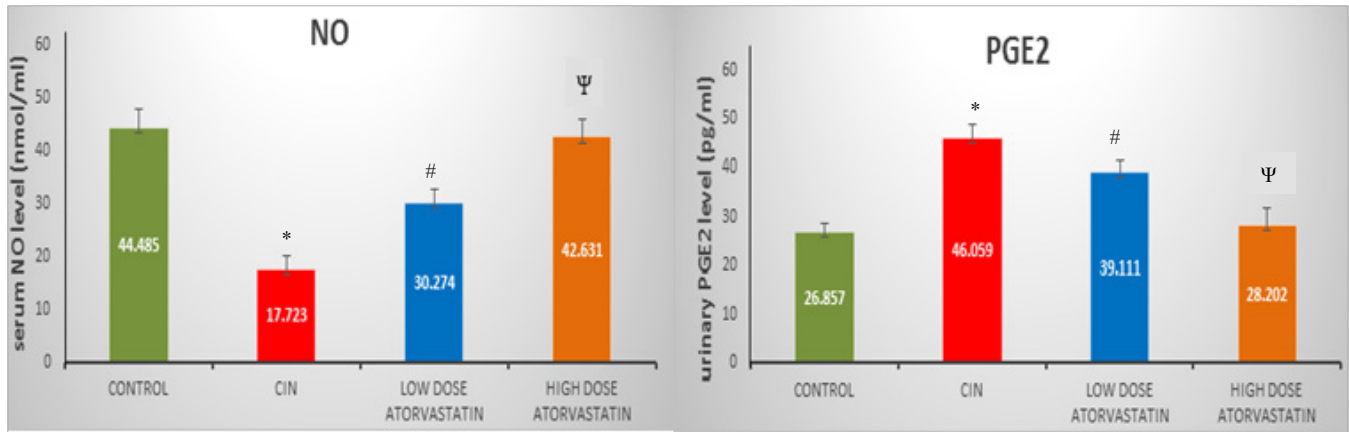
\* significant difference, comparing CIN group with other groups (for CrCl, MAU and lipocalin-2) and with control and high dose atorvastatin group (for serum urea).

# significant difference, comparing low dose atorvastatin group with other groups (for CrCl and MAU) and with control and CIN groups (for lipocalin-2).

Ψ significant difference, comparing high dose atorvastatin group with CIN and low dose atorvastatin (for CrCl) and with CIN groups (for serum urea and lipocalin-2) and with other groups (for MAU).

P-value  $< 0.05$  considered significant difference.

CIN= contrast induced nephropathy, CrCl= creatinine clearance, MAU = microalbuminuria, n = number of rats per group.



**Figure 2:** Effect of atorvastatin on endothelial function markers after CIN. Data were expressed as M±SEM.

\* significant difference, comparing CIN group with other groups (for NO) and with control and high dose atorvastatin group (for urinary PGE2)

# significant difference, comparing low dose atorvastatin group with other group.

Ψ significant difference, comparing high dose atorvastatin group with CIN and low dose atorvastatin groups.

P-value < 0.05 considered significant difference.

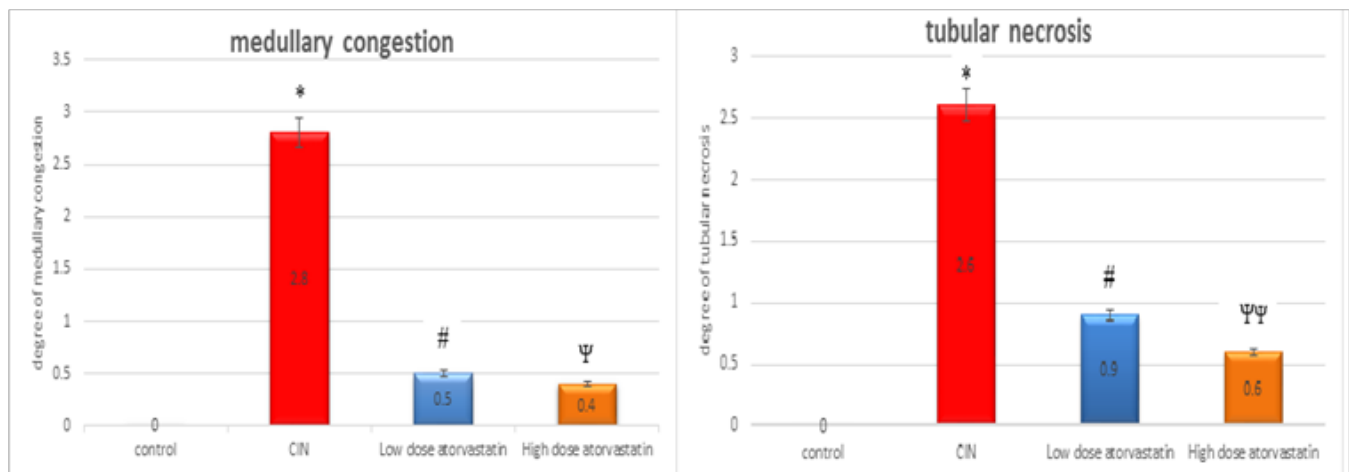
NO= nitric oxide, PGE2= prostaglandin E2, CIN= contrast induced nephropathy, n= number of rats per group

dose atorvastatin group ( $p < 0.05$ ). In low dose atorvastatin group, serum urea levels decrease but not significantly when compare with the CIN group ( $p = 0.13$ ). Meanwhile, high dose atorvastatin reduces serum urea levels significantly compared with CIN group ( $p < 0.05$ ) and approach to control group ( $p = 0.715$ ), as shown in Figure 1.

Regarding MAU levels, the CIN group elevates MAU levels significantly compared with other groups ( $p < 0.05$ ). Low dose atorvastatin reduces MAU levels significantly compared with CIN group, but still higher than control group and high dose atorvastatin group. Meanwhile, high dose atorvastatin group significantly reduced MUA levels compared with the CIN group and low dose atorvastatin group ( $p < 0.05$ ), but also still

higher than the control group ( $p < 0.05$ ), as shown in Figure 1.

Considering serum lipocalin-2, its level significantly elevated by the CIN group compared with other groups ( $p < 0.05$ ). Low dose atorvastatin group significantly reduced values of this marker compared with CIN group ( $p < 0.05$ ), but still significantly higher than the control group ( $p < 0.05$ ) and approximate to the levels of high dose atorvastatin group ( $p = 0.088$ ). Meanwhile, a high dose atorvastatin group significantly reduced serum lipocalin-2 levels compared with CIN group and approach to the control and low dose atorvastatin groups ( $p = 0.282$  and  $0.088$ , respectively), as shown in Figure 1.



**Figure 3:** Effect of atorvastatin on medullary congestion and tubular necrosis after CIN. Data were expressed as M±SEM.

\* significant difference, comparing CIN group with other groups.

# significant difference, comparing low dose atorvastatin group with control and CIN group.

Ψ significant difference, comparing high dose atorvastatin group with CIN group.

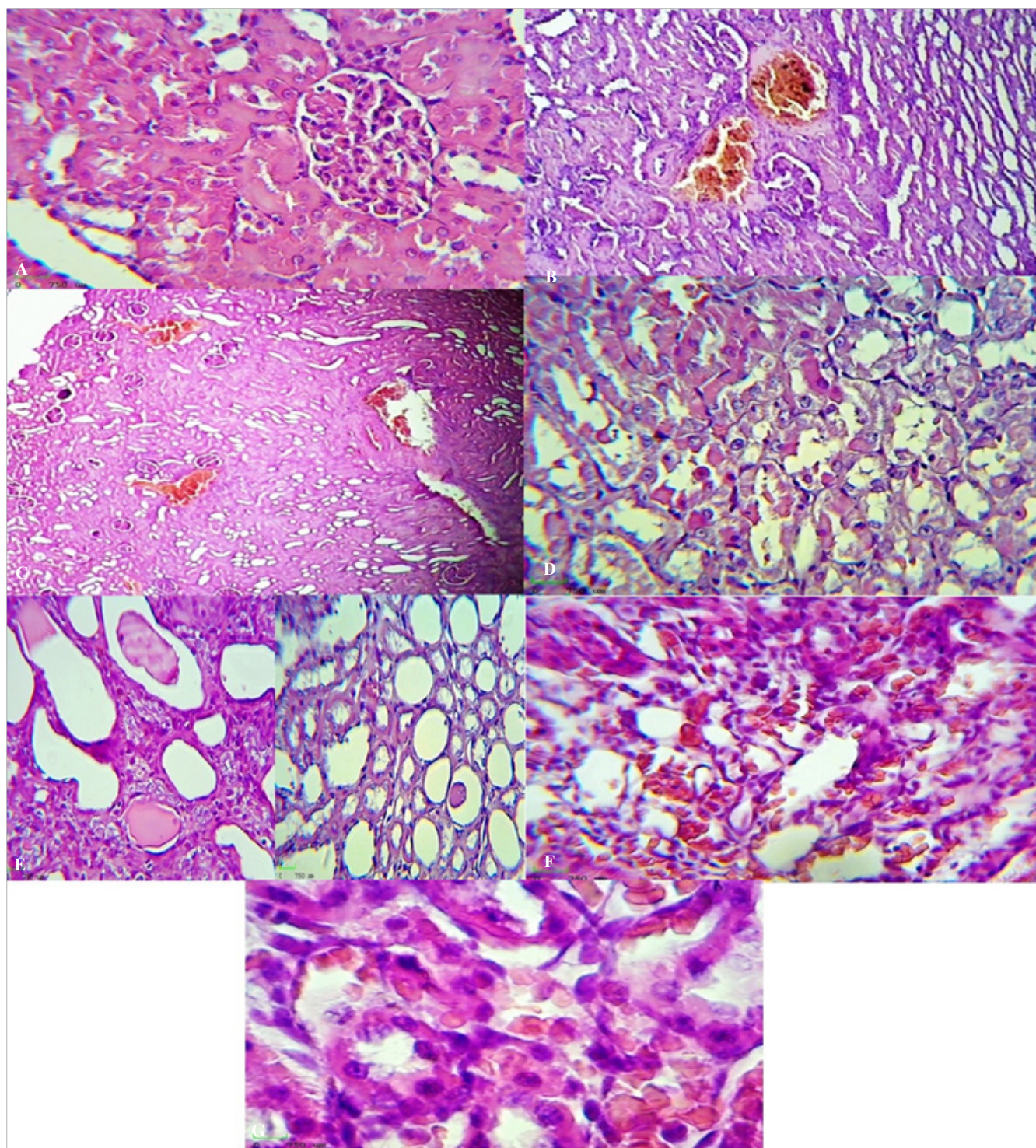
ΨΨ significant difference, comparing high dose atorvastatin group with control and CIN group.

P-value < 0.05 considered significant difference.

Histopathological scores/change: no change (0), mild (1), moderate (2), severe (3) and very severe (4)

CIN= contrast induced nephropathy.





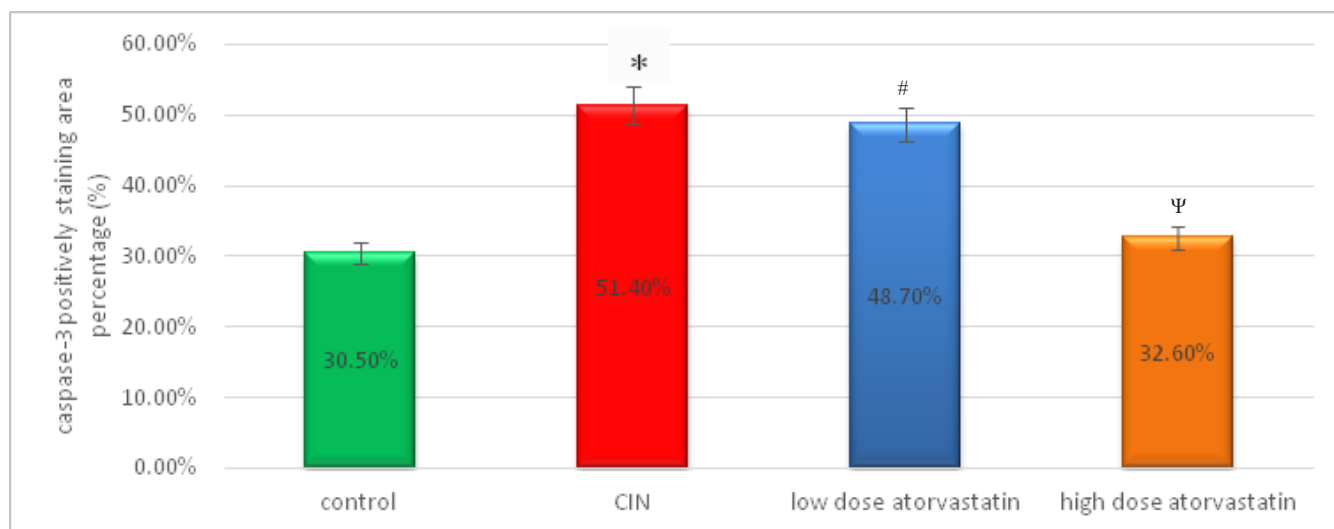
**Figures 4A to G:** Effect of atorvastatin on histopathology after CIN. (A) Control group, shows normal renal tissue, magnification X400; (B) CIN group, shows severe medullary congestion, magnification X100; (C) CIN group, shows very severe medullary congestion, magnification X40; (D) CIN group, shows tubular necrosis, magnification X400; (E) CIN group, shows proteinaceous cast, magnification X400; (F) Low dose atorvastatin group, shows moderate medullary congestion, magnification X200; (G) High dose atorvastatin group, shows mild medullary congestion, magnification X400.

#### **Effect of Atorvastatin on Endothelial function markers after CIN.**

Regarding the effect of atorvastatin on endothelial function markers after exposure to CIN, NO levels reduced significantly in the CIN group compared with other groups ( $p < 0.05$ ). Low dose atorvastatin group elevated NO levels significantly

compared with CIN group, but still significantly lower than the control group and high dose atorvastatin group ( $p < 0.05$ ). Meanwhile, high dose atorvastatin significantly increased NO levels compared with low dose atorvastatin and CIN groups ( $p < 0.05$ ) and approach to control group ( $p = 0.672$ ), as shown in Figure 2.





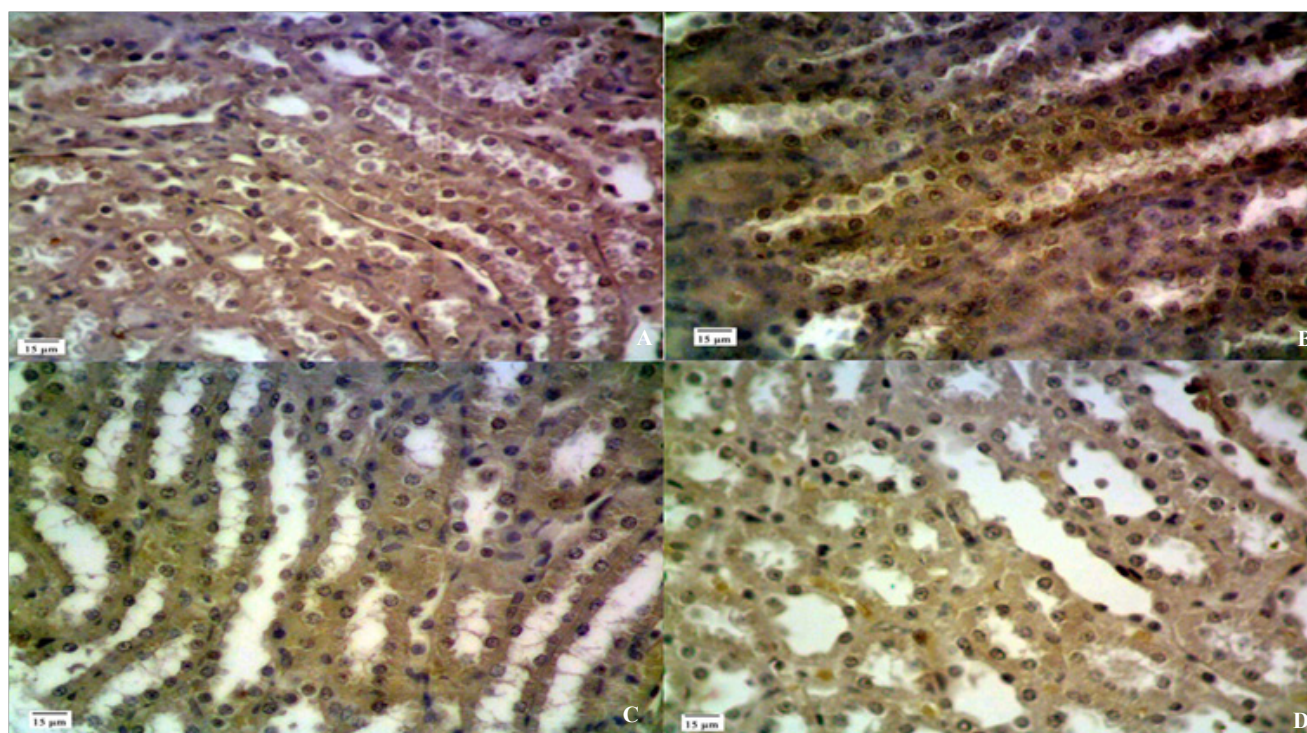
**Figure 5:** Effect of atorvastatin on caspase-3 positively staining area percentage after CIN. Data were expressed as  $M \pm SEM$ .

\* significant difference, compared with control and high dose atorvastatin groups.

Ψ significant difference, comparing high dose atorvastatin with CIN and low dose atorvastatin groups.

p-value < 0.05 considered significant difference.

CIN= contrast-induced nephropathy.



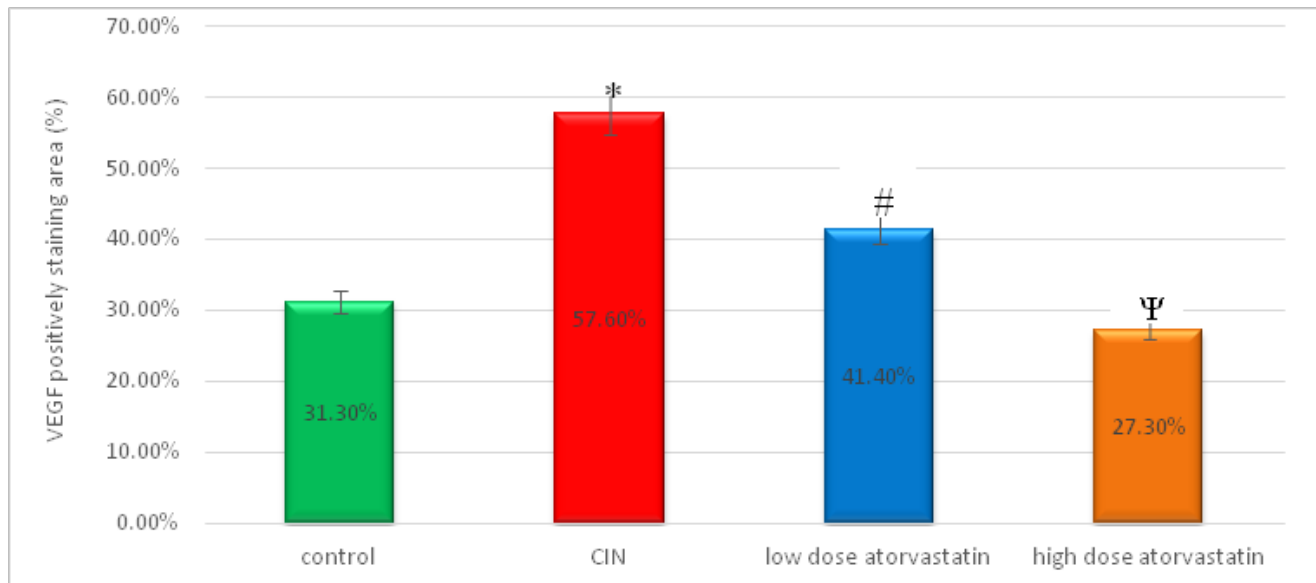
**Figures 6A to D:** Microphotograph of immunohistochemical (IHC) staining of caspase-3 in renal rat tissue (outer medulla), magnification: 400X. (A) Control group, representing 53.3% positively staining with +1 staining intensity; (B) CIN group, representing 100% positively staining with +3 staining intensity; (C) Low dose atorvastatin group, representing 100% positively staining with +2 staining intensity; (D) High dose atorvastatin group, representing 25% negatively staining and 75% positively staining with +1 staining intensity.

Considering the urinary PGE2 marker, its levels increased significantly in the CIN group compared with control and high dose atorvastatin groups ( $p < 0.05$ ). Low dose atorvastatin group reduced PGE2 levels, but not significantly compared with CIN group ( $p = 0.073$ ). High dose atorvastatin significantly reduced PGE2 levels compared with low dose atorvastatin and CIN groups ( $p < 0.05$ ) and approach to control

group ( $p = 0.722$ ), as shown in Figure 2.

#### Effect of Atorvastatin on Histopathology after CIN.

Histopathological examination shows normal renal tissues (glomeruli and tubules) in the control group (Figures 3 and 4). In contrast, the CIN group develop severe and very severe medullary congestion, tubular necrosis and proteinaceous



**Figure 7:** Effect of atorvastatin on VEGF positively staining area percentage after CIN. Data were expressed as M $\pm$ SEM

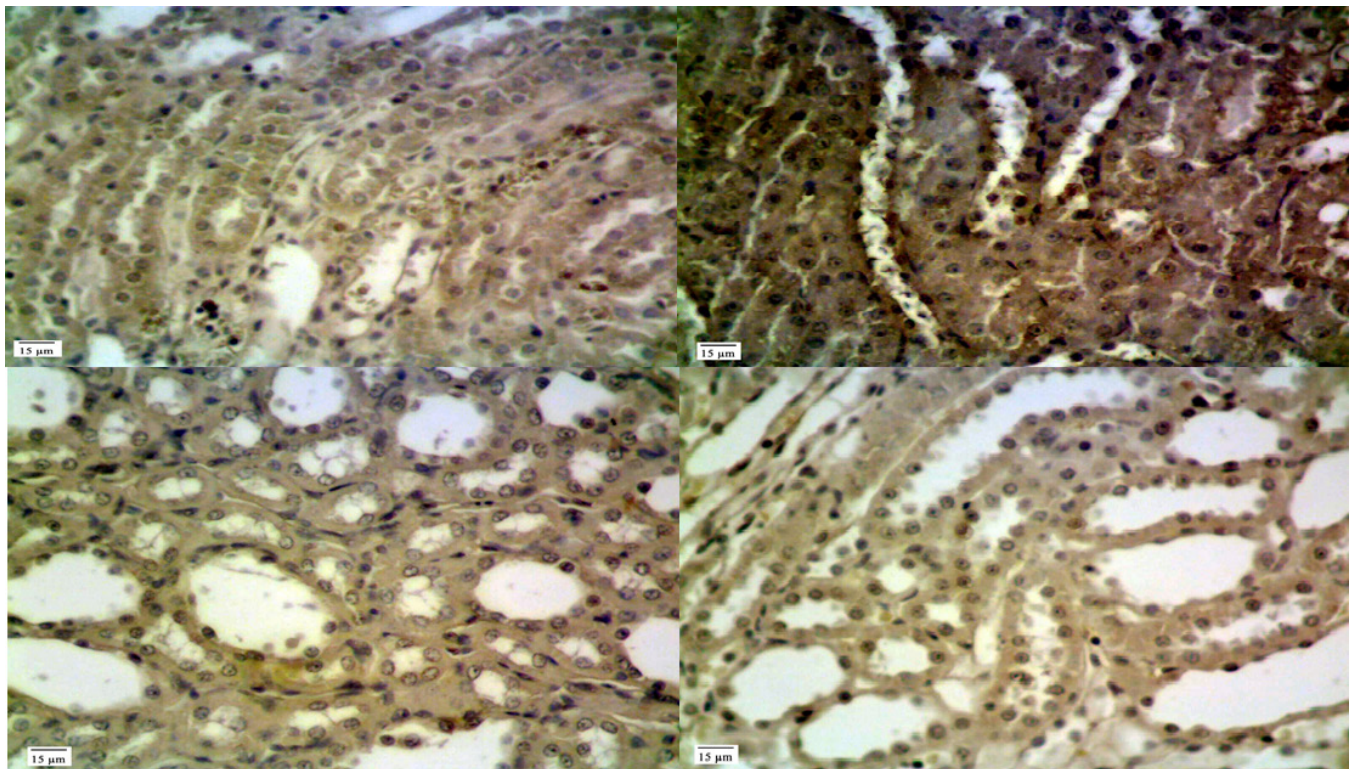
\* significant difference, comparing CIN group with others group.

# Significant difference, comparing low dose atorvastatin group with CIN and high dose atorvastatin groups.

Ψ significant difference, comparing high dose atorvastatin group with CIN and low dose atorvastatin groups.

p-value < 0.05 considered significant difference.

VEGF= vascular endothelial growth factor, CIN= contrast-induced nephropathy.



**Figure 8:** Microphotograph of immunohistochemical (IHC) staining of VEGF in renal rat tissue (outer medulla), magnification: 400X.

**A:** control group, representing 40% negatively staining and 60% positively staining with +1 staining intensity.

**B:** CIN group, representing 100% positively staining with +3 staining intensity.

**C:** Low dose atorvastatin group, representing 100% positively staining with +2 staining intensity.

**D:** High dose atorvastatin group, representing 37.5% negatively staining and 62.5% positively staining with +1 staining intensity.



casts. Meanwhile, treatment with low dose atorvastatin significantly ( $p < 0.05$ ) reduces medullary congestion, tubular necrosis, and disappear proteinaceous casts compared with CIN group. Also, high dose atorvastatin shows significantly ( $p < 0.05$ ) reduced medullary congestion and tubular necrosis compared with CIN group and there is no significant difference between low and high dose atorvastatin on histopathological changes. Figures 3 and 4 show the effect of atorvastatin on tubules and medulla.

### Effect of Atorvastatin on Caspase-3

#### Immunohistochemical staining after CIN.

Regarding caspase-3 staining intensity, in CIN and low dose atorvastatin groups, the staining intensity was significantly high and equal 51.4% (Figures 5 and 6) and 48.7% (Figures 5 and 6) respectively when compared with other two groups ( $p < 0.05$ ). Meanwhile, in high dose atorvastatin, the staining intensity was 32.6%, as shown in Figure 5 and 6 and approach to the control group 30.5%, as demonstrated in Figures 5 and 6 ( $p = 0.764$ ).

#### Effect of atorvastatin on VEGF immunohistochemical staining after CIN.

The immunohistochemical staining intensity for VEGF was significantly high in the CIN group (57.6%), compared with other groups ( $p < 0.05$ ). Meanwhile, in low dose atorvastatin group the staining intensity was 41.4%, and it was significantly lower than CIN group ( $p < 0.05$ ), but still significantly higher than high dose atorvastatin group ( $p < 0.05$ ) and start to approach to the control group ( $p = 0.069$ ). Moreover, in the high dose atorvastatin group, the staining intensity was 27.3%, and it was significantly lower than CIN and low dose atorvastatin groups ( $p < 0.05$ ) and approximate more to the control group (31.4%) ( $p = 0.796$ ). The percentage of staining intensity for VEGF demonstrated in Figure 7 and 8.

## DISCUSSION

Acute kidney injury (AKI) is a rapid loss of renal function within 7 days.<sup>20</sup> The acute kidney injury is detected by laboratory diagnosis such as increase serum creatinine (reduce creatinine clearance) and urea levels, also, reduce the ability of the kidney to excrete sufficient amounts of urine.<sup>21</sup> Although the kidneys have various functions, creatinine, urea, and urine volume are unique to the renal functions and easily measured, so routinely used in the evaluation of acute and chronic renal injuries.<sup>22</sup>

In this study, CrCl was markedly reduced in the CIN group compared with the control group, and pretreatment with atorvastatin reversed the depletion in CrCl and increased it by 118% and 321% in low and high dose atorvastatin group, respectively, comparing with CIN group.

These results agree with Deng *et al.* study (2015), who was found that rosuvastatin treated group significantly increase CrCl<sup>23</sup> and with Wang *et al.* (2017), who was demonstrated that treatment with atorvastatin reduced serum creatinine<sup>12</sup> and consist with Xuyu He *et al.* (2016), who was indicated that atorvastatin have renoprotective effect by increasing CrCl in treated atorvastatin group<sup>13</sup> and with El-Moseelhy *et*

*al.* (2014), who was found that pretreatment with atorvastatin significantly reduced serum creatinine against doxorubicin-induced renal toxicity.<sup>24</sup>

In the current study, serum urea levels reduced by 26% in the high dose atorvastatin group comparing with the CIN group. This result match with Jinzi Su *et al.* (2014), who was found that atorvastatin reduce blood urea nitrogen in contrast media group<sup>25</sup> and agree with El-Moseelhy *et al.* study (2014), who demonstrated that pretreatment with atorvastatin significantly reduced blood urea nitrogen against doxorubicin-induced kidney damage<sup>24</sup> and disagree with Haitham Galal *et al.* (2015), who was reported no significant difference between low and high dose of atorvastatin in reduction of serum urea levels in diabetic patients undergoing percutaneous coronary intervention.<sup>26</sup>

In this study, the MAU level highly elevated in the CIN group by 177%, compared with the control group; while in low and high dose atorvastatin groups, the MAU level reduced significantly by 21% and 56% respectively, comparing with CIN group. These results were similar to Xuyu He *et al* study (2016), who was mentioned that atorvastatin reduce MAU level in a dose-depended manner<sup>13</sup> and in line with Jie Deng *et al.* study (2015), who was found that levels of MAU reduced by administration of rosuvastatin<sup>23</sup> and consist with Vasilios G Athyros *et al* study (2015), who was demonstrated that atorvastatin has more renoprotective effect than rosuvastatin in maintaining GFR and reducing MAU levels.<sup>27</sup>

Neutrophil gelatinase-associated lipocalin (lipocalin-2) is a 25 KDa protein which is bound covalently to gelatinase from secondary granules of neutrophils. Previous studies on animal models showed lipocalin-2 as one of the very early upregulated genes after acute kidney injury.<sup>28,29</sup> In another study, proteomic analyses Akt that lipocalin-2 as one of the highly induced protein after renal ischemia or nephrotoxicity in animal models.<sup>30</sup> Prasad Devarajan (2010) was confirmed the lipocalin-2 as a sensitive biomarker to establishes acute kidney injury in humans. He demonstrated that levels of lipocalin-2 in serum and urine related with serum creatinine levels and kidney biopsies in patients with acute kidney injury, also, he found an accumulation of the high amount of lipocalin-2 in cortical tubules.<sup>31</sup>

In the current study, serum lipocalin-2 level markedly elevated in the CIN group by 245%, compared with the control group. These results consist of Vassilis Filiopoulos *et al.* (2014), who was considered that lipocalin-2 level in serum and urine as an ideal biomarker to diagnose early acute kidney injury for CIN in children and adults after coronary angiography.<sup>28</sup> In the present study, atorvastatin in low and high dose reduced serum lipocalin-2 levels by 32% and 56% respectively, compared with CIN group. These results agree with Liu WJ *et al.* (2013), who found that administration of statins with alprostadil in patients undergo coronary angiography significantly lowered serum lipocalin-2 levels, compared with non-treated patients,<sup>32</sup> also match with Akbar Ahmad *et al.* (2012), who demonstrated that pretreatment with apocynin (a natural plant compound with antioxidant properties) significantly reduced lipocalin-2 levels in urine and plasma, compared with contrast media group.<sup>33</sup>

In the present study, serum NO levels significantly decreased in the CIN group by 60%, compared to a control group. These results agree with previous studies, which demonstrated that



contrast media could reduce renal nitric oxide bioavailability by generating free radicals such as superoxide which interact rapidly with NO to generate another free radical "peroxynitrite" and damaged endothelial layer by these free radicals which responsible for NO synthesis.<sup>34,35</sup>

In the current study, treatment with atorvastatin before contrast media administration increased serum NO level in a dose-dependent manner by 70.8% and 140% in low and high dose respectively, compared with CIN group. Akt Ota *et al.* (2010) found that treatment with statins can increase endothelial nitric oxide synthase (eNOS) activity by inducing phosphatidylinositol three kinases/ Akt pathway through phosphorylation of Akt at ser473, which in turn upregulate eNOS activity.<sup>37</sup>

Prostaglandin (PG) plays a central role in regulating glomerular filtration rate (GFR) and renal blood flow. Prostaglandin E2 is the major renal PG subtype and generated in all renal cells which have a PGE2 synthase enzyme. In normal physiological conditions, the effect of PG on GFR and renal blood flow is little, but in the pathophysiological state, especially in reduced circulatory blood volume, the normal renal functions depend mainly on prostaglandin action.<sup>38</sup>

In this study, the urinary PGE2 level significantly increased by 41.7% in the CIN group, compared with the control group. These results match with Norregaard *et al.* findings (2015), who reported that pathophysiological conditions such as congestive heart failure, chronic kidney disease, and liver cirrhosis, can lead to severe fall in circulating blood volume and in turn activating adrenergic nervous system and renin-angiotensin system, all these lead to vasoconstriction of renal blood vessels and diminished renal functions. These conditions lead to endogenous stimulation of vasodilatory PGs synthesis to reverse vasoconstriction effects and maintain renal functions.<sup>39</sup>

In the current study, urinary PGE2 levels significantly reduced in the high dose atorvastatin group by 38.7%, compared with the CIN group. These results match with Maraqa *et al.* study (2016), who found that renal PGE2 levels elevated in diabetic rats, compared with control rats, and this elevation reversed by atorvastatin treatment.<sup>40</sup>

When the pro-inflammatory cytokines IL-1 $\beta$  and IL-6 activated, this leads to induction of cyclooxygenase-2 (COX2) which in turn stimulates PGE2 production. Atorvastatin can reduce PGE2 levels by its anti-inflammatory and vasodilating effects.<sup>41</sup>

Outer medullary congestion of the kidney is one of the vascular hallmarks of CIN. Tubular obstruction by proteins also associated with CIN. One study has shown that after diatrizoate (a type of contrast media) administration, five of the six rats developed tubular protein cast.<sup>18</sup>

In the current study, histopathology showed severe tubular necrosis, very severe medullary congestion, and numerous proteinaceous casts formation in renal tissue after exposure to contrast media. This study has used a hard and invasive induction model of CIN and this may explain why we have seen severe tubular necrosis and medullary congestion in the CIN group.

Administration of atorvastatin as pretreatment of

contrast media significantly reduced the tubular necrosis and medullary congestion scores and disappeared proteinaceous casts, although statistically no significant difference was seen between a low and high dose of atorvastatin. These results agree with Deng *et al.* study (2015), who was found that treatment with rosuvastatin significantly reduced histological scores of renal tissue damage, compared with contrast media group<sup>23</sup> and consist with Wang *et al.* findings (2017), who demonstrated that treatment with atorvastatin and rosuvastatin significantly reduced medullary congestion and tubular necrosis, compared with contrast media group.<sup>12</sup>

Apoptosis is a programmed cell death that happens in different cellular organisms; it's one form of cell death, causing distinctive cellular changes.<sup>41</sup> Apoptosis initiated by two main pathways: intrinsic and extrinsic, both pathways causing activation and upregulation of specific protease enzymes called caspases (caspase-3 and-7). Nephrotoxic drugs activated apoptosis mainly by the intrinsic pathway.<sup>42</sup>

Iodinated contrast media induce apoptosis in renal cells by activating p38 and JNK1/2 pathways via increasing intracellular levels of ROS. The p38 and JNK1/2 pathways are mainly activated by different environmental stress conditions such as UV radiation, shock, osmotic load, proinflammatory cytokines, and oxidative stress. So, p38 and JNK1/2 always act together and referred to as stress-activated protein kinases (SAPKs).<sup>43</sup> The initiation of apoptosis occurred with sustained activation of p38 and subsequent activation of JNK1/2.<sup>44</sup> In stress conditions, JNK1/2 is phosphorylated, activated, and released Bcl-2 protein that is sequestered in cells. Then, Bcl-2 activates Bax by dissociation from its anchor in the cytoplasm. Bax translocates to the mitochondria and undergo oligomerization and release cytochrome c by loss of mitochondrial membrane potential and initiate caspase cascade reactions.<sup>45</sup>

In the current study, caspase-3 significantly overexpressed in renal tissue of the CIN group, compared to the control group, while pretreatment with a high dose of atorvastatin significantly decreases tissue staining intensity by 36.5%, compared with CIN group. This can explain that contrast media have an apoptotic effect on renal tubular cells, which can be attenuated by pretreatment with atorvastatin.

These results match with Buyuklu *et al.* (2014), who observed that intense specific staining of caspase-3 in tubular cells significantly overexpressed in contrast media group, compared with other groups<sup>46</sup> and agree with Deng *et al.* (2015), who found that contrast media-induced apoptosis by activating caspase-3, which can notably reduced by rosuvastatin as pretreatment<sup>23</sup> and consist with Xuyu He *et al.* findings (2016), who was reported that expression levels of caspase-3 significantly raised in contrast media group, while significantly reduced in a dose-dependent manner by atorvastatin.<sup>13</sup>

Contrary to what is known about the beneficial effects of VEGF, there is a harmful effect of VEGF on renal diseases, especially in diabetic nephropathy in which VEGF plays an important role in pathogenesis. Levels of VEGF have been found elevated in kidneys of diabetic patients with Types 1

and 2. The high levels of VEGF have been found responsible for inducing glomerular hyperfiltration, urinary albumin excretion, and glomerular and tubular hypertrophy.<sup>47</sup> In the current study, the expression of VEGF was increased by 45.6% in the CIN group, compared with the control group. These results agree with Kedah *et al* study (2012), who found that expression of VEGF was highly elevated in contrast media groups, compared with control. Homles *et al* (2007) reported that VEGF is a part of the system that restores oxygen supply to the tissue when oxygen supply inadequate to meet the metabolic needs of tissues such as hypoxia.<sup>48</sup> So, hypoxia is one of the main pathogenesis in CIN.<sup>49</sup>

In the present study, atorvastatin reduced the expression of VEGF by 28.6% and 52.6% in low and high dose respectively, compared with CIN group. These results agree with Ari *et al* study (2012), who demonstrated that VEGF was highly expressed in tubular cells after exposure to contrast media due to hypoxia, this elevation can be reversed by paricalcitol treatment (vitamin D2 analog) which improve renal hypoxia and reduce oxidative stress conditions,<sup>50</sup> also consist with Chen *et al* study (2012), who was demonstrated that high levels of reactive oxygen species (ROS) in non-small cell lung cancers required for inducing VEGF expression, while treatment with atorvastatin significantly reduced VEGF expression in vivo and in vitro by inhibiting ROS generation and upregulating antioxidants glutathione peroxidase and catalase activity.<sup>51</sup>

## CONCLUSION

From this data, one can conclude that atorvastatin may act as a prophylactic agent to prevent CIN in a dose-dependent manner. The proposed mechanisms of this nephroprotective activity involved improving endothelial function, anti-apoptotic and angiogenic effect, ending with improving histomorphological changes.

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