Evaluation of the Effects of Azithromycin on the Kidney of Adult Albino Rats and the Possible Protective Role of Vitamin C Using Histological and Immuno-Histochemical Studies

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Abstract

Background: Azithromycin (AZ) is a part of the azalide subclass of macrolides, it has a potential antiviral and anti-inflammatory effects and it has been used to treat COVID-19.Vitamin C (VIT. C); L-Ascorbic acid is an antioxidant that can combat free radicals in the body, thereby decreases inflammation. It is also required for collagen complex and speed wound healing.

Objectives: This work aimed to study the histopathological changes in the kidney of adult albino rats induced by AZ and the possible protective role of VIT. C.

Material and Methods: Forty five adult albino rats were used in this study. They were divided into equal 3 groups; group I(Control),group II (AZ) and group III(AZ + VIT. C). Drugs were administrated via intragastric route by stomach tube daily for successive two weeks. Rats were sacrificed and tissue samples were collected and processed for histopathological and immunohistochemical (IHC) examination.

Results: Histological evaluation of AZ group showed abnormal structure of the renal cortex, some renal corpuscles revealed dilatation of renal spaces, cells of the proximal and distal convoluted tubules showed marked vacuolations in their cytoplasm and shrinkage of nuclei, some empty spaces, hemorrhages and mononuclear cellular infiltration. IHC examination showed significant increase in both Caspase 3 and tumor necrosis factor-α(TNF-α) immune stain. Group III showed restoration of kidney structure in H&E and IHC stained sections.

Conclusion: Azithromycin induced renal adverse side effect, VIT.C can reduce this nephrotoxicity, due to its antioxidant, anti-inflammatory, and anti- fibrotic properties.

Keywords: Azithromycin; Nephrotoxicity; Vitamin C.

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Introduction

Azithromycin is a well-tolerated macrolide that can be given once daily, doctors usually advised AZ in children and adults with respiratory tract infections as it has a potent anti-inflammatory effect, the latest data proved that AZ is very potent in the treatment of cystic lung fibrosis by direct inhibition of excessive inflammation **(Florescu et al., 2009).**

AZ has a long half-life duration and a high affinity to tissue penetration and accumulation; it was FDA approved since1991 **(McMullan and Mostaghim, 2015; Fohner et al., 2017).**

The largest tissue concentration of AZ was observed in the liver, followed by the kidney, spleen, lung and the heart in order **(Yoshida and Furuta, 1999).**

Some studies were done by the French government in March 2020 to test the efficacy of combination of AZ and the anti-malarial drug hydroxychloroquine in treatment of COVID 19.The results showed good improvement and all patients recovered within 6 days of treatment, although larger studies are needed to confirm them**(Gautret et al., 2020).**

Azithromycin has broad-spectrum antibacterial activities due to its mechanism of action which is by inhibition of protein synthesis of bacteria that prevent their growth. It binds to the 50S subunit of the bacterial ribosome, so inhibits translation of mRNA **(Dinos,2017).**

Some renal adverse effects as acute interstitial nephritis and renal failure were associated with AZ treatment. According to case reports and cohort studies, these renal changes were considered to be due to the direct effect of the drug and its metabolites on ribosomal functions and long duration of these derivatives in tissues **(Baronas et al., 2007 ; Persicoet al., 2011).**

Vitamin C (L-ascorbic acid) is a water soluble vitamin and also one of the major natural antioxidants originates from the natural sources of daily food intake, the natural antioxidants have an important role in the prevention of oxidative damage that is a part of the pathophysiology and histopathology of many health disorders **(Heistad,2005 ; Budin et al., 2011).**

Vitamin C is a potent antioxidant agent in biological systems that lower harmful oxygen-derived species that causes damage of the cell membrane and DNA; as hydroxyl radical, hydrogen peroxide (H_2O_2), and singlet oxygen **(Hacışevki,2009)**.

This work aimed to study the histopathological changes in the kidney of adult albino rats induced by AZ and the possible protective role of VIT. C.

Materials and Methods

A. **Drugs:** 1- AZ with a trade name of Zithromax (500 mg) tablet produced by Pfizer Company, 2-VIT. C oral drops manufactured by the Universal Pharmaceutical Industries Company. They were brought from local pharmacy.
B. **Animals:** Forty five (45)adult albino rats were used for this study, with average weight 170-250 gm. They were housed and obtained from Faculty of Medicine, Sohag University, Sohag, Egypt. They were kept on

a 12-hour light/12-hour dark cycle at an environmental temperature 23± 1°C and humidity of 55±5% .They were fed with rat chow and free access to water.They were adapted for one week to the experimental site.

Experimental Design

The experiment was done in agreement with the guidelines of Sohag University Committee for Animal Care and Use. Rats were randomly divided into 3 equal groups (15 each) as follows**:**

• **Group I (Control group**); 15 rats received normal saline by oral gavage.

• **Group II (AZ group):** 15 rats were treated with AZ in a dose of 30mg/kg by oral gavage daily for two successive weeks **(Atli et al., 2015).**

• **Group III (AZ + VIT. C):** 15 rats were treated with AZ as the previous group +VIT. C (20 mg/kg) by oral gavage daily for two successive weeks **(Viswanatha Swamy et al., 2011).**

Twentyfour hours after the end of the experiment, rats were anesthetized, sacrificed and dissected.Samples from the kidney were taken for histological and IHC studies.

Histological studies

Preparation of the specimens for light microscopic examination: The specimens were fixed in 10% neutral buffered formalin for at least 24 h and was embedded in paraffin wax (melting point 56–58°C),then were cut at 5-7 µm tissue thickness and subjected to:

I. Light microscopic examination:

A. Stained with Haematoxylin and Eosin (H&E) **(Bancroft and Gamble,2002).**

B. Stained with Masson trichrome stains (MT) (**Chen et al., 2017).**

II. IHC staining: Sections were boiled in 10 mm citrate buffer (AP9003) at pH 6 for 10 minutes to retrieve antigen, then incubated for one hour with the following antibodies;

a. Caspase-3 (Rabbit polyclonal antibody, ab13847) for apoptosis (purchased from Abcam, MA, USA) **(Mohamed and Kassem,2018**).

b. TNF-α (stained with avidin-biotin peroxidase technique) **(Hora et al., 2005).**

Sections were counterstained with Mayer's Haematoxylin stain (TA060-MH). Positive reaction appeared as brown discoloration, Citrate buffer, UltraVision detection system and Mayer's hematoxylin were purchased from LabVision Thermo Scientific, Fremont, California, USA. Antibody against TNF-α was purchased from (Minneapolis, Minnesota, USA).

Morphometric and statistical study

The following measures were taken:

a. Area of collagen density **(Schipke et al., 2017).**

b. Area of Caspase-3 immunoreaction density was measured using an objective lens of x40 magnification **(Mansour et al., 2021).**

Fifteen (15) non-overlapping fields for each section were examined. This was done using Image J software (version 1.51k, Wayne Rasband, National Institutes of Health, and USA). From each variable the mean ± standard deviation (Mean ± SD) was

measured using SPSS program version 16. One-way analysis of variance (ANOVA) and a post-hoc test were used to find the statistical difference between groups. ANOVA was statistically significant if p value ≤0.05 **(Eid1 et al.,2020).**

Results

1. Histological examination

A. By H&E stain

- **Group I (Control Animals):**

Histological examination of sections of the kidney of adult albino rats revealed normal structure of the renal corpuscles and tubules. Renal corpuscles showed a thin walled cup like swelling called the Bowman's capsule and a capillary tuft named glomerulus, that is embedded into the Bowman's capsule and separated from it by a space called sub-capsular space or Bowman's space, Bowman's capsules outer layer is formed of simple squamous epithelium, the proximal convoluted tubules had a pyramidal cells lining with deeply stained (eosinophilic) cytoplasm and narrow lumen, the distal convoluted tubules had a wider lumen lined by cuboidal cells with faint acidophilic cytoplasm **(Figure 1.a).**

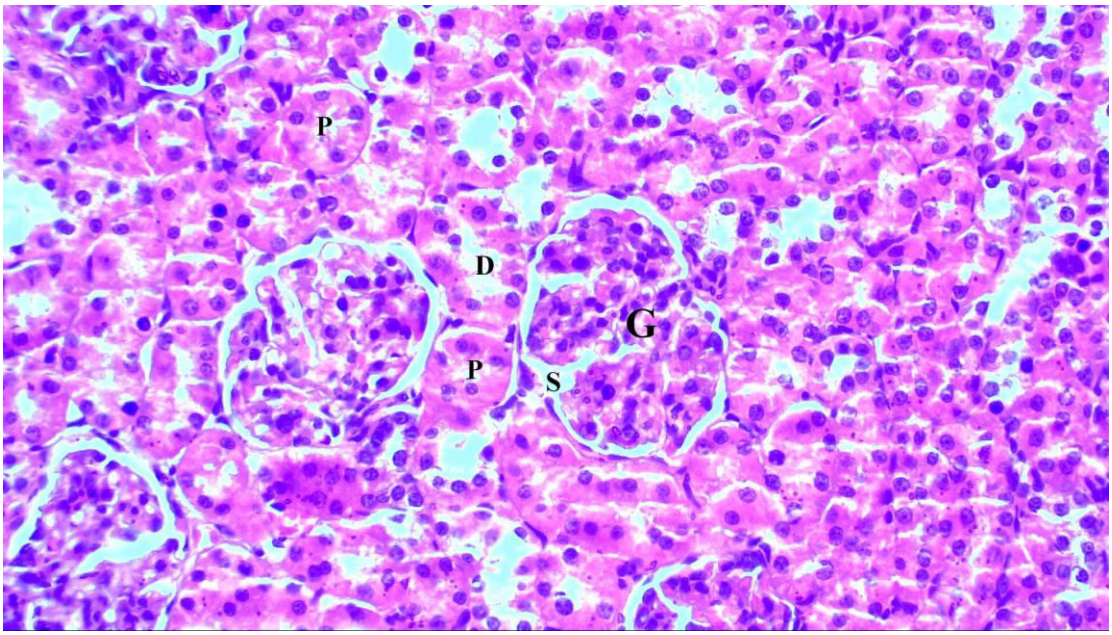


Figure 1a: A photograph of the control group of section in renal cortex stained with H&E showing normal glomerulus (G), Bowman's space (S), proximal convoluted tubules (P) with narrow lumen lined by cuboidal cells with darkly stained cytoplasm. The distal convoluted tubules (D) had dilated lumen lined by a round cell with pale cytoplasm (**H&E, x400).**

Group II (AZ treated animals)

In AZ treated group abnormal structure of the renal cortex, shrinked renal corpuscles with renal spaces dilatation was founded. The lining epithelium of the convoluted tubules showed numerous vacuolations in the cytoplasm, small shrinked nuclei, empty spaces, hemorrhages and mononuclear cellular infiltration **(Figure 1.b).**

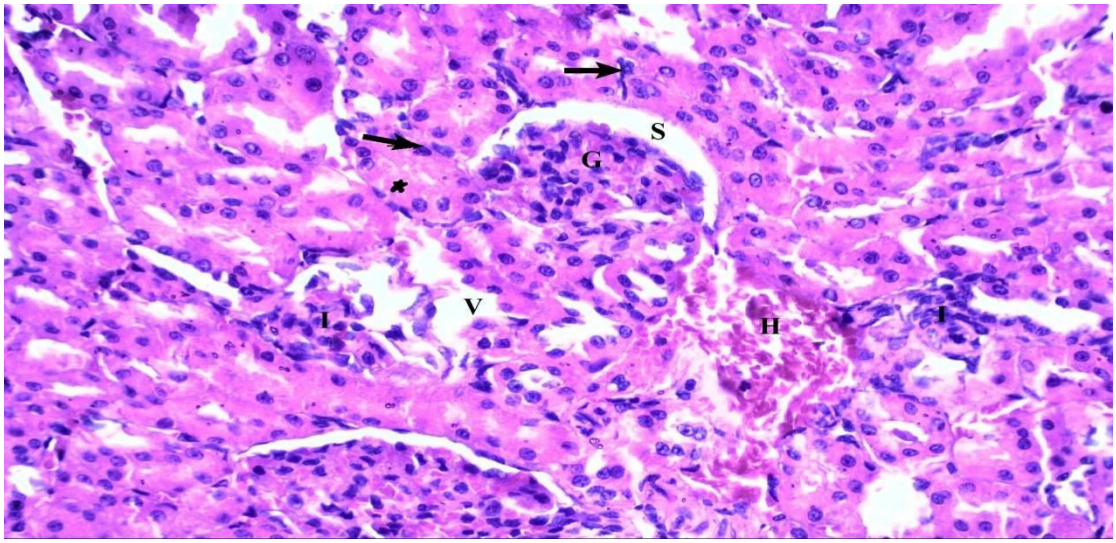


Figure 1b: A photograph of the AZ group of section of renal cortex stained with H&E showed dilated renal space(S) in glomerulus (G), swollen cells (star) of proximal and distal convoluted tubules. Most of them had pyknotic nuclei (arrows), areas of hemorrhages (H), vacuolations (v) and mononuclear cellular infiltrations (I) (H&E, x400).

Group III (AZ+VIT. C treated animals)

This group showed nearly normal renal corpuscles with some pyknotic nuclei, some areas of mononuclear infiltrates and **(Figure 1.c).**

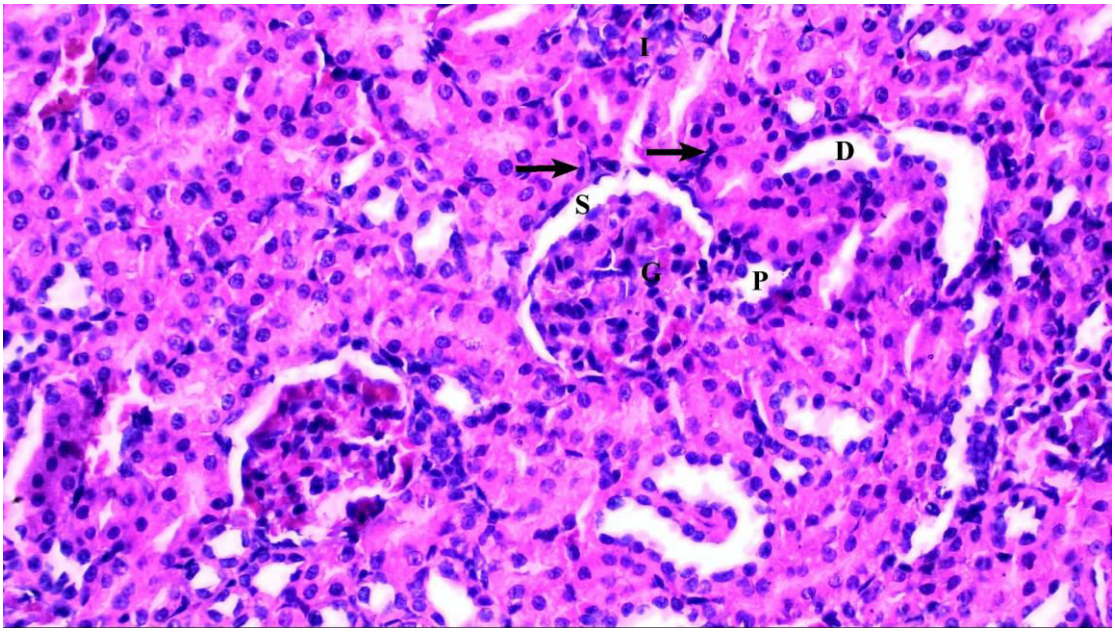


Figure 1c: A photograph of renal cortex of AZ and VIT. C group renal section stained with H&E showed apparently normal renal cortex, glomerulus (G), nearly normal proximal tubule (P) and distal tubule (D).Some cells had pyknotic nuclei (arrows) and some mononuclear cellular infiltrates (I) (H&E, x400).

B. By Masson trichrome stain (MT stain)

The control group showed minimal collagenous deposition in the interstitial tissue without fibrosis **(Figure 2.a).** In the renal cortex of the AZ treated group, the stain showed obvious severe collagenous deposition within the basement membrane of the glomeruli, around the Bowman's

capsule and the interstitial tissue between tubules of kidney **(Figure 2 .b).**Whereas, sections of co-treated group of AZ and VIT. C showed nearly normal collagen deposition **(Figure 2.c).**

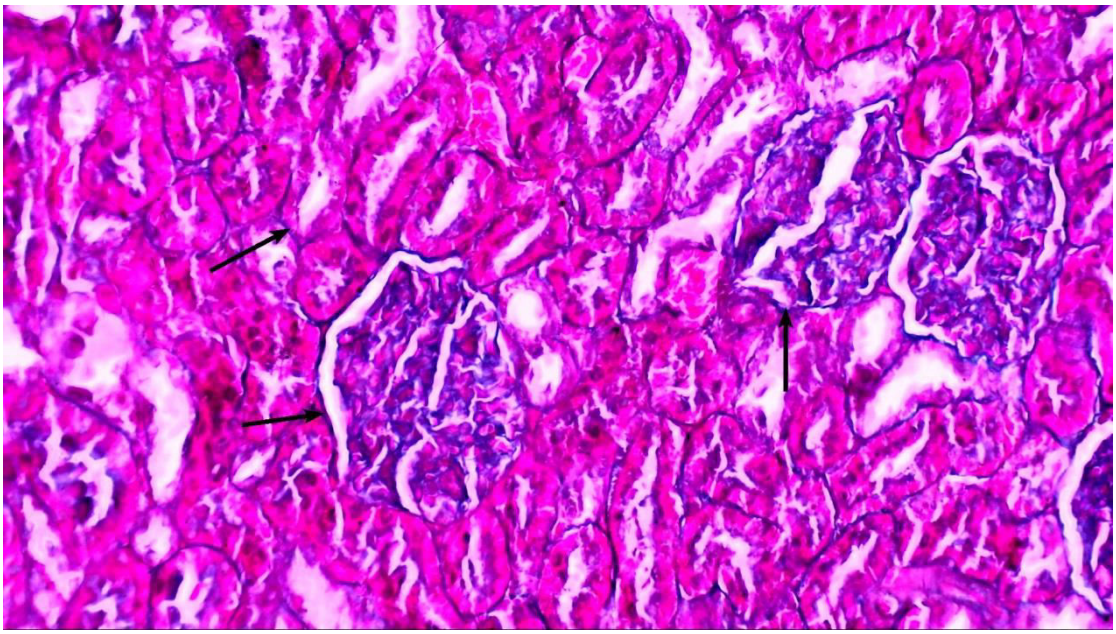


Figure 2a: A photograph of a section of renal cortex of the control group showed minimal collagenous deposition in the kidney corpuscles and in-between renal tubules (arrows) **(MT, x400)**

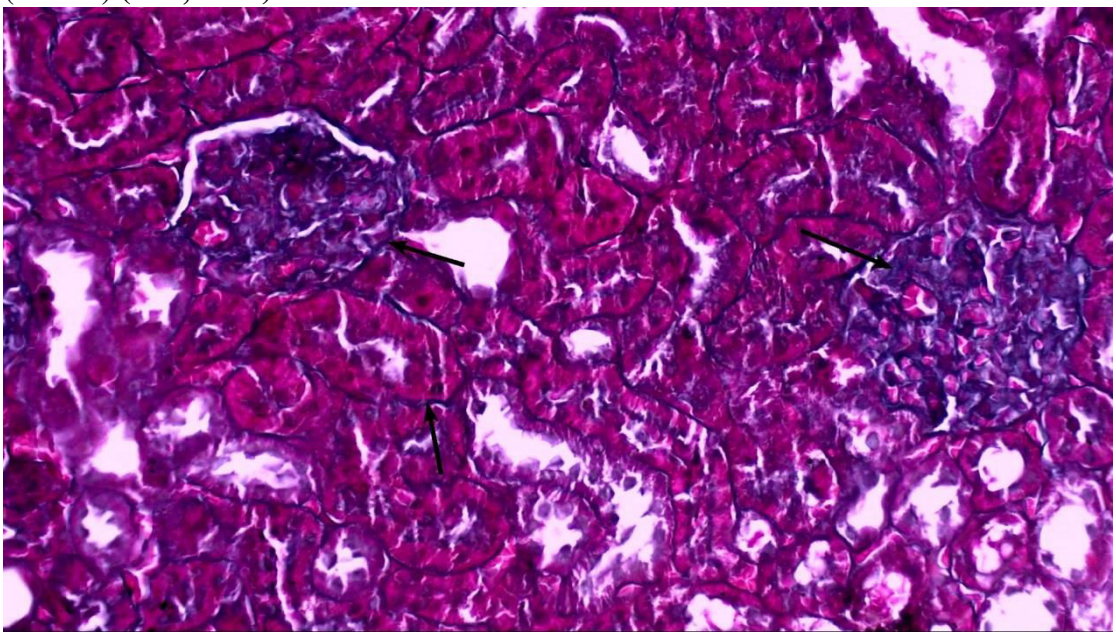


Figure 2b: A photograph of a renal section of azithromycin group showed an apparent thick collagen fibers deposition in the kidney corpuscle and in between renal tubules(arrows)(**MT, X400).**

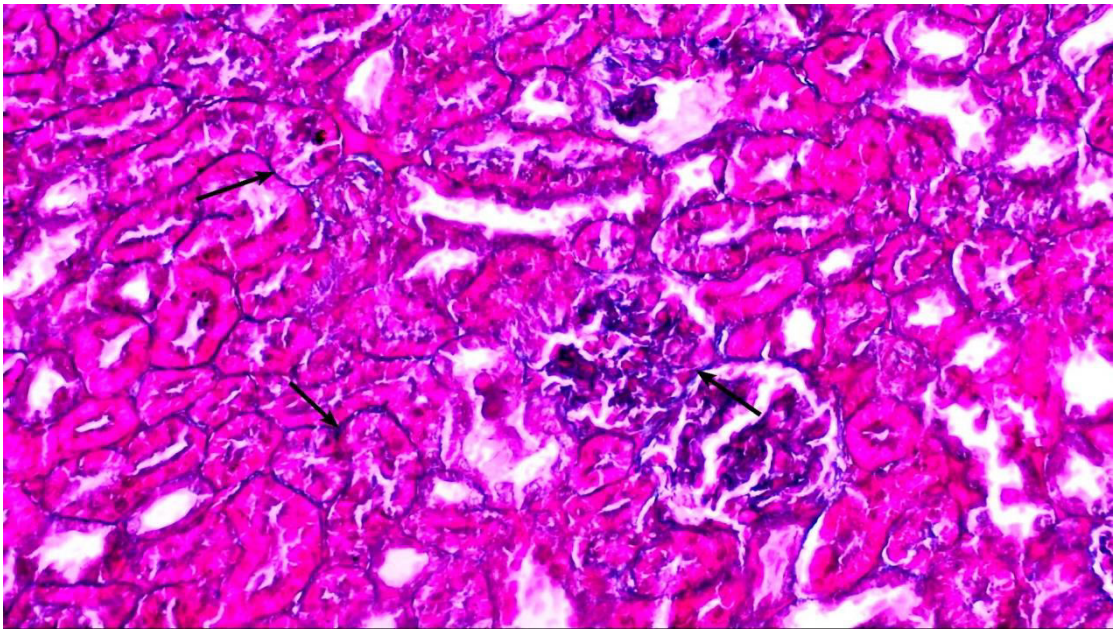


Figure 2c: A photograph of a section of azithromycin +vitamin c group showed an apparent minimal collagen fibers deposition in the renal corpuscle and in between tubules of kidney(arrows)(MT, x400).

2. IHC reaction

A. Caspase-3:

In the control group the staining of the cytoplasm of the epithelium of renal convoluted tubules with anti Caspase-3 showed negative reaction **(Figure 3.a).**Whereas, group II(AZ group) showed highly positive Caspase-3 reaction in the convoluted tubules (**Figure 3.b),** In group III(AZ and VIT. C co-treated group) IHC studies revealed slightly positiveCaspase-3 reaction in the cytoplasm **(Figure 3.c).**

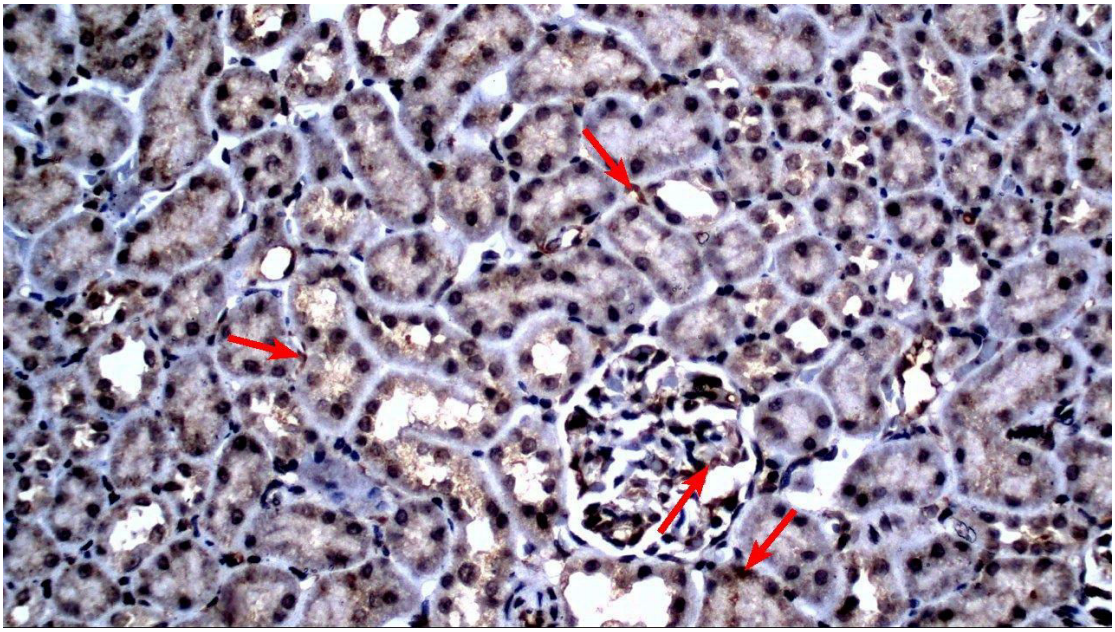


Figure 3a: A photograph of Caspace 3 IHC stained section of kidney of control group showing weak cytoplasmic reaction in the cells of kidney glomeruli and tubules (arrows) (x400).

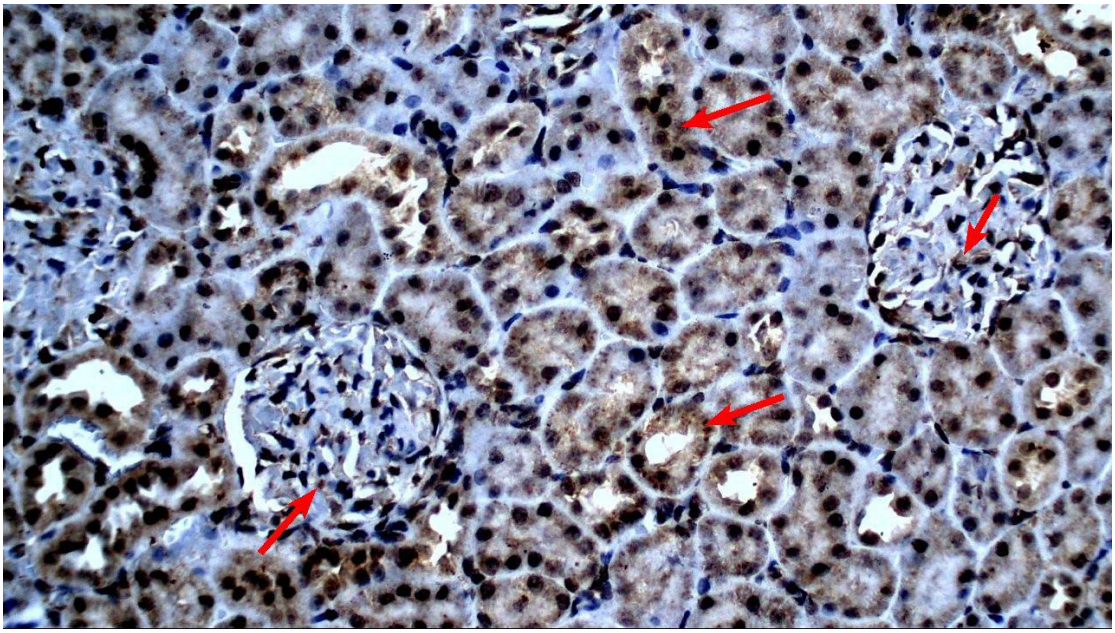


Figure 3b: A photograph of Caspace 3-IHC stained section form the cortex of AZ treated group showed widespread immunoreactivity reactions for caspace 3 in the cells of kidney glomeruli and tubules (arrows) (x400).

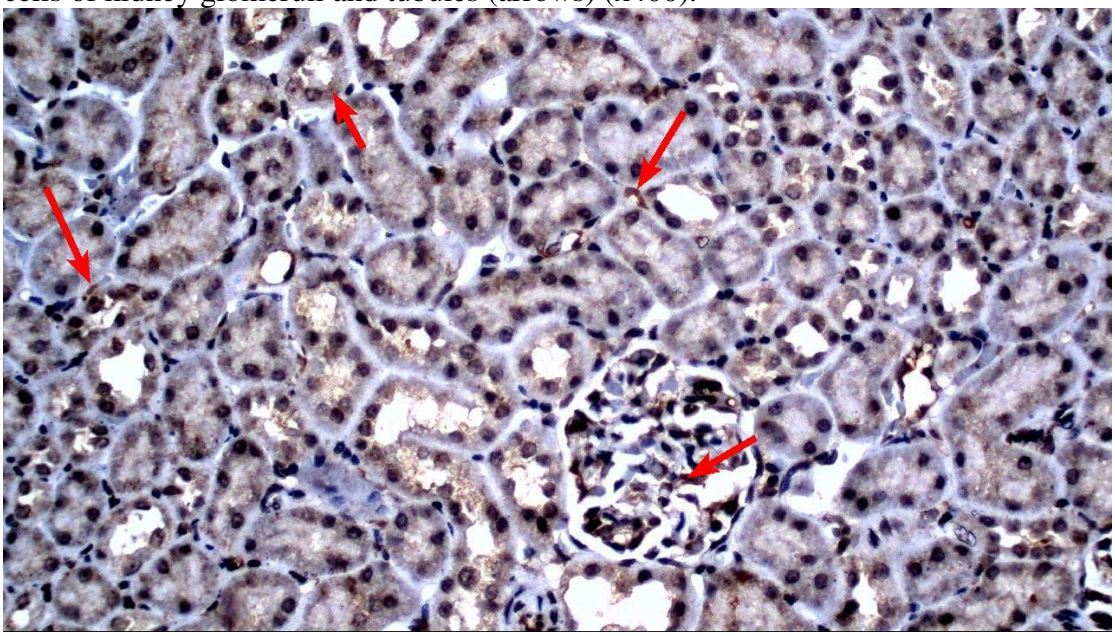


Figure 3c: A photograph of Caspace 3-IHCstained section from the cortex of AZ and VIT. C treated group showed weak cytoplasmic Caspace 3 reaction in the cells of kidney glomeruli and tubules (arrows) (x400).

B. TNF-α:

The renal cortex of control group using TNF-α showed weak cytoplasmic reaction in the cells of renal cortex (renal tubules and glomeruli) **(Figure 4.a),** IHC studies of the renal cortex of group 2using TNF revealed widespread immunoreactivity reactions in cells of glomeruli and renal tubules **(Figure 4.b),** in azithromycin and vitamin c group IHC staining using TNF-α revealed weak cytoplasmic reaction in

the cells of renal cortex (renal tubules and glomeruli) (**Figure 4.C).**

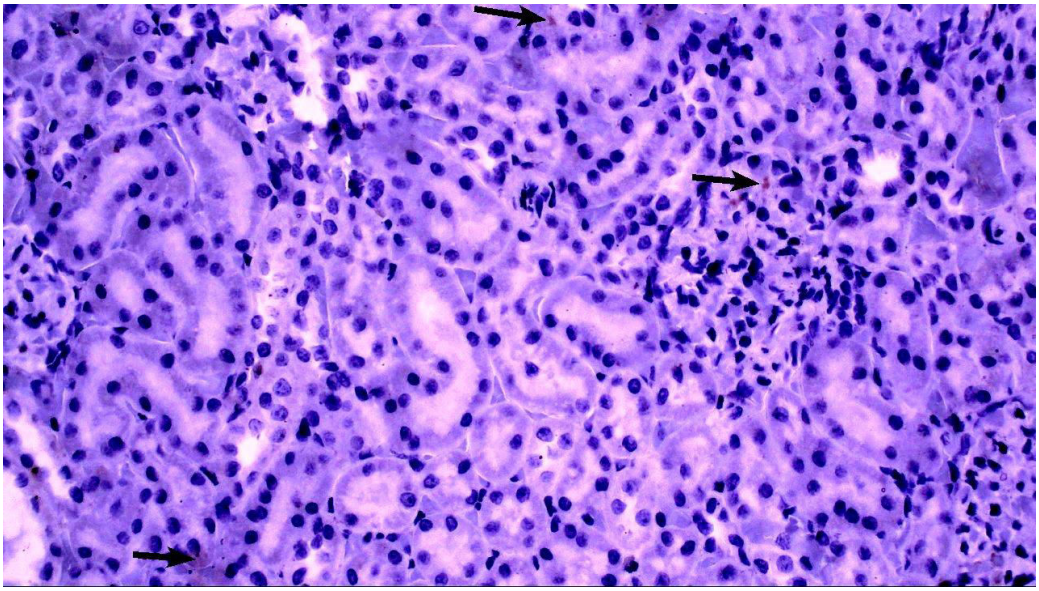


Figure 4a: A photograph of TNF-α-IHC stained section from the cortex of control group showed weak cytoplasmic TNF-α reaction in the cells of renal glomeruli and tubules (arrows) (x400).

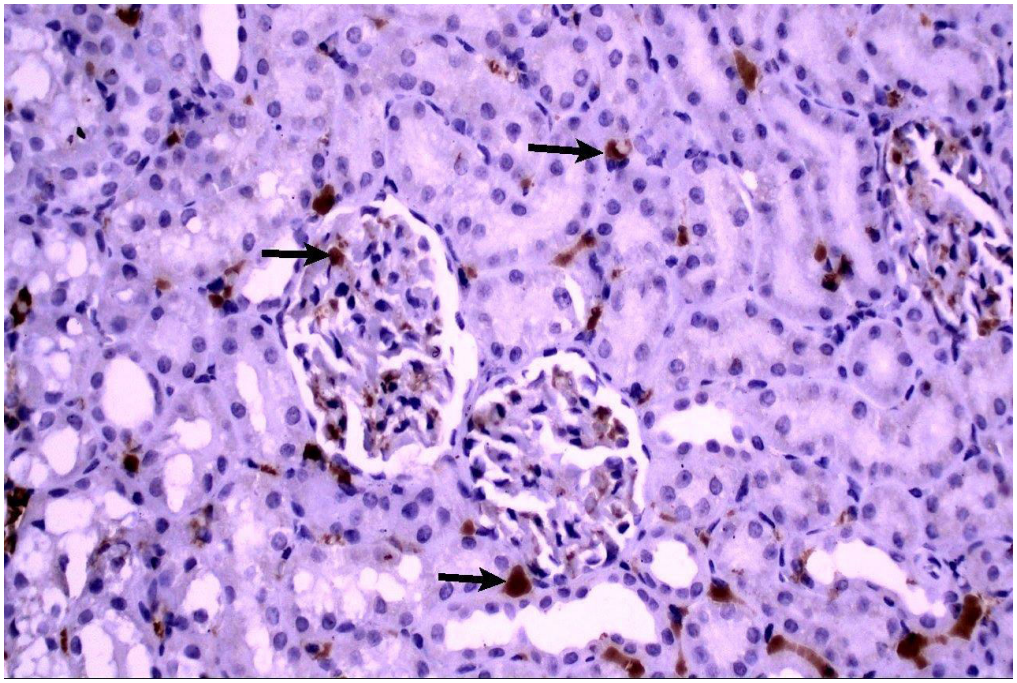


Figure 4b: A photograph of TNF-α IHC stained section from renal cortex of AZ treated group showing widespread cytoplasmic immunoreactivity reactions for TNF-α in cells of glomeruli and kidney tubules (arrows) (x400).

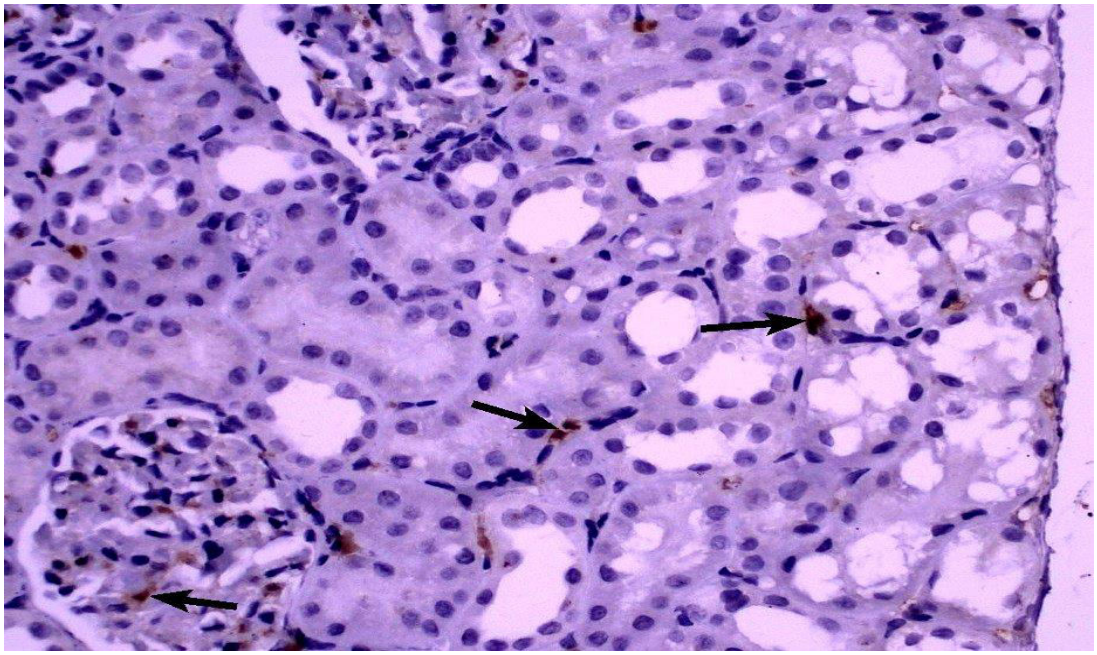


Figure 4c: A photograph of TNF-α-IHC stained section for cortex of AZ and VIT. C treated group showed weak reactions in the cells of renal glomeruli and tubules (arrows) (x400).

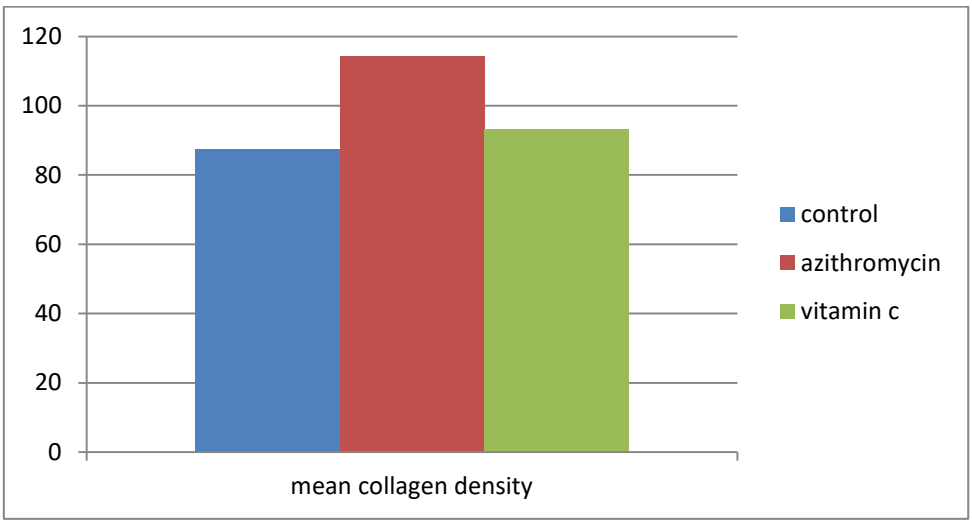
3. Morphometric studies

A. Collagen fibers density

The average collagen fibers density (arbitrary units, pixels) in the control group was (87.3±1.4), while in group II it was (114.2±4.8). In group III, the amount of collagen fibers was (93.2±4.2) with significant difference between the different groups ($p<0.001$)**(Table.1 and Histogram.1).**

Table 1. Collagen and Caspace 3 immunoreaction density in all groups

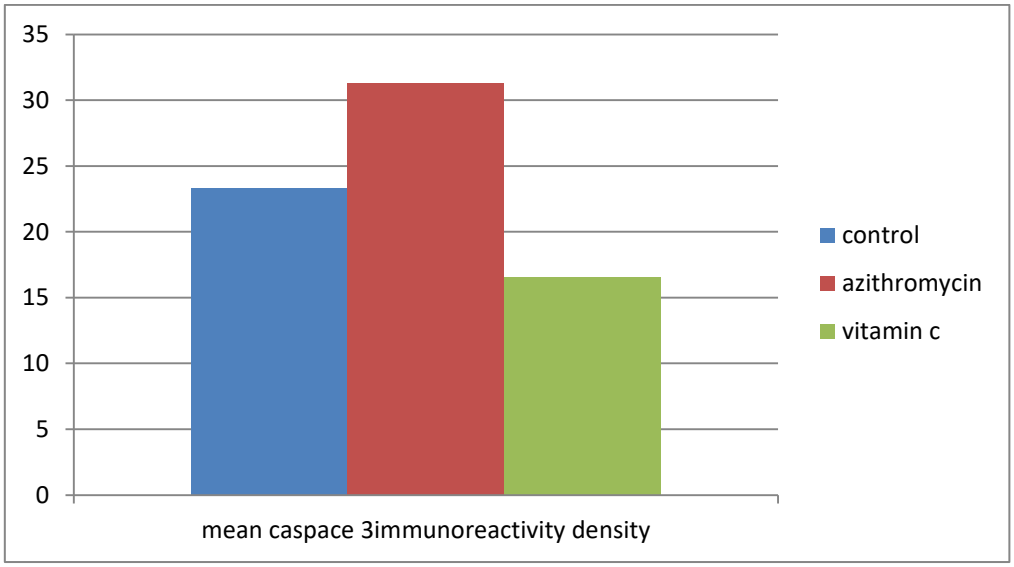
Variables	Control	AZ	VIT. C	P value
Collagen fibers density	87.3±1.4	114.2±4.8	93.2±4.2	**0.001***
Caspase 3 immunoreactivity	23.3±3.9	31.3±2.3	16.5±2.4	**0.001***



Histogram 1. Mean collagen density for all groups

B. Caspase-3 immunoreaction density

The average Caspase-3 immunoreaction density (arbitrary units, pixels) in the control group was (23.3±3.9),while in group II it was (31.3±2.3).In group III, the amount of collagen fibers was(16.5±2.4) with significant difference between the different groups (p <0.001)(**Table 1 and Histogram 2).**



Histogram.2: Caspace 3 immunoractivity for all groups

Discussion

Azithromycin was used with a great commitment for use with or without hydroxychloroquine during the initial phase of the COVID-19 pandemic in agreement with **Bogdanić et al.(2022).**AZ has an antibacterial activity, an immunomodulating effect, and some antiviral activity as was suggested by **Sultana et al..(2020).**

Perazella (2009) stated that the kidneys has an important role in elimination of many hydrophilic xenobiotics, including drugs, toxins, and endogenous compounds. **Solidoro et al. (2013)**proved that AZ a member of the macrolide (azalide) family can remain in the tissues up to one month even following a single dose administration and its blood serum concentration can be ≥ 100-fold that in body tissues. In this study examination of H&E stained renal sections of AZ treated group showed abnormal structure of the renal cortex. Some renal corpuscles showed renal spaces dilatation. The lining epithelium of the convoluted tubules showed numerous vacuolations inside the cytoplasm, shrinked nuclei, some empty spaces, hemorrhages and mononuclear cellular infiltration consistent with the previous report of **EL. Sayed et al. (2019).**They approved that oral administration of AZ 45 mg/kg body weight for 5 days resulted in marked liver and renal abnormalities in both histopathological and biochemical concerns.

Sakurai et al. (2018) reported that histopathological examination of kidneys from AZ treated showed mild vacuolations in the renal tubules, glomerulus, and transitional epithelium of the kidney due to induction of

phospholipidosis by AZ treatment in rat kidneys.

Martinez et al. (2015) and Usadadia et al. (2020) also advocated that other organs as the liver and heart of AZ treated rats showed severe liver sinusoidal hemorrhages, congestion and vacuolar changes. The heart showed increased spaces between cardiac muscle fibers with inflammatory cells infiltration of and multiple areas of hemorrhages.

Olayinka and Ore (2014) supposed that the causes of hepatotoxic and nephrotoxic effects of AZ were due to the formation of highly reactive free radicals as a result of oxidative threat caused by AZ which disrupt the normal cellular functioning of the liver and kidney.

In the current study the cortex of the kidney of AZ treated group showed severe apparent collagenous depositions within the basement membrane of glomeruli, around Bowman's capsule and in the interstitial tissue compared with the control by Masson's trichrome staining.

Pacher et al.(2005) approved that collagen deposition may be due to induction of oxidative damage of cellular lipids, proteins, and DNA as a result of increased free-radical formation. **Persico *et al.,* (2011) and Woodruff *et al.,* (2015)** also that, AZ induced acute interstitial nephritis with mixture of cellular infiltrate including lymphocytes, plasma cells, eosinophils, neutrophils with interstitial edema and tubular damage. **Elkomy et al. (2018)** suggested that the AZ drug caused degeneration and necrosis in the tubular lining epithelium of kidney of fetuses and caused fibrosis in between the atrophied renal tubules.

In this study we found by IHC studies of the cortex of AZ treated group marked Caspase-3 immunoreactivity in the convoluted tubules than in the control group in agreement with **EL. Sayed et al. (2019)** who reported that kidney of AZ treated rats showed necrosis of renal tubular cells with pyknosis of their nuclei and vacuolation of their cytoplasm. **Persico et al. (2011)** mentioned that the histology of the kidney of patient treated by AZ showed cellular infiltrate mixture in the form of plasma cells, eosinophils, neutrophils lymphocytes with interstitial edema and tubular damage,

El-Shitany & El-Desoky (2016) and **Mansour et al. (2021),**reported that IHC studies on heart of rat models treated with AZ showed production of oxidative stress, inflammation, and apoptosis of the myocardial tissue leading to ECG changes, myocardial infarction and death.

Medina and Moreno-Otero (2005) founded that oxidative stress is a pathogenic process participating in initiation and progression of cell damage. **Oboh** and **Ogunruku (2010)** and **Ryan et al. (2010)** stated that the damage induced byreactive oxygen species (ROS) includes alterations of cellular macromolecules such as membrane lipid, DNA, and/or protein, leads to changes in intracellular calcium or intracellular pH, and eventually can lead to cell death.

Markowitz and Perazella, (2005) reported that drugs that cause tubular cell toxicity act this by impairing mitochondrial function,

increasing oxidative stress, or via producing free radicals.

In this study, TNF-α -IHC of the cortex of AZ treated group revealed widespread immunoreactivity in the cells of glomeruli and renal tubules, this was in agreement with **(El-Shitany and El-Desoky, 2016)** who suggested that AZ increases the inflammatory response as it increases IL-1β and TNF-α**.**

Shin et al. (2002) and **Cai et al. (2013)** also, reported that TNF-α is responsible for regulating the production of some mediators that increasing the inflammatory reactions. TNF-α was known to attract leukocytes to the inflammatory sites so increasing the generation of ROS.

Odigie et al. (2007) and **Idogun and Ajala (2005)** stated that VIT. Cis a potent antioxidant agent, which mediates its antioxidant effect by scavenging free ROS and via inhibition of free radicals generation.

In this study AZ and VIT. C co-treated rats showed diminishing of most of hazards done by AZ and this was obvious in H&E stain, MT stain and IHC markers examined with light microscopic. This was in agreement with **Adeneye and Olagunju (2009)** who reported that VIT. C used as a protective and as a prophylactic agent against drug-induced nephrotoxicity. Also, **Abdel-Daim et al. (2015)** proved that VIT. C has anti-inflammatory and antiapoptotic cardioprotective mechanisms against AZ –induced cardiotoxicity. It also decreases the elevated IL-1β and TNF-α and decreasesCaspase-3 expression.

Conclusion

Azithromycin induces severe renal adverse side effect in rats, VIT. C reduces AZ-induced nephrotoxicity, and these effects may be related to antioxidant, anti-inflammatory, ant apoptotic and anti- fibrotic properties. Further studies are required to confirm the efficacy of VIT. C as a protective agent in human AZ induced nephrotoxicity.

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