Original Article

# Evaluation of the Protective Effect of Vitamins E and C on Acute Tubular Damage Induced by Colistin in Rat Model

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## ABSTRACT

**Objective:** This study was performed to investigate the protective effect of vitamins E and C against tubular damage induced by the high dose, 450 000 IU/kg/day, of colistin methanesulfonate (CMS) in rat model.
**Methods:** Thirty six rats were randomly divided into six groups (n = 6) treated with: sterile saline, CMS, CMS + olive oil (OO), CMS + vitamin C (vit C), CMS + vit E or CMS + vit E + vit C. Urine N-acetyl-b-D-glucosaminidase (NAG) and gamma-glutamyl-transferase (GGT) levels, plasma level of creatinine (Cr), vit E and vit C, and renal tissue levels of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), as well as renal histology were performed.
**Results:** CMS induced an acute tubular necrosis, increased the NAG, GGT and MDA levels and reduced the levels of vit E, vit C, SOD, CAT and GPx. Co-treatment with vitamins E and C, alone or in combination, restored all biochemical parameters cited above and improved the histopathological damage, with a superior nephroprotective effect of combined vitamins.
**Conclusion:** Tubular damage induced by colistin is at least partly due to oxidative stress. Nephro-protective effect of vitamins E+C is partially mediated through its antioxidant properties and the higher protection of combined vitamins is related to its synergistic effects.

**Keywords:** Colistin, Tubular necrosis, Oxidative stress, Vitamins E and C.

## INTRODUCTION

Colistin, clinically available in the form of its prodrug, colistin methanesulfonate (CMS), is increasingly used to treat infections caused by multidrug-resistant gram negative bacteria that are resistant to conventional antibiotics[1,2]. CMS is hydrolyzed *in vivo* or *in vitro* to a series of methanesulphonated derivatives plus the microbiologically active and toxic parent compound, colistin[3].

The administration of CMS/colistin has been associated with nephrotoxicity which is the major dose-limiting adverse effect impacting its clinical use[4]. Although, the mechanism of tubular toxicity still remains unknown, oxidative damage has been proposed implicated in nephrotoxic effect of colistin[5-7]. Therefore, there is an urgent need to investigate approaches to ameliorate colistin-induced nephrotoxicity, thereby widening the therapeutic window to allow administration of higher doses of CMS.

Antioxidants have been reported to reduce and remove free radicals and lipid peroxidation. Vitamin E (vit E) is well known antioxidants as a putative radical scavenger which is probably an important inhibitor of membrane lipid peroxidation. It is a lipid soluble agent which can readily cross cell membranes and exerts its effect both on cells and membranes[8]. On the other hand, ascorbic acid, which exerts powerful antioxidative properties on the hydrophobic compartments, can scavenge chain initiation by removing aqueous radicals[9]. Moreover, ascorbic acid potentiates the antioxidant activities of vit E by reducing tocopheroxyl radical[10]. Vit C in combination with vitamin E can play an important role in the regeneration of vit E[11,12].

The present study was therefore performed to evaluate the nephroprotective effect of vit E, vit C and combined vitamins E+C on colistin-induced nephrotoxicity in rats.

## MATERIELS AND METHODS

### Chemical products

Colistin methanesulphonate (CMS) was obtained from Aventis-France (1 million IU/vial). Vitamins E (α-tocopherol acetate) and C were purchased from Sigma (St. Louis, MO, USA). Glutathione (oxidized and reduced), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), 5-5'-dithio-bis-2-nitrobenzoic acid (DTNB) and thiobarbituric acid (TBA) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and were purchased from standard commercial suppliers.

### Animals

Male *wistar* rats weighing 250 ± 20 g were purchased from the breeding centre of the Central Pharmacy (SIPHAT). All animal procedures were conducted in strict conformity with the local Institute Ethical Committee Guidelines for the Care and Use of laboratory animals of our institution: they were kept in an environmentally controlled breeding room (temperature: 22±2°C, humidity: 60±5%, 12 h dark/light cycle) and given free access to alimentation and water. Standard diet composed of corn, soya and VMC (vitamins minerals compound). The characteristic of the standard diet[13] was illustrated in the Table 1.

### Experimental design

In our previous study on rats[14], the treatment with 450 000 IU/kg/day of CMS led to severe tubular damage. The cotreatment with vit E alone provided only a partial protection[15]. For this reason, we investigated the effect of combination of two

antioxidants, vitamins E and C, to attain a total protection.

Animals were randomly divided into 6 groups (n = 6) as follow: (1) treated with 1 ml/kg/day of sterile saline, (2) treated with 450 000 IU/kg/day of CMS; (3) as group 2 and co-treated with 1 ml/kg/day of olive oil (OO), (4) as group 2 and co-treated with 100 mg/kg/day of vit C, (5) as group 2 and co-treated with 100 mg/kg/day of vit E and (6) as group 2 and co-treated with 100 mg/kg/day of vit C + 100 mg/kg of vit E, respectively, for 7 days.

The administration of saline solution and CMS was given by intramuscular way, in twice daily doses (12 h apart). Vitamins E and C were administrated by oral gavage in once daily dose after dissolved in 1 ml/kg of OO and 1 ml/kg of distilled water, respectively. These dosages of vitamins E and C were reported to protect from marked nephrotoxicity caused by vancomycin[16] and gentamicin[17] in rats, respectively.

### Preparation of urine, blood and renal tissues samples

At the end of experiment period, animals were housed in individual metabolic cages and 12 h urine samples were collected and centrifuged at 1000 g for 5 min[14,18]. The supernatant was aliquoted into eppendorf tubes for determination of NAG and GGT levels.

Thereafter, animals were anesthetized, euthanized, and blood samples were collected from the heart in heparin tubes and centrifuged at 2500 g for 15 min[14,19]. The plasma was aliquoted into eppendorf tubes for determination of Cr, urea, vit E and vit C levels.

Then, the kidneys were removed, 500 mg were homogenized in 5 ml of lysis buffer (50 Mm Tris, 150 mM NaCl adjusted to pH 7.4) and centrifuged at 8000 g for 10 min[15,13]. The supernatant was collected for the determination of MDA, SOD, CAT and GPx levels.

### Biochemical assays

### Estimation of urine NAG and GGT levels

The level of N-acetyl-β-D-glucosaminidase (NAG) in urine was determined by colorimetric assay (Roche Applied Science, 68298 Mannheim Germany) and that of γ-glutamyl-transferase (GGT) was determined by enzymatic methods using commercial reagent kits (Ref. 94-1328) from Biomaghreb.

### Estimation of plasma Cr level

The levels of creatinine (Cr) in plasma were estimated spectro-photometrically using commercial diagnostic kits (Ref. 304331) from Biomagreb.

### Estimation of plasma vit E and vit C levels

The vit E level was essayed by the extraction method of Katsanidis and Addis[20] using high-performance liquid chromate-graphy (HPLC) and that of vit C was performed as described by Jagota and Dani[21].

### Protein quantification

Kidney protein contents were assayed by the method of Bradford[22].

### Lipid peroxidation marker in kidney

The renal tissue malondialdehyde (MDA) level, a lipid peroxidation marker, was determined spectrophotometrically according to Draper and Hadley[23].

### Antioxidant parameters in the renal tissues

The superoxide dismutase (SOD) activity was estimated according to Beauchamp and Fridovich[24], glutathione peroxidase (GPx) was measured according to Flohe and Gunzler[25], and catalase (CAT)

activity was assayed by the method of Aebi[26].

### Histopathological examination

For light microscopic examination, kidneys removed from the control and tested rats were cleaned and fixed in 10% buffered formalin solution. Then they were embedded in paraffin and stained with hematoxylin–eosin for histopathological studies. All sections were evaluated for the degree of tubular and glomerular injury and necrosis[14,19].

### Statistical analysis

Data are expressed as mean ± SD (standard deviation). The statistical significance between experimental groups was assessed by one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. A P value, 0.05 was considered significant.

## RESULTS

### Urine NAG and GGT levels

The urine NAG activity increased significantly by 40% in group treated with 450 000 IU/kg/day of CMS, compared to the control. The co-treatment with vit C, vit E or combined vitamins reduced the NAG activities by 15%, 17% and 23% respectively, compared to group received only the CMS (tab. 2).

Similarly, the urine GGT activity increased significantly by 23% after treatment with 450 000 IU/kg/day of CMS, compared to the control. The simultaneous treatment with vit C, vit E or combined vitamins attenuated the GGT activities by 12%, 11% and 16%, respectively, compared to CMS group (tab. 2).

### Plasma Cr level

Plasma Cr increased in the CMS group but without significant change, compared to the control. Similar, there was no significant variation in Cr level in all co-treated groups with vitamins, compared to CMS group (tab. 2).

### Plasma vit E and vit C levels

Plasma vit E and vit C levels also decreased by 46% and 53%, respectively, after treatment with 450 000 IU/kg/day of CMS, in comparison to those of the control. These changes were improved when the rats were co-treated with vitamins, compared to those of CMS group (tab. 3).

### Lipid peroxidation in kidney homogenates

The renal tissue level of MDA increased significantly by 61% in the CMS group, compared to the control. The co-treatment with vit C, vit E or combined vitamins reduced significantly the renal tissues lipid peroxidation by 18%, 22% and 31%, respectively, compared to those of treated rats only with CMS (fig. 1).

### Antioxidant enzymes in kidney homogenates

The treatment with CMS decreased significantly the renal tissues activities of SOD, CAT and GPx by 45%, 47% and 34%, respectively, relative to those of the control. These activities were restored significantly when animals were co-treated with vit C (by 38%, 41% and 26%, respectively), vit E (47%, 52% and 29%, respectively) or combined vitamins (60%, 74% and 44%, respectively) (fig. 2 a-c).

### Kidney histopathology

Light microscopic examination of the kidney in the control revealed a normal structure (fig. 3A). In the 450 000 UI/kg/day of CMS treated rats, histological sections showed a severe tubular necrosis but with glomerular preservation (fig. 3B). Co-administration of vit C or vit E plus CMS provided partial tubular protection (fig. 3C/D). However, the simultaneous administration of both vitamins plus CMS

seems more efficient; kidney exhibited only slight tubular dilatations (fig. 3E).

## DISCUSSION

The present study indicated that the treatment with CMS at 450 000 IU/kg/day for 7 days, led to severe renal damage described in an acute tubular necrosis, accompanied with significant biochemical impairment. The increase of the urinary GGT and NAG excretion in colistin-treated rats is an indication of tubular cell injury[27]. NAG, a proximal tubule lysosomal enzyme, is among the most extensively studied and best characterized urinary enzymes. It has proven to be a sensitive and robust marker of acute tubular injury. Increased NAG levels have been reported with nephrotoxicant exposure[28,29]. In contrast, after co-treatment with vitamins, the urinary NAG and GGT excretion decreased across the 7 days. This improvement, as demonstrated by the pathological findings, explains the protection effect of vitamins against tubular cell damage caused by colistin. We noted equally a significant perturbation of oxidative stress markers in renal tissue of colistin group. The MDA augmentation proves the implication of lipid peroxidation. Moreover, the SOD, CAT and GPx activities are significantly declined. Hence, these observations reveal that oxidative stress may be the underlying mechanism of nephrotoxicity due to colistin. Oxidative stress has been reported to have a central role in tubular toxicity caused by many drugs, including gentamicin, amikacin, vancomycin, and cisplatin, reactive oxygen species (ROS) generated via mitochondria have been shown to initiate renal cell apoptosis, ultimately leading to renal dysfunction[30]. On the other hand, we found that co-treatment with vitamins E and C, alone or in combination, reinforce the resistance of renal tissue to colistin aggression. The combination of vitamins E and C (100 mg/kg/day body wt. each) brought about a better protection with respect to their single dose. Thus, the results demonstrate that vitamins decrease the nephrotoxic effect of colistin and support the previous hypothesis.

These finding are in concordance with our histological results. Light microscopic analyses revealed that co-treated animals with combined vitamins plus CMS exhibit only a minor tubular dilatation. The co-treatment only with vit E or vit C plus the same dose of CMS revealed a slight tubular necrosis that not exists in the combination of vitamins, which confirmed the synergistic action of these vitamins as mentioned previously by others studies on gentamicin[17] and cisplatin[31]. Therefore, a combination therapy of vitamins E and C at 100 mg/kg/day each can be effective in protecting against colistin-induced renal damage. The equivalent human dose corresponding to 100 mg/kg/day (approximately 500 mg in an adult of 60 kg b.w) of vit C (2 g/day) or vit E (1 g/day), as recommended by the Food and Nutrition Board of the U.S. Institute of Medicine, was found to be less than the tolerable upper intake concentration (UL). Our results revealed that these doses of vitamins restored the plasmatic concentration of vit E and vit C in co-treated group with CMS plus vitamins; we observed compared values to those of the control. Thus these doses of vitamins E and C are excessive and could protect the kidneys against the deleterious effect of colistin.

In conclusion, our experiments revealed that nephrotoxicity induced by colistin could be related in part to oxidative damage. The combination of vitamins E and C could effectively protect against colistin-induced renal damage than the co-treatment with each vitamin alone. The protection is partially mediated through the antioxidant ant synergistic effect of the vitamins.

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### Competing Interests

None declared.

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**Table 1:** Composition of basic food (Society of Animals Nutrition "SNA", Sfax, Tunisia). This food consists of corn, soya, VMC (vitamins minerals compound) with the following characteristics.

| Nutritional properties (%) | |
|---|---|
| Moisture (maximal) | 14 |
| Fibers (maximal) | 5 |
| Proteins (minimal) | 18 |
| Fat (maximal) | 3 |
| Ash (maximal) | 13.5 |
| Carbohydrate | 46.5 |
| Calorific value (kcal/kg) | 2846 |
| **Amino acid (%)** | |
| Methionine | 0.36 |
| Cysteine | 0.26 |
| Threonine | 0.62 |
| Tryptophan | 0.2 |
| Mineral mix (mg/kg) | |
| Manganese | 80 |
| Fer | 48 |
| Cuivre | 18 |
| Zinc | 64 |
| Selenium | 0.28 |
| Cobalt | 0.2 |
| Iode | 2 |
| **Vitamin and antioxidant (mg/kg)** | |
| Vitamine A | 11,2 |
| Vitamine D3 | 2800 |
| Vitamine H | 25 |
| Antioxidant (BHA–BHT) | 100 |

**Table 2:** The variation of plasma Cr and urinary GGT and NAG levels in the control and the different treated groups of rats over 7 days.

| | Cr (mg/dl) | GGT (U/g Cr) | NAG (U/l) |
|---|---|---|---|
| Control | 0.47 ± 0.11 | 665.22 ± 32.11 | 25.15 ± 3.61 |
| CMS | 0.53 ± 0.12 | 824.44 ± 46.33$^{**}$ | 35.38 ± 5.45$^{**}$ |
| CMS + OO | 0.51 ± 0.18 | 801.44 ± 41.75$^{**}$ | 33.76 ± 4.62$^{**}$ |
| CMS + vit C | 0.47 ± 0.12 | 727.23 ± 36.28$^{\#}$ | 30.12 ± 5.13$^{\#}$ |
| CMS + vit E | 0.48 ± 0.15 | 735.53 ± 32.15$^{\#}$ | 29.27 ± 4.15$^{\#}$ |
| CMS+ vit E+ vit C | 0.45 ± 0.10 | 694.83 ± 38.23$^{\#\#}$ | 27.16 ± 3.66$^{\#\#}$ |

Values are expressed as mean ± SD (n = 6). $^{**}$ $p < 0.01$ vs control; $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$ vs CMS group. CMS = colistin methanesulfate of sodium; vit: vitamin; OO: olive oil; Cr: creatinine; NAG: N-acetyl-β-D-glucosaminidase; GGT: γ-glutamyl-transferase.

**Table 3:** Plasma level of vit E and vit C in the control and the different experimental treated rats over 7 days.

| | Vit E (µg/ml) | Vit C (µg/ml) |
|---|---|---|
| Control | 6.21 ± 1.65 | 13.22 ± 3.25 |
| CMS | 3.34 ± 0.62** | 6.21 ± 2.30** |
| CMS + OO | 3.73 ± 0.81** | 7.81 ± 2.53** |
| CMS + vit C | 5.15 ± 1.15# | 11.23 ± 3.15# |
| CMS + vit E | 5.53 ± 0.85# | 8.58 ± 3.45# |
| CMS + vit E + vit C | 6.52 ± 2.12## | 12.75 ± 3.22## |

Values are expressed as mean ± SD (n = 6). **p < 0.01 vs control; #p < 0.05 and ##p < 0.01 vs CMS group.
CMS = colistin methanesulfate of sodium; Cr: creatinine; OO = olive oil; vit: vitamin.

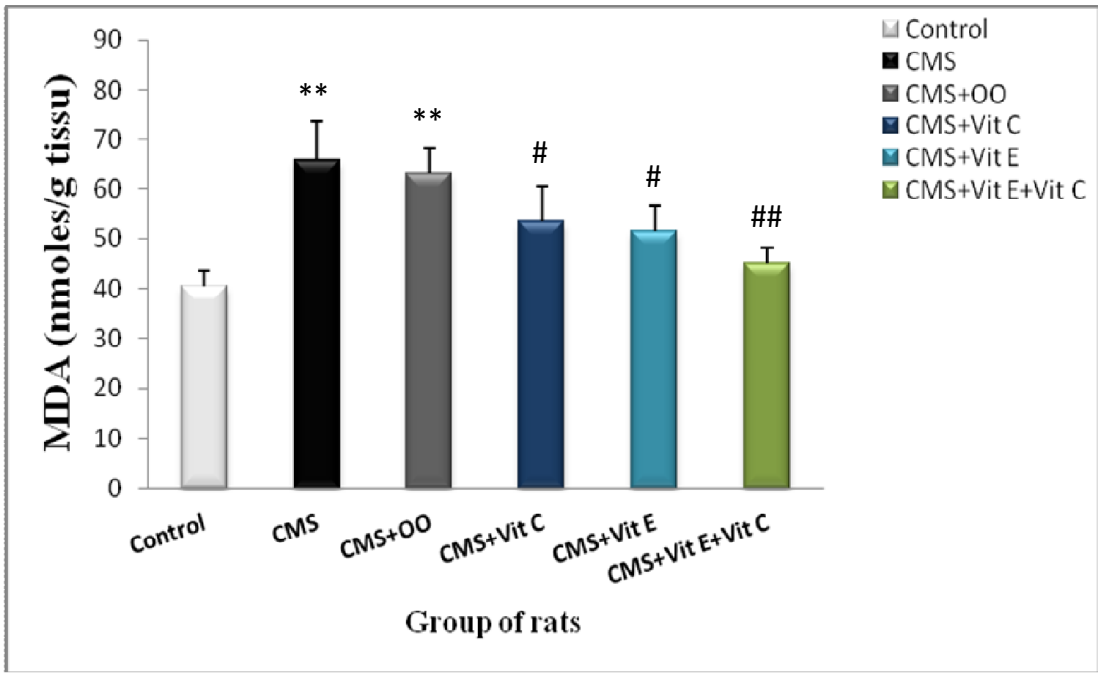


**Figure 1:** MDA level in kidney of treated rats for 7 days with sterile saline, CMS, CMS + vit E, CMS + vit C or CMS + vit E + vit C. Values are given as means ± standard deviation (SD) of six rats. **p < 0.01 compared to control; #p < 0.05 and ##p < 0.01 compared to CMS group.

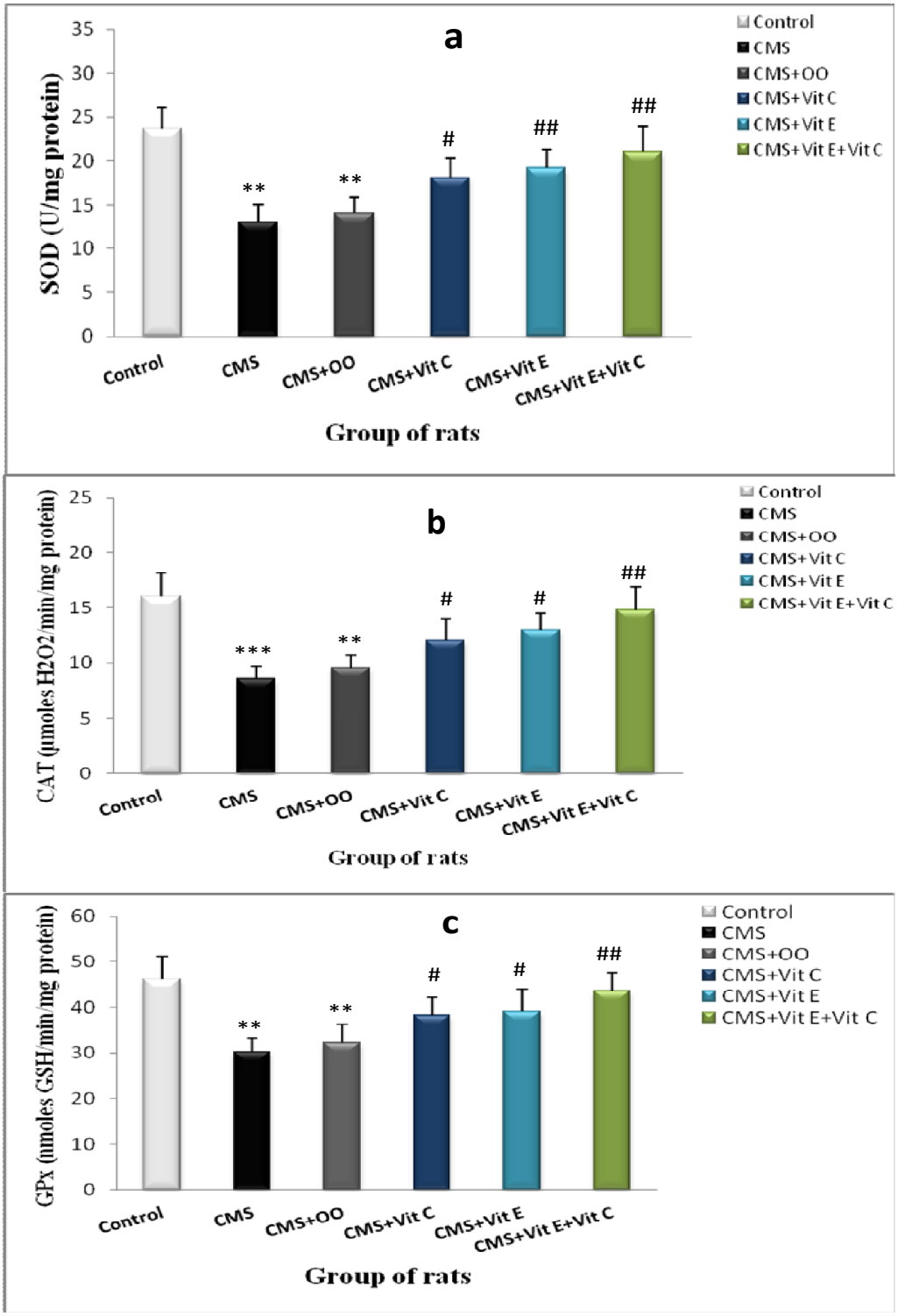


**Figure 2:** SOD (**a**), CAT (**b**) and GPx (**c**) activities in kidney of treated rats for 7 days with sterile saline, CMS, CMS + vit E, CMS + vit C or CMS + vit E + vit C. Data are expressed as mean ± SD (n=6). $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ as compared with CMS group.

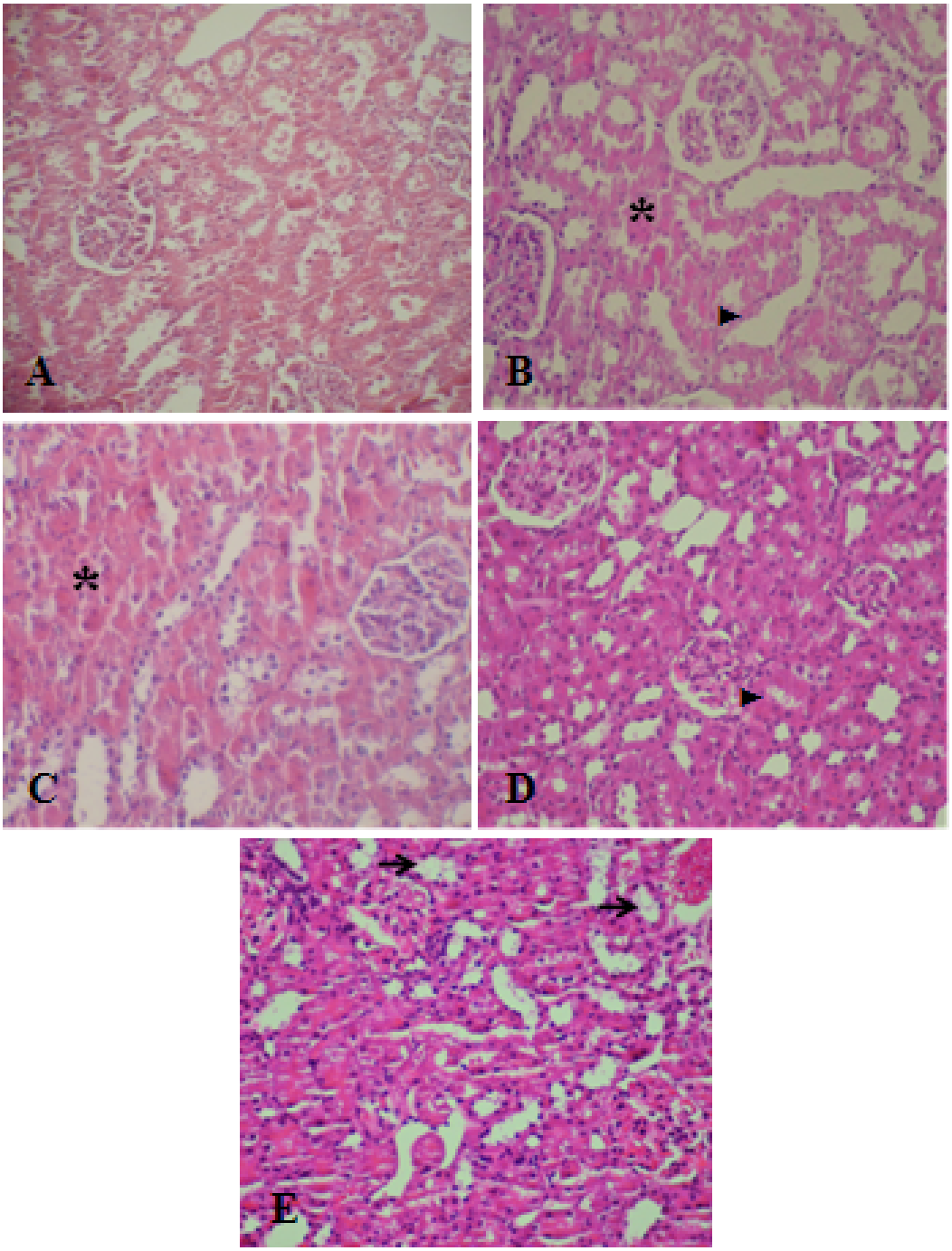


**Figure 3:** Kidney histological sections (Hematoxylin and Hosin, 250 X) of: (**A**) control rat showing normal renal cortex, (**B**) treated rat with CMS showing an acute tubular dilatation (►) and necrosis (*), co-treated rat with CMS + vit C (**C**) or vit E (**D**) showing moderate tubular dilatation and necrosis, (**E**) co-treated rat with CMS + vit E + vit C showing slight tubular dilatation (➔).