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Evaluation of reno-protective and anti-inflammatory effects of doxycycline in carrageenan-induced renal inflammation

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ABSTRACT

The present study was designed to investigate biochemical changes induced by doxycycline treatment of carrageenan-induced renal inflammation. For this purpose, 60 rats divided into four groups. Group (1) used as a control and administrated distilled water (1 ml/kg, i. p.) daily for 5 days. Group (2) carrageenan group: rats administrated 2% λ- Carrageenan (25 µl/ kg, i. p.) as one dose at first day of experiment. Group (3) doxycycline rats were received doxycycline (100 mg/kg, i. p./day/5 days). Group (4) carrageenan+ doxycycline rats were received doxycycline (100 mg/kg, i. p.), after an hour 2% λ- carrageenan (25 µl/kg, i. p.) as one dose at first day of experiment. The experiment was continued for 5 days. Collection of samples was performed on 1st, 3rd, and 5th day of experiment. Serum was used for evaluation of kidney function (urea, creatinine, Na^+ and K^+) and plasma proteins (total protein, albumin, and globulin), as well as inflammatory markers and cytokines (TNF-*α*, IL-6, CRP, and NO). Kidney tissue used for estimation of antioxidant parameters (MDA and GPx), and histopathology. The results revealed that there were significant increases in urea, creatinine, MDA, TNF-*α*, IL-6, CRP, and NO in carrageenan group, while GPx, Na^+ and K^+ showed significant decreases. Moreover, histological examination of group 2 showed degenerative changes in the lining epithelium of renal tubules associated with peritubular mononuclear cells infiltration compared with control group. Therefore, it could be concluded that doxycycline has anti-inflammatory role.

1. INTRODUCTION

The kidneys are one of the more important organs in the body. It has a major role in the filtration, remove waste products and excess fluid from the body. Kidney also produces hormones that affect the function of other organs such as, erythropoietin hormone which stimulates red blood cell production. So, a wide range of spontaneous renal lesions may be observed (Khan et al., 2018). Chronic progressive nephropathy may be exacerbated by chemical administration. Carrageenan is linear sulfated polysaccharides, obtained by extraction from certain species of red seaweeds is widely used to induce inflammation of the tissues (Zia et al., 2017). Doxycycline (Dox) is a semisynthetic tetracycline used to treat many bacterial infections. Dox is becoming increasingly popular due to pleiotropic functions independent of its antimicrobial activity (Brown et al., 2004). Dox can scavenge free radicals and has stabilizing effect. Moreover, it has anti-inflammatory and immunomodulatory effects (Yiğit et al., 2017). The objective of present study was to evaluate the reno-protective, anti-inflammatory and antioxidant effects of doxycycline in acute kidney inflammation induced by carrageenan through the investigation of renal functions (urea, creatinine, Na^+ and K^+), and serum protein (total protein, albumin, and globulin), antioxidant parameters (MDA and GPx), pro-inflammatory markers and cytokines (TNF-α, IL-6, CRP, and NO) and histopathological examination of kidney

2. MATERIAL AND METHODS

2.1. Animals:

Sixty male albino rats with a weight of between 100-120 gm were obtained from the Animal House, Faculty of Veterinary Medicine, Benha University, Egypt. All animals were caged and maintained on a standard diet, with free access to tap water and were acclimatized for 1 week before starting the experiments. They were kept at normal atmosphere condition and regular period of natural light/darkness daily cycle. The study performed with approval from the institutional review board for animal experiments of the Faculty of Veterinary Medicine, Benha University.

2.2. Chemicals:

Doxycycline was obtained from EL-Nile Company for pharmaceutical and chemical industries, Cairo, Egypt according to method of Kogawa and Salgado, (2012). 2%λ-Carrageenan was obtained from Special Ingredients Ltd, 4 Foxwood Industrial Park Chesterfield, United Kingdom.

2.3. Experimental design:

In this study, rats were categorized into four classes. Group (1) was used as a control and received distilled water (1

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ml/kg, i.p.) daily for 5 days. Group (2) carrageenan group: rats administrated 2% λ- carrageenan (25 µl/kg i. p.) as one dose at first day of experiment. Group (3) Doxycycline rats were received doxycycline given (100 mg/kg, i. p./day/5 days). Group (4) carrageenan + doxycycline rats were received doxycycline (100 mg/kg, i. p.), after one hour 2% λ- carrageenan given (25 µl/kg, i. p.) as one dose at first day of experiment. The experiment was continued for 5 days.

2.4. Sampling:

Blood samples from the retro-orbital plexus were obtained after overnight fasting from all groups after 1st, 3rd, and 5th day of the experiment. 1 ml blood samples were collected in gel tubes and by centrifugation at 2500 rpm for 15 minutes serum was separated, then serum was stored at -20 °C for estimation of biochemical parameters. After collection of blood samples, rats were sacrificed by cervical decapitation and kidney specimen was perfused with cold saline to exclude the blood cells. The kidney was divided into two parts. The first part (1 gm) was suspended in 4 ml physiological saline for homogenization then centrifugation. The supernatants were kept at -20ºC till the time of determination of oxidative/antioxidant parameters (Yang et al., 2010). For histopathological examinations the second part was fixed in 10 % formalin solution.

2.5. Measurement of biochemical parameters:

Serum total proteins were determined by the reaction described by Burits et al. (1999). Colorimetric determination of albumin was done by technique according to Doumas et al. (1971). The serum globulin was calculated by subtracting obtained albumin level from the obtained total protein level. Colorimetric determination of urea was done according to Henry et al. (1974). Quantitative Kinetic determination of creatinine was done by technique of Fabiny and Ertingshausen, (1971). Quantitative determination of sodium ion IVD was done according to Henry et al. (1974). Quantitative determination of potassium ion IVD was done according to the technique of Young, (1997). Immunoassay method for the quantitative determination of TNF-α was carried out according to Aggarwal and Natarajan, (1996). Quantitative determination of nitric oxide was done according to Kelm et al. (1988). Quantitative determination of (CRP) was done according to Clyne et al. (1999). We used ELISA kit for quantitative detection of IL-6 according to the method of Ferguson-Smith et al. (1988).

2.6. Antioxidant parameters determination:

Malondialdehyde was determined by the reaction described by Ohkawa et al. (1979) and GPX is determined by the reaction described by Plgia and Velentine (1967).

2.7. Histopathological examinations:

Small tissue specimens from the kidney were collected from animals of different groups then fixed in 10% neutral buffered formalin. After proper fixation, were dehydrated in alcohol, cleared xylol and embedded in paraffin wax. Then, 5 µm tissue-paraffin sections were prepared and stained with H&E stain (Bancroft and Layton, 2013).

2.8. Statistical analysis:

The statistical analysis was carried out using one-way ANOVA with SPSS, ver. 22 (IBM Corp. Released 2013). Data of serum biochemical and antioxidant assay were treated as a complete randomization design based on Steel and Torrie (1980). Multiple comparisons were made using the Tukey test. The level of significance was set at $p< 0.05$.

3. RESULTS

3.1. Biochemical analysis:

The results presented in table (1) in 1st check point, table (2) in 2nd check point and table (3) in 3rd check point revealed that carrageenan injected rats in group 2 showed a significant increases in blood urea nitrogen and creatinine with a significant decreases in Na^+, K^+, total protein, albumin and globulin when compared with non-treated control group.

In contrast, comparing with carrageenan group carrageenan injected rats and treated with doxycycline in group 4 significant decreases in blood urea nitrogen and creatinine with significant increases in Na^+, K^+, total protein, albumin and globulin were recorded.

Table 1 Changes in serum biochemical parameters in different groups at the 1st check point of the experiment.

Treatments	Creatinine (mg/dl)	Urea (mg/dl)	Na^+ (mmol/l)	K^+ (mmol/l)	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)
Control	0.56±0.01^c	31.70±1.08^b	148.50±1.04^a	5.05±0.10^a	6.66±0.19^a	3.38±0.05^a	3.38±0.15^a
Carrageenan	1.06±0.04^a	62.43±1.28^a	131.75±1.11^c	3.40±0.09^c	4.57±0.11^c	2.43±0.10^d	2.23±0.12^c
Doxycycline	0.57 ±0.01^c	32.00±0.56^b	147.20±1.32^a	4.90±0.09^a	5.64±0.09^b	3.17±0.04^b	2.55±0.04^b
Carrageenan +Doxycycline	0.81±0.01^b	26.69±3.2^c	142.00±0.91^b	3.98±0.13^b	5.32±0.06^b	2.69±0.06^c	2.56±0.07^b

a, b & c: There is no significant difference (P>0.05) between any two means, within the same column have the same superscript letter.

Table 2 Changes in serum biochemical parameters in different groups at the 2nd check point of the experiment.

Treatments	Creatinine (mg/dl)	Urea (mg/dl)	Na^+ (mmol/l)	K^+ (mmol/l)	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)
Control	0.61±0.01^c	24.68±1.29^c	138.75±1.65^a	4.98±0.15^a	6.68±0.06^a	3.41±0.06^a	3.33±0.02^a
Carrageenan	1.17±0.05^a	54.61±2.20^a	132.00±1.08^b	3.40±0.07^c	4.89±0.07^d	2.57±0.09^d	2.33±0.12^b
Doxycycline	0.63±0.01^c	24.71±1.06^c	135.25±0.85^{ab}	4.94 ±0.11^a	6.41±0.05^b	3.18±0.05^b	3.27±0.04^a
Carrageenan +Doxycycline	0.70±0.01^b	30.42±1.04^b	139.25±3.09^a	4.05±0.06^b	5.45±0.11^c	2.93±0.03^c	2.47±0.09^b

a, b & c: There is no significant difference (P>0.05) between any two means, within the same column have the same superscript letter.

Table 3 Changes in serum biochemical parameters in different groups at the 3rd check point of the experiment.

Treatments	Creatinine (mg/dl)	Urea (mg/dl)	Na^+ (mmol/l)	K^+ (mmol/l)	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)
Control	0.54±0.01^c	26.89±1.42^c	139.50±1.04^{ab}	5.18±0.09^a	6.68±0.19^a	3.49±0.10^a	3.44±0.11^a
Carrageenan	1.10±0.05^a	58.75±1.05^a	132.00±1.08^c	3.28±0.11^c	4.63±0.09^b	2.04±0.07^b	2.69±0.10^b
Doxycycline	0.56±0.02^c	40.30±0.94^b	137.50±1.04^b	5.16 ±0.12^a	6.32±0.07^a	2.99±0.13^a	3.32±0.18^a
Carrageenan +Doxycycline	0.65±0.03^b	34.94±1.56^b	141.75±1.55^a	4.45±0.41^b	6.56±0.27^a	3.15±0.32^a	3.33±0.31^a

a, b & c: There is no significant difference (P>0.05) between any two means, within the same column have the same superscript letter.

3.2. Antioxidant and pro-inflammatory parameters determination:

The results presented in table (4) in 1st check point, table (5) in 2nd check point and table (6) in 3rd check point revealed that carrageenan injected rats in group 2 showed a significant increase in MDA, TNF-α, IL-6, CRP and NO, with a significant decrease in GPx when compared with non-treated control rats. In contrast, comparing with

carrageenan group carrageenan injected rats and treated with doxycycline in group 4 revealed a significant decreases in MDA, TNF-α, IL-6, CRP, and NO, with significant increase in GPx.

Table 4 Changes in antioxidants and pro-inflammatory parameters determination in different groups at the 1st check point of the experiment.

Treatments	GPx (ng/mg)	MDA (nmol/mg)	Interlukin-6 (Pg/ml)	CRP (ng/ml)	NO (U/mg)	TNF-α (Pg/ml)
Control	27.13±1.05^{a}	28.25±1.49^{c}	3.65±0.05^{c}	23.00±3.42^{c}	15.00±1.29^{c}	8.00±1.08^{c}
Carrageenan	8.84±0.22^{d}	178.25±7.05^{a}	9.06±0.13^{a}	244.50±8.59^{a}	124.75±2.43^{a}	65.5±3.75^{a}
Doxycycline	23.81±0.73^{b}	26.25±2.39^{c}	3.11±0.14^{d}	18.25±2.53^{c}	12.00±0.91^{c}	10.50±0.65^{c}
Carrageenan + Doxycycline	19.69±0.37^{c}	95.75±2.32^{b}	6.52±0.18^{b}	179.25±4.71^{b}	101.00±4.95^{b}	41.50±1.32^{b}

a, b & c: There is no significant difference (P>0.05) between any two means, within the same column have the same superscript letter.

Table 5 Changes in antioxidants and pro-inflammatory parameters determination in different groups at the 2nd check point of the experiment.

Treatments	GPx (ng/mg)	MDA (nmol/mg)	Interlukin-6 (Pg/ml)	CRP (ng/ml)	NO (U/mg)	TNF-α (Pg/ml)
Control	25.94±0.78^{a}	31.5±3.1^{c}	4.05±0.18^{c}	9.81±0.81^{c}	18.75±1.11^{c}	6.25±1.11^{c}
Carrageenan	9.35±0.43^{d}	174.75±3.25^{a}	9.85±0.35^{a}	194.25±3.42^{a}	181±10.89^{a}	71.25±2.53^{a}
Doxycycline	21.44±0.41^{b}	25.25±1.7^{c}	3.21±0.21^{d}	11.50±0.65^{c}	12.50±2.10^{c}	9.75±1.49^{c}
Carrageenan +Doxycycline	16.75±0.85^{c}	88.5±1.32^{b}	5.46±0.25^{b}	91.00±2.48^{b}	93.00±3.49^{b}	41.75±1.11^{b}

a, b & c: There is no significant difference (P>0.05) between any two means, within the same column have the same superscript letter.

Table 6 Changes in antioxidants and pro-inflammatory parameters determination in different groups at the 3rd check point of the experiment.

Treatments	GPx (ng/mg)	MDA (nmol/mg)	Interlukin-6 (Pg/ml)	CRP (ng/ml)	NO (U/mg)	TNF-α (Pg/ml)
Control	27.19±1.7^{a}	28.75±2.17^{b}	4.26±0.17^{b}	13.38±2.10^{c}	19.00±1.47^{c}	8.50±1.19^{c}
Carrageenan	11.19±0.2^{c}	151.75±7.81^{a}	9.56±0.25^{a}	208.00±16.27^{a}	167.75±9.07^{a}	69.25±3.94^{a}
Doxycycline	23.88±0.5^{b}	27.5±1.71^{b}	3.23±0.27^{c}	11.25±1.11^{c}	11.00±0.41^{c}	16.50±2.66^{c}
Carrageenan +Doxycycline	22.81±1.3^{b}	27.75±2.53^{b}	3.85±0.26^{bc}	61.75±2.29^{b}	79.50±1.85^{b}	32.25±6.1^{b}

a, b & c: There is no significant difference (P>0.05) between any two means, within the same column have the same superscript letter.

3.3. Histopathology:

The examined kidneys of non- treated control rats showed normal renal glomeruli and tubules (Fig. 1-A). Carrageenan injected rats revealed marked confluent degenerative changes within the lining epithelium of some renal tubules associated with peritubular mononuclear cells infiltration and (Fig. 1-B). Kidney of rats treated with doxycycline showed normal renal glomeruli and tubules (Fig. 1-C). While the examined kidneys of carrageenan injected rats and treated with doxycycline showed mild congestion and degeneration of the renal tubules (Fig. 1-D).

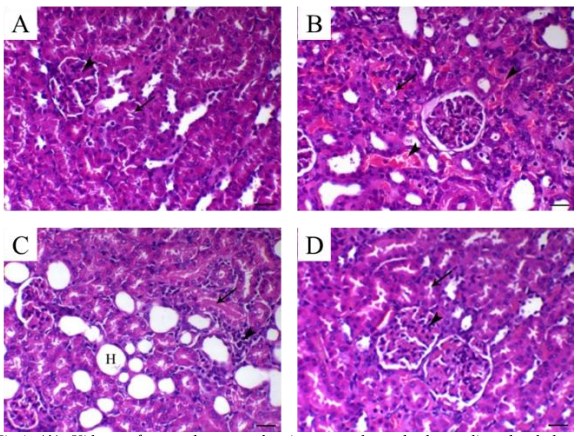

Fig.1 (A) Kidney of control group showing normal renal glomeruli and tubules (arrowhead and arrow respectively), H&E, bar= 50 µm. (B) Kidney of carrageenan injected rats showing cystic dilatation of some renal tubules with flattening of their lining epithelium (arrow) associated with peritubular mononuclear cells infiltration (arrowhead) and hydronephrosis (H), H&E,. (C) Kidney of rats administrated doxycycline showing normal renal glomeruli and tubules (arrowhead and arrow respectively), H&E. (D) Kidney of carrageenan injected rats and treated with doxycycline showing congestion of the renal capillaries (arrowheads), H&E stain X200

4. DISCUSSION

Regarding to biochemical parameters, rats injected with carrageenan showed a significant increase in blood urea nitrogen and creatinine when compared with control group with a significant decrease in Na and K. These results agree with Dilipkumar (2018). In addition, these results could be attributed to the early stage of carrageenan administration which is associated with the development of immediate inflammatory mediators such as histamine, bradykinin, leukotrienes, platelet-activating factor, and cyclo-oxygenase products in the inflamed tissue (Bignotto et al., 2009). In contrast, the treated group with doxycycline significantly reduced the elevated blood urea nitrogen and creatinine. These results agree with Nakagawa et al. (2018). These results may be due to doxycycline prevented inflammation and ROS activation in the kidney (Labossiere et al., 2015). Also, Nakagawa et al. (2018) found that Dox has several effects, including antitumor, anti-inflammatory, antioxidant, and matrix metalloproteinase (MMP)-inhibiting activities by binding or reducing mRNA expression of their enzymatic active sites and protecting against acute kidney injury. Regarding to the serum proteins at different check points in carrageenan injected rats, compared with control group significant decrease in total protein, albumin, and globulin. These results are in line with Li et al. (2013) who attributed that the carrageenan-induced inflammation was followed by major metabolic changes incorporating biosynthesis of acute phase proteins and metabolisms of amino acids, TCA cycle, glycolysis, fatty acids, ketone bodies, and choline in acute phase. A typical acute phase reaction (APR) with these proteins known as "acute phase" glycoproteins is the detection of carrageenan-induced major elevations of (NAG) N-acetyl-β-D-glucosaminidase in blood plasma.

Inflammatory hypoalbuminemia is a common finding in hospitalized people, and is exacerbated in malnourished patients or with renal disorders (Charlie-Silva et al., 2019). Regarding to carrageenan injected rats which treated with doxycycline significant increase in total protein, albumin, and globulin were recorded at different check points when compared with carrageenan group. These results are in line with Mahbub et al. (2011). During inflammatory conditions, the increase in total proteins can be due to doxycycline suppressing cytokine output from neutrophils and macrophages and contributing to the overall immune-regulatory function by scavenging reactive oxygen species and thus preventing or minimizing the destruction of pathological tissue (Di Caprio et al., 2015).

Concerning to antioxidants parameters, carrageenan injected rats showed a significant increase in MDA compared to rat non-treated in all check points. This result agrees with Mitrea et al. (2020), who attributed that to carrageenan administration produced lipid peroxidation in kidneys. Malondialdehyde (MDA) arises due to the

peroxidation of polyunsaturated fatty acids in cells (Dahiya et al., 2013).

In present study, the antioxidant enzyme GPx in carrageenan injected rats was significantly decrease in all check points compared to non-treated control group. This result agrees with Petrov et al. (2018). The decrease of GPx may be due to the ROS harmful effects which increased after the inflammation by carrageenan. The sophisticated antioxidant protection mechanism consisting of an enzyme system involved in converting ROS to less reactive molecules such as O2 and water has been formed by the cells (Zouari Bouassida et al., 2018).

Regarding to carrageenan injected rats and treated with doxycycline significant decrease in MDA and significant increase in GPx were recorded. These results are in accordance with Lai et al. (2010). These results may explained by doxycycline has been reported to have an effect on the antioxidant enzymes levels in experimental periodontitis in rats. Sub-antimicrobial dose of doxycycline inhibited both systemic and local oxidative stress (Trivedi and Lal, 2017). Doxycycline administration also significantly reduces mRNA expressions of pro-inflammatory cytokines in rats with periodontitis (Castro et al., 2011).

Regarding to the inflammatory markers and cytokines CRP, No, TNF-α and IL-6 at different check points in carrageenan injected rats, significant increase in CRP, No, TNF-α and IL-6 were recorded comparing with, compared with control group . These results are in accordance with that obtained by Petrov et al. (2018). The elevation in both inflammatory markers and cytokines could be to, after induction of inflammation, activated neutrophils have been shown to secrete enzymes (myeloperoxidase, elastase, proteases) and release even more oxygen radicals, further encouraging inflammation and increasing levels of TNF- α, IL-1, IL-6, and malondialdehyde (Yaşar et al., 2010).

Carrageenan injected rats and treated with doxycycline showed a significant decrease in CRP, No, TNF-α and IL-6. These results are in harmony with Hoyt et al. (2006). These findings may be due to the anti-inflammatory effects of doxycycline by reducing the expression of genes encoding proinflammatory cytokines, TNF-alpha and IL-1β.18. It may also regulate the expression of TIMP-1 (Yaşar et al., 2010). Also, doxycycline decreases NO output through iNOS by iNOS mRNA destabilization through decreased p38 MAPK expression (Hoyt et al., 2006). Concerning to histopathological changes, carrageenan causes confluent degeneration of the lining epithelium of within the renal tubules associated with peritubular mononuclear cells infiltration. Also, in second sacrifice showed marked increase of the lesion. The results presented are in line with Mansouri et al. (2015). Carrageenan induced inflammation in the kidney (Mitrea et al., 2020). There is also a lot of evidence that the development of ROS at the inflammation site, such as hydrogen peroxide, superoxide and hydroxyl radicals, leads to tissue damage (Cuzzocrea et al., 2000). The current results revealed that administration of doxycycline in treated group was shown mild renal tubular degeneration and marked decrease in tissue damage. This result was in harmony with Cortes et al. (2018).

5. CONCULOSIONS

The current study findings indicated that doxycycline has anti-inflammatory and ren protective effects in acute carrageenan-induced inflammation.

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