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Ketorolac- and warfarin-induced renal toxicity: ultrastructural and biochemical study

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Abstract

Background: Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used drug classes worldwide. Although, these drugs have a potent analgesic and anti-inflammatory effects associated with nephrotoxicity. Factors such as advanced age and comorbidities may contribute to increase the risk of NSAID-related nephrotoxicity. The current study was designed to study the dual effect of non-NSAIDs and anticoagulants on the rat kidney.

Methods: Thirty-two adult male albino rats were divided into four groups ($n = 8$): first, served as the control group which was orally administered with distilled water; second, served as the ketorolac (KT) group which received a daily intramuscular injection of ketorolac tromethamine (3 mg/kg/day); third, served as the warfarin (WR) group which was orally administered with racemic warfarin (0.3 mg/kg/day); and fourth, served as the combined (KT/WR) group which received an oral administration of WR (0.3 mg/kg/day) followed by an intramuscular injection of KT (3 mg/kg/day). After six consecutive days of daily treatment, all animals were sacrificed and their blood and tissue samples were collected.

Results: A significant decrease in serum level of creatinine was observed in WR- and KT/WR-treated groups. However, urea increased significantly in the serum of the combined group, KT/WR. Furthermore, both WR- and KT/WR-treated groups showed a significant increase in malondialdyhed (MDA) level; however, WR treatment induced a significant decrease in reduced glutathione (GSH) level. The total antioxidant capacity (TAC) of rat kidney showed a significant decrease in all treated groups. Electron microscopic investigations of kidney cortex localized variable degrees of focal degeneration, vacuolation, and vascular congestion in KT- and WR-treated groups that are more vigorously attacking the cortical tissues in KT/WR-treated rats.

Conclusions: We can conclude that the combined therapy of anti-inflammatory and anticoagulant drugs must be avoided or at least minimized, particularly in the elderly people, who mostly had other complications, in order to avoid the severe side effects on kidney structure and function.

Keywords: NSAIDs, Anticoagulants, Kidney, Histopathology, Oxidative stress

Background

The most widely used drug classes worldwide are non-steroidal anti-inflammatory drugs (NSAIDs). Recent studies point to NSAIDs as the most effective treatment for reducing moderate or severe pain. The vigorous development of their uses reflects the great clinical need they address to control of pain and fever, the two common manifestations of broad spectrum of diseases (Utzeri & Usai, 2017). Among these drugs, ketorolac tromethamine being a potent NSAID analgesic inhibits the synthesis of prostaglandins and may relate to adverse effects which can be serious, such as gastrointestinal ulcers and bleeding, heart attack, liver toxicity, and kidney disease (Aly, Mahmoud, Hassan, & Fahmy, 2015; Bally et al., 2017; Ilic et al., 2011; Lanas & Chan, 2017; Tomic et al., 2008). There are interactions between NSAIDs and several classes of anti-thrombotic drugs, including WR being under interest. WR represents the most widely prescribed oral anticoagulant and is used by

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patients with chronic atrial fibrillation, mechanical valves, deep vein thrombosis, and recurrent stroke (Eriksson & Wadelius, 2012; Li et al., 2016; Liu et al., 2012). It was previously reported to induce acute kidney injury (Brodsky et al., 2009; Brodsky et al., 2010; Brodsky et al., 2011; Ozcan et al., 2012). Many patients, elderly in particular, are highly sensitive to WR and require very low doses to achieve and maintain anticoagulation (Pirmohamed & Park, 2003). It is an antagonist of vitamin K, which inhibits the conversion of oxidized vitamin K epoxide into its reduced form, which is required for gamma carboxylation of the coagulation factors and anticoagulant proteins C and S (Juurlink, 2007). The independent pharmacological action of KT on a separate limb of the hemostatic mechanism from that which is affected by WR raises the potential of a clinically important interaction occurring when WR and KT are co-administered. Since kidneys are among the target organs affected by these drugs, the present investigation was undertaken to investigate the co-administration adverse effects of KT and WR, paying particular attention to prospective nephrotoxicity accompanied with such overlapped administration.

Methods

Experimental animals

Thirty-two adult male Wistar albino rats weighing 150–180 g were purchased from the animal house colony in Research Institute of Ophthalmology (Giza, Egypt), kept in a controlled environment, and allowed slandered laboratory diet and drinking water ad libitum throughout the experimental period in accordance with the guidelines of the Ethical Committee.

The rats were divided into four groups of eight animals each. Group 1 was orally administered with distilled water and served as the control group. Group 2 served as the ketorolac (KT) group which was daily injected intramuscularly with ketorolac tromethamine (3 mg/kg/day) (Buckley, Davidson, & Das, 1993). Group 3 served as the warfarin (WR) group which was orally administered with racemic warfarin (0.3 mg/kg/day) (Ozcan et al., 2012). Group 4, the combined group (KT/WR), animals received an oral administration of WR (0.3 mg/kg/day) followed by an intramuscular injection of KT (3 mg/kg/day). At the end of the experiment, after six consecutive days of daily treatment, all animals were sacrificed by cervical dislocation and their blood and tissue samples were collected.

Sample collection

Sera were separated and stored in aliquots at − 70 °C till used for estimation of serum urea and creatinine levels. Then, the abdomen was opened and the kidneys were removed, washed three times in ice cold saline, and blotted individually on ash-free filter paper.

Preparation of tissue homogenates for biochemical analysis

Specimens from each kidney tissue were separated into two parts. Each piece was weighted and homogenized separately with a tissue homogenizer. One part was homogenized in phosphate buffer saline (PBS) 50 mM, pH 7.4, for the estimation of total antioxidant capacity (TAC) and reduced glutathione (GSH) level; the second was homogenized in potassium phosphate buffer 10 mM, pH 7.4, for the estimation of malondialdyhed (MDA). The crude tissue homogenate was centrifuged at 10,000 rpm for 15 min in cold centrifuge, and the resultant supernatant was used for the different estimations.

Biochemical analysis

The level of clinical biochemistry for urea and creatinine was measured using commercially available kits according to the manufacturer's instructions (SPECTRUM Diagnostics kit was procured from the Egyptian Company for Biotechnology (S.A.E), Obour city industrial area, block 20008 piece 19 A. Cairo, Egypt).

Measurement of tissue malondialdehyde

The extent of lipid peroxidation was monitored through measuring the level of MDA using commercial kits as described by Ohkawa, Ohishi, and Yagi (1979). The method depends on the interaction of thiobarbituric acid with MDA in acidic medium at a temperature of 95 °C to form thiobarbituric acid reactive product, and the absorbance of the resultant pink product was measured at 534 nm.

Measurement of tissue reduced glutathione

GSH concentration was determined by the method described by Beutler, Duron, and Kelly (1963) in tissue homogenate. The method was based on the reduction of 5,5′ dithiobis-2-nitrobenzoic acid with GSH to produce a yellow compound. The reduced chromogen was directly proportional to GSH concentration, and its absorbance was measured at 412 nm. A parallel standard was also maintained.

Measurement of tissue total antioxidant capacity

The TAC was determined using commercial kits purchased from Biodiagnostic based on the method described by Koracevic, Koracevic, Djordjevic, Andrejevic, and Cosic (2001). The determination of the antioxidative capacity was performed by the reaction of antioxidants in the sample with a defined amount of exogenously provided hydrogen peroxide (H_2O_2). The antioxidants in the sample eliminated a certain amount of the provided hydrogen peroxide. The residual H_2O_2 was determined colorimetrically by an enzymatic reaction which involves the conversion of 3,5 dichloro-2-hydroxy benzensulphonate to a colored product. The color was measured spectrophotometrically at 505 nm. Protein content in tissue homogenate was measured according

Table 1 Kidney function and oxidative stress parameters in ketorolac and warfarin treated rats.

Groups	Urea (mg/dl)	Creatinine (mg/dl)	GSH (μmol/ g tissue)	MDA (nmol/g tissue)	TAC (μmol/mg protein)
Control	45.3 ± 1.7 (8)	0.79 ± 0.019 (8)	0.26 ± 0.007 (8)	58 ± 1.4 (8)	1.0 ± 0.03 (8)
KT	50.1 ± 3.6 (8)	0.81 ± 0.006 (8)	0.24 ± 0.012 (8)	58.4 ± 2.9 (8)	0.77 ± 0.02[a] (8)
WR	54.2 ± 6.45 (8)	0.67 ± 0.016[a] (8)	0.21 ± 0.02[a] (8)	76.5 ± 2.0[a] (8)	0.43 ± 0.03[a] (8)
KT/WR	57.5 ± 5.2[a] (8)	0.7 ± 0.05[a] (8)	0.26 ± 0.007 (8)	64.4 ± 1.14[a] (8)	0.69 ± 0.02[a] (8)

The values represent the means ± SE with the number of animals between parentheses
The different letters represent statistically significant values while the same letters represent statistically non-significant values

to the method of Lowry, Rosebrough, Farr, and Randall (1951).

Electron microscopic preparations

Ultrastructural studies were done on the renal cortical tissue of rats which were sacrificed after 6 days of treatment. Approximately, 1 mm^3 pieces of kidney cortex were immediately fixed in 3% phosphate-buffered glutaraldehyde (pH 7.3) at room temperature for 2 h, and then post-fixed in 1% buffered osmium tetroxide for 1–2 h at 4 °C, dehydrated in ascending grade of ethanol, and embedded in Epon. Semi-thin sections (1 μm) were stained with toluidine blue and examined with a light microscope. Ultrathin sections (70 nm) were obtained from the selected blocks, mounted on copper grads, and stained with uranyl acetate and lead citrate. The stained grads were then examined by a JEOL-JEM.1400 transmission electron microscope.

Statistical analysis

Data obtained from specimens were analyzed by using the one-way ANOVA. Statistical analyses were performed using statistical package program SPSS 15.0 (IBM Corp., Armonk, NY), and statistical significance was determined when p values were less than 0.05.

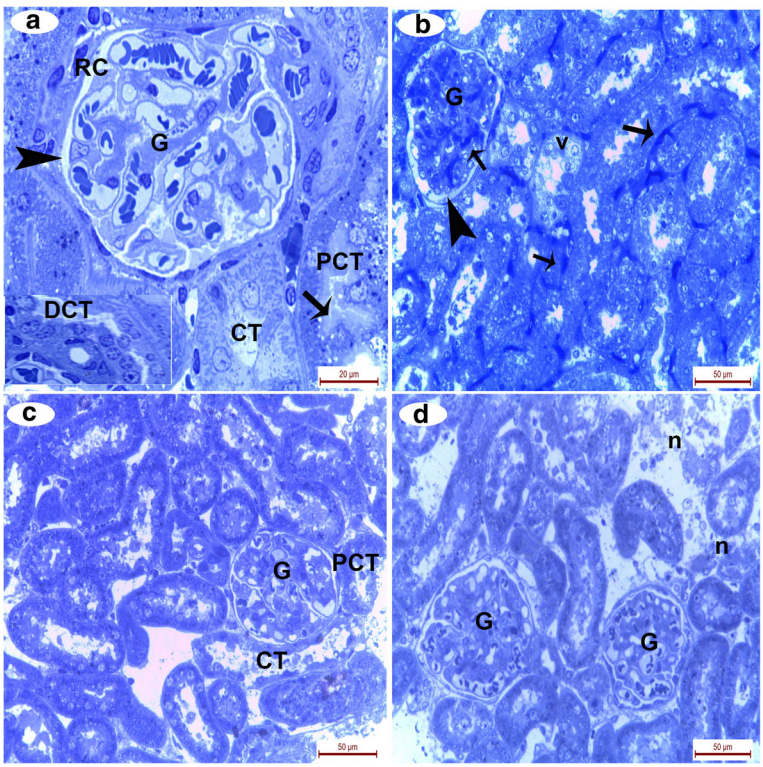

Fig. 1 Photomicrographs of renal cortex of rat. **a** Control group showing normal architecture of cortical parenchyma, renal corpuscle (RC), glomerulus (G), Bowman's space (arrow head), proximal convoluted tubule (PCT), brush border (arrow), and distal convoluted tubule (DCT) (inserted photo; scale bar = 20 μm). **b** KT group showing congested blood capillaries inside the glomeruli and in between the renal tubules (arrows), Bowman's space filled with casts (arrow head), hypertrophic glomerulus (G), and vacuolated and degenerated tubular cells (V) (scale bar = 50 μm). **c** WR group showing focal degeneration in PCT and CT and hypertrophic glomerulus (G) (scale bar = 50 μm). **d** KT/WR group showing focal necrosis (n) and hypertrophic glomerulus (G) surrounded by narrow Bowman's space (scale bar = 50 μm). Semi-thin sections, toluidine blue stain

Results

Biochemical effects

As compared to the control group, Table 1 shows the variable degrees of alteration in kidney function and oxidative stress parameters of treated rats. Urea was significantly increased in KT/WR treated rats; however, creatinine levels showed a significant decrease in both WR and KT/WR groups. As regards to the oxidative stress parameters, MDA showed a significant increase of renal tissue level in both WR and KT/WR treated rats; however, the GSH level showed a significant decrease in rats which received WR. Besides, TAC level records a significant decrease in KT, WR, and KT/WR treated groups.

Histopathological effects

The cortical parenchyma of the rat kidney contained renal corpuscles, blood vessels, and tubules. Light microscopic investigation showed the normal renal corpuscles as dense, rounded glomeruli surrounded by narrow Bowman's spaces, and different tubules vary in their diameter and staining affinities; these are proximal convoluted tubule (PCT), distal convoluted tubule (DCT), and collecting tubule (CT) (Fig. 1 a). In all treated groups (KT, WR, and KT/ WR) densely stained, hypertrophic glomeruli were seen surrounded by smaller Bowman's spaces filled with casts (Fig. 1b–d). Besides, congested blood vessels and varying degrees of tubular degeneration were also seen in KT group (Fig. 1b). Sections of co-administrative KT/WR group showed multiple necrotic foci in kidney tubules (Fig. 1d). Compared to the control sections (Fig. 2a–d), electron microscopic investigation localized vascular congestion and severe vacuolation in the glomerulus (Fig. 3a) and tubular cells of KT group (Fig. 3b–d). PCT appeared with damaged mitochondria and fragmented microvilli and basal enfolding and many lysosomes (Fig. 3b). Ultrathin sections of the WR group showed severe glomerular and tubular vacuolation (Fig. 4a–d), damage and disorganization of mitochondria, and basal enfolding (Fig. 4a, c). Clear cells of the CT appeared with fragmented cellular membrane (Fig. 4d). The co-administrative group (KT/WR) revealed dilated blood capillaries and damaged plasma membranes of renal glomeruli (Fig. 5a). Figure 5b and c showed the severe vacuolation and degeneration of PCT and DCT lining cells. Figure 5d showed damaged and disorganized mitochondria, many lysosomes, and fragmented basal enfolding of the tubular cells.

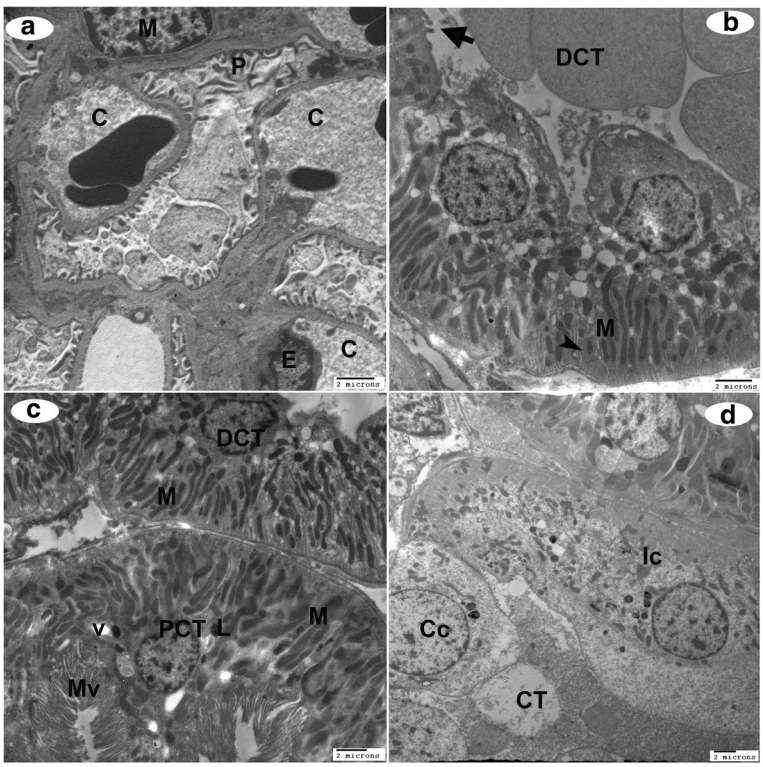

Fig. 2 Electron micrographs of the control rat cortex. **a** Glomerulus with several capillary loops (C), mesangial cell with dense matrix (M), and several podocyte processes (P) and endothelia cell nucleus (E). **b** DCT with elongated mitochondria (M), basal enfolding (arrow head), and very few microvilli (arrow). **c** PCT with characteristic microvilli (Mv), elongated mitochondria (M) in between the basal enfolding, lysosomes (L), and vacuoles (V). **d** CT with poorly stained clear cell (Cc) and more electron dense intercalated cell (Ic). Scale bar = 2 μm)

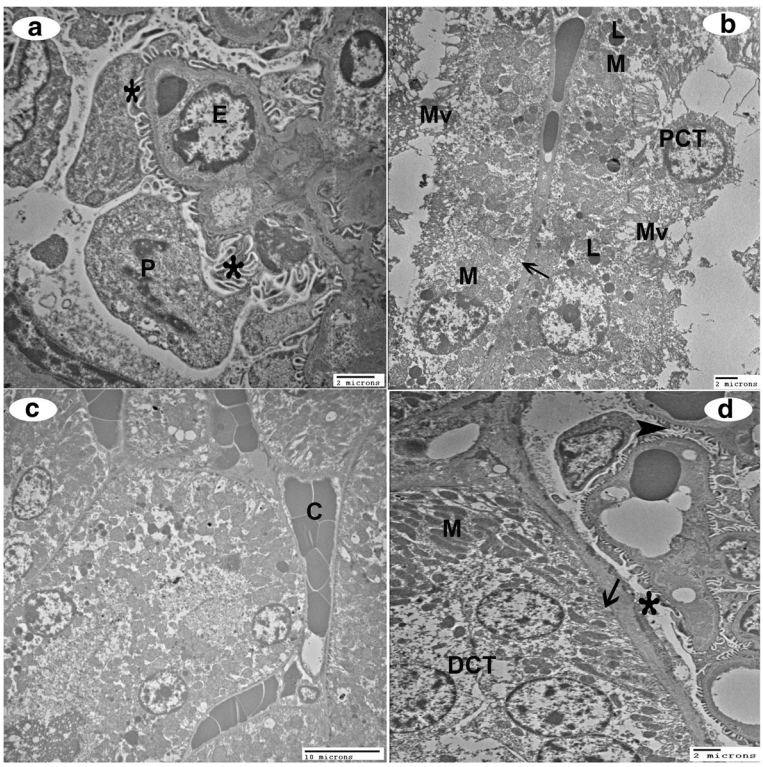

Fig. 3 Electron micrographs of the rat cortex of KT group. **a** Glomerular vacuolation in the nuclei and cytoplasm of endothelial cell (E) and podocyte (P), note the hypertrophic processes of podocytes (asterisk). **b** PCT with severe vacuolation, fragmented microvilli (Mv), damaged mitochondria (M), fragmented basal enfolding (arrow), and many lysosomes (L). **c** Congested blood capillaries (C) in between the vacuolated kidney tubules. **d** Severe vacuolation in the DCT, disorganized mitochondria (M), and fragmented basal enfolding (arrow), note the narrow Bowman's space (asterisk) and hypertrophic processes of podocytes (arrow head) (scale bar = 2 μm)

Discussion

Ketorolac is considered a potent analgesic with moderate anti-inflammatory effects (Schwier & Tran, 2016), although it has certain harmful effects on vital body organs such as the liver, kidney, and gastrointestinal tract (Aly et al., 2015; Bally et al., 2017; Tomic et al., 2008). During the recent years, an increasing number of studies have been published concerning the nephrotoxic effects of NSAIDs and anticoagulants (Chana et al., 2014; Lanas & Chan, 2017; Ozcan et al., 2012; Sriuttha, Sirichanchuen, & Permsuwan, 2018). The present investigation was constructed to study the co-administrative effect of KT and WR on histological picture and some biochemical parameters of rat kidney.

The current work clearly illustrated that the administration of 3 mg/kg body weight of KT together with 0.3 mg/kg body weight of WR for six consecutive days induced vascular congestion together with variable degrees of degeneration and vacuolation in the glomeruli and tubular cells of all treated groups, being more vigorously attacking the cortical tissues in KT/WR treated rats. The major mechanisms for WR-induced renal toxicity were previously reported as glomerular hemorrhage and tubular dysfunction by obstruction due to RBC casts (Mendonca, Gupta, Valsan, & Tewari, 2017; Ozcan et al., 2012). Similar to Chana et al. (2014) who revealed that KT induced acute kidney injury, another NSAID, diclofenac sodium, was found to induce the congestion in the blood vessels, the degeneration in nephrons, and the increase in the connective tissue in rat kidneys (Khoshvakhti et al., 2015). Thus, the combined treatment of KT and WR may be responsible for the accelerated progressive events of acute kidney injury obtained in the current study.

The serum levels of urea and creatinine are screening tests of renal function; their values remain within the normal range until more than 50% of renal function is lost (Mendonca et al., 2017). At all stages of renal insufficiency, the creatinine is a much more reliable indicator of renal function than the urea because the urea is far more likely to be affected by dietary and physiologic conditions not related to renal function. As kidney function declines, the urinary excretion of urea and creatinine also declines and blood concentration of both increases. However, in acute kidney injury, plasma creatinine and urea levels do not necessarily rise in tandem and the ratio of urea

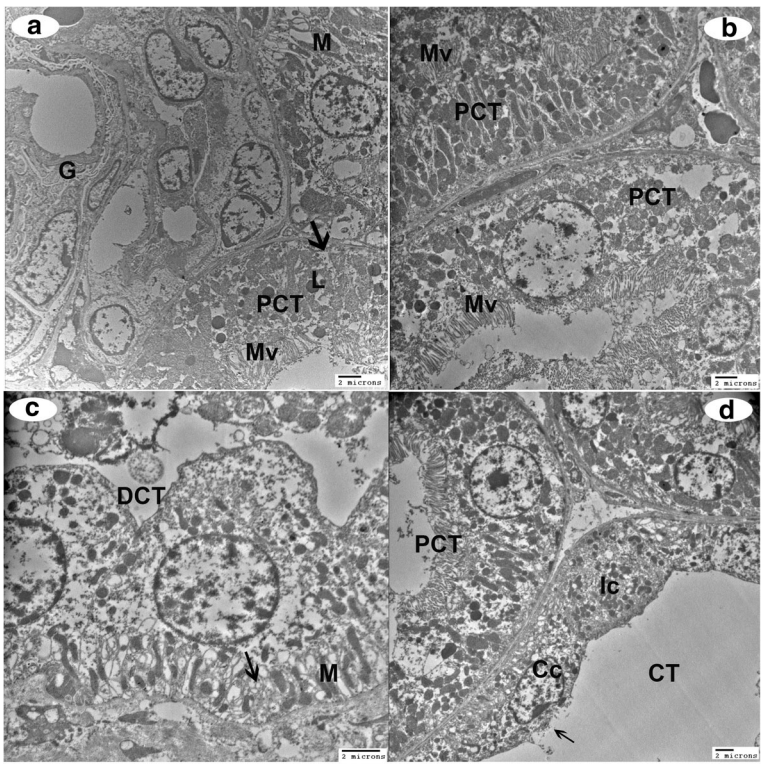

Fig. 4 Electron micrographs of the rat cortex of WR group. **a** Severe vacuolation in the glomerulus (G) and PCT, fragmented microvilli (Mv), damaged mitochondria (M), fragmented basal enfolding (arrow), and many lysosomes (L). **b** Lining cells of PCT with varying degrees of vacuolation and microvilli fragmention (Mv). **c** Cellular vacuolation of DCT, few and damaged mitochondria (M), and disorganized basal enfolding (arrow). **d** Varying degrees of cellular vacuolation in the PCT and CT, clear cell (Cc) with fragmented membrane (arrow), and intercalated cells (Ic) with disorganized and damaged mitochondria (scale bar = 2 μm)

to creatinine may be sometimes increased as urea increased or creatinine decreased (Uchino, Bellomo, & Goldsmith, 2012). Results obtained in the present study revealed mild alterations in the kidney function, reflected as a significant increase in urea of KT/WR-treated rats and a significant decrease in the creatinine level of both WR and KT/WR-treated groups. These altered parameters were in accordance with several reports reflecting a disturbed kidney function (Aly et al., 2015; Hörl, 2010; Kim & Joo, 2007; Pelligand et al., 2015) More recently, Lucas et al. (2018) reported that NSAIDs increase the risk of developing nephrotoxicity and acute tubular necrosis and they recommended that this condition be well evaluated. The authors concluded that NSAIDs, selective and non-selective, directly interfere with renal function due to prostaglandin inhibition, and suggested acute sodium retention, which is the main cause of the overfilling effect due to arterial hypertension and edema (Chana et al., 2014). This edema was clearly localized as severe vacuolation in the kidney tubules of co-administrated group, KT/WR, of the present work. Also, Sabry, Sakr, and Shahin (2014) indicated that treatment with NSAIDs during pregnancy could cause renal dysgenesis in neonates. Cao et al., 2014 reported an acute interstitial nephritis and acute tubule-interstitial disease among older-aged treated Chinese patients. In addition, Ozcan et al. (2012) reported that warfarin treatment resulted in a dose-dependent increase in serum creatinine. This increase following warfarin treatment was greater at 3 and 19 weeks after the ablative surgery. Morphologically, the authors localized the acute tubular injury with RBC and RBC casts in the kidney tubules of treated rats, the picture of which was also visualized in the kidney of KT- and KT/WR-treated rats of the current study. WR as a vitamin K antagonist interferes with vitamin K actions and causes critical adverse side effects on the bone and vascular health (Danziger, 2008; Fusaro et al., 2012, 2015). In 2016, Li et al. indicated that the dose-dependent variations of warfarin are markedly influenced by pharmacokinetic aspects that are determined by genetic and environmental factors. These effects of WR may be responsible for increased renal toxicity when co-administrated with KT in the present study.

As regards the antioxidant status of rat kidney exposed to KT and/or WR, compared to the control animals in the current study, MDA levels increased significantly in WR- and KT/WR-treated rats. However,

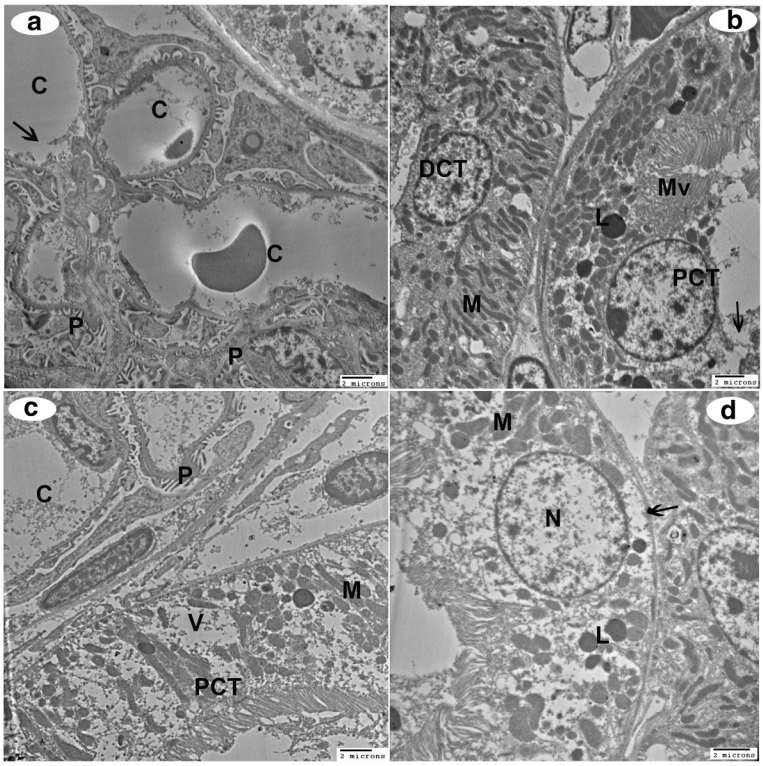

Fig. 5 Electron micrographs of the rat cortex of KT/WR group. **a** The glomerular podocyte with hypertrophic processes (P), dilated blood capillaries (c), and damaged plasma membranes (arrow). **b** Lining cells of PCT and DCT appeared with varying degrees of vacuolation and fragmentation, microvilli (Mv), mitochondria (M), lysosomes (L), and damaged plasma membrane (arrow). **c** PCT with severe vacuolated cells (V) and damaged mitochondria (M), and glomerulus with dilated blood capillary (C) and stretched electron dense podocytic processes (P). **d** Higher magnification of PCT with vacuolated nucleus (N), damaged disorganized mitochondria (M), many lysosomes (L), and fragmented basal enfolding (arrow) (scale bar = 2 μm)

the levels of GSH and TAC were significantly declined in WR and in all treated groups, respectively. This altered antioxidant status is in accordance with those obtained by other investigators with KT and other NSAID treatments (Adachi et al., 2007; Cantoni et al., 2003; Galar-Martínez et al., 2014). Also, several reports proved that NSAIDs strongly induce oxidative stress with the concomitant production of reactive oxygen species (ROS) (Adachi et al., 2007; Galar-Martínez et al., 2014; Galati, Tafazoli, Sabzevari, Chan, & O'Brien, 2002; Minami et al., 2005). This increased ROS together with the attenuated antioxidant defense system as regarded by decreased GSH and TAC levels of the current study may be responsible not only for the deleterious effects on membrane lipids related to the increased MDA level, but also to the deterioration of proteins and nucleic acids. This in turn leads to renal toxicity, dysfunction, and cellular damage. The present electron microscopic investigation confirmed that KT and/or WR has degenerative effects on rat kidney that could disrupt the normal renal structure and function.

Browne et al. (1999) investigated the pro-oxidant properties of some NSAIDs, including KT in rat hepatic mitochondria. The authors affirmed that these drugs were mostly associated with the disruption of lipid metabolic pathways and mitochondrial function. These obtained data were concomitant with a decrease in ATP production, increased ROS and elevated oxidative stress (Grosch, Tegeder, Niederberger, Brautigam, & Geisslinger, 2001; Hickey, Raje, Reid, Gross, & Ray, 2001; Maity et al., 2008, 2009; Mingatto, Santos, Uyemura, Jordani, & Curti, 1996; Pal et al., 2010, 2012), and induced renal injury. On the other hand, warfarin via direct or indirect effects (vitamin K-dependent proteins) may affect the glomerular filtration barrier, which results in glomerular hemorrhage with subsequent occlusive red blood cell cast formation and acute kidney injury. Based on this hypothesis, oxidative stress exerted by KT/WR treatment may play a significant role in both glomerular filtration barrier damage and acute tubular injury of rat kidney obtained in the present study.

Ware et al. (2013) explained the following mechanisms of increased oxidative stress in an animal model of warfarin-related nephropathy. First, free hemoglobin released by RBC in the tubular lumen affects tubular epithelial cells by generating reactive oxygen species and increasing lipid peroxidation. Second, free hemoglobin

incorporates into the tubular epithelial cells via several surface receptors, such as megalin-cubilin receptors (Tracz, Alam, & Nath, 2007). Intracellularly, free hemoglobin activates caspases and induces apoptosis (Homsi, Janino, & de Faria, 2006). Third, the intracellular hemoglobin dissociates into globin and heme, while the latter is also a potent oxidant and activates pro-inflammatory pathways (Tsiftsoglou, Tsamadou, & Papadopoulou, 2006).

Conclusions

From the present biochemical and histopathological data, it can be concluded that the combined treatment of anti-inflammatory and anticoagulant drugs induced renal injury and histological alterations through the suppression of the antioxidant defense system and enhancement of oxidative stress. Thus, we recommend that the use of these combined drugs must be restricted, especially in the elderly who suffer from renal diseases and other comorbidities.

Abbreviations

CT: Collecting tubule; DCT: Distal convoluted tubule; GSH: Reduced glutathione; H2O2: Hydrogen peroxide; KT: Ketorolac; MDA: Malondialdyhed; NSAIDs: Non-steroidal anti-inflammatory drugs; PBS: Phosphate buffer saline; PCT: Proximal convoluted tubule; RBC: Red blood cell; ROS: Reactive oxygen species; TAC: Total antioxidant capacity; WR: Warfarin

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Availability of data and materials

The data are available from the authors.

Authors' contributions

AIO conceived and designed the study and collected and analyze the histological data. AAG and AMM collected and analyzed the biochemical data and then wrote the results. AIO wrote the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

Animals were treated in accordance with the guidelines of the Ethical Committee for animal care of the "Faculty of Science–Cairo University" with approval number CU-I-F-70/18.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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