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The renoprotective effect of Adipose mesenchymal stem cell-derived exosomes combined with Roflumilast in chronic kidney disease induced by Doxorubicin

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Abstract: Background: Chronic kidney disease (CKD) is a global health concern characterized by the presence of irreversible kidney damage. The renoprotective role of adipose mesenchymal stromal cell-derived exosomes (ADMSCs-Exos) and roflumilast (ROF) in the management of acute kidney damage has been demonstrated in preclinical studies.

Objectives: The current study was conducted to investigate the potential therapeutic efficacy of Exos combined with ROF in the management of CKD.

Material and methods: Isolating and characterizing exosomes involved using electron microscopy and flow cytometry techniques. Rats were randomly separated into seven groups, Control group: rats were injected with 0.9% saline. ROF group: The rats received an oral administration of ROF at a dosage of 1.2 mg/kg. Exos group: rats were given two injections of 100 µg Exos. DOX group: rats were subjected to two injections on day 1 and day 14, at a dosage of 4 mg/kg of DOX. DOX + ROF group: rats were administered ROF for a duration of 7 days following each injection of DOX. DOX + Exos group: rats were administered exosomes five days after receiving both doses of DOX. DOX + ROF+ Exos group: rats were administered both ROF and Exos subsequent to each DOX injection. At the end of the experiment, kidney functions, antioxidant status and renal histology was detected.

Results: The isolated exosomes exhibited the characteristic cup-shaped morphology and expressed the surface proteins CD81, CD9, and CD63. The administration of ROF or Exos resulted in a notable enhancement in both renal functions and structural integrity when compared to the DOX group ($p < 0.05$). Furthermore, the administration of ROF or Exos resulted in an increase in the levels of the antioxidant GSH, while the level of MDA was shown to decrease compared to the DOX group ($p < 0.05$) with the combination group showing the most significant improvement.

Conclusion: The study findings demonstrate for the initial instance that the administration of ROF and Exos may confer protection against DOX-induced CKD by ameliorating both renal function and morphology.

Keywords: *chronic kidney disease, Doxorubicin, Exosomes, Roflumilast*

Introduction

Chronic kidney disease (CKD) is widely acknowledged as a global epidemic. Proteinuria is a crucial indicator of renal impairment and serves as the foundation for establishing the definition and classification of renal disorders [1]. Numerous clinical studies have demonstrated that the presence of chronic proteinuria is a significant risk factor for the progression to end-stage renal disease [2]. The production of proteinuria is commonly attributed to two main factors: disruption to the glomerular filtration barrier or impaired reabsorption of the proximal tubule [3].

The Doxorubicin (DOX) model has been extensively utilized in preclinical studies to simulate the progression of CKD. This model mostly exhibits proteinuria and a decline in renal function [4]. DOX, an anthracycline antibiotic, is widely recognized as a very efficacious chemotherapeutic agent. The application of this treatment modality is prevalent in the management of diverse human neoplasms, including neurofibromatosis, nephrotoxicity, and hepatocellular carcinoma [5]. Nevertheless, the utilization of DOX has been limited as a result of its detrimental impact on several organs and tissues [6]. The precise mechanism underlying the nephrotoxicity induced by DOX yet to be fully elucidated. However, numerous researchers have proposed that DOX elicits the production of reactive oxygen species (ROS) [7].

Oxidative stress refers to a state when there is an inequilibrium between the production of reactive oxygen species (ROS) and the ability of cells or tissues to eliminate or neutralize them. Multiple studies have provided evidence indicating that the dysregulation of the redox state in the kidney facilitates the activation of fibrogenic pathways, ultimately resulting in the development of renal failure [8]. The apoptotic pathway in the kidney is activated as a result of the rise in oxidative stress and the reduction of endogenous antioxidants [9]. The existing laboratory findings indicate that DOX has the potential to cause nephropathy, characterized by several effects such as heightened permeability of glomerular capillaries, atrophy of glomeruli, and the buildup of DOX within the glomerulus [5]. Therefore, it is imperative to design a novel therapeutic strategy for DOX.

Recent research investigations have provided evidence about the impact of mesenchymal stem cells (MSCs) on the promotion of tissue regeneration [10]. These studies have specifically highlighted the pro-regenerative characteristics of adipose derived mesenchymal stem cells (ADMSCs) when transplanted into the damaged kidney [11]. Yet, there are several constraints that persist in the treatment of MSCs, such as the need for invasive collection methods, a limited quantity of isolated cells, dependence on the age of the donor, and the requirement for extensive in vitro multiplication [12]. Extensive investigations have been conducted to explore viable substitutes for stem cells, focusing on the restoration of impaired tissues and the facilitation of tissue regeneration. Recent research has provided evidence that MSCs play a role in the microenvironment around injured tissues through the release of exosomes (Exos). In the interim, Exos have a role in the regulation of cellular processes such as proliferation and differentiation within injured tissues. Consequently, these Exos contribute to the indirect repair of the aforementioned lesions [13]. MSCs-derived exosomes have the capacity to elicit an immunological response and facilitate a two-way modulation of immune tolerance [14]. Hence, the use of Exos mitigates the potential hazards associated with the malfunction and alteration of transplanted stem cells, so presenting itself as a viable non-cellular therapeutic approach for the purpose of tissue regeneration [15].

Phosphodiesterases (PDE) are a class of hydrolytic enzymes that play a crucial role in the process of degrading cyclic nucleotides. The PDE comprise a total of 11 subfamilies [16]. Phosphodiesterase-4 (PDE4), a member of PDE subfamily, is found to be expressed in some leukocytes and has been associated with several inflammatory disorders [17]. Roflumilast (ROF) is classified as a second-generation phosphodiesterase 4 (PDE4) inhibitor, and its ability to suppress PDE4-mediated inflammatory reactions in humans has been documented [18]. Prior research has demonstrated that administration of PDE4 inhibitors to mice and rats prior to exposure to hazardous substances effectively mitigated renal dysfunction and injury [19, 20]. Nevertheless, the impact of ROF on a preexisting renal damage and renal function remains uncertain.

Currently, the available pharmacological options for impeding the progression of CKD are constrained. Hence, the formulation of a treatment methodology that specifically focuses on the underlying pathways associated with CKD holds great potential in addressing its etiology. The hypothesis suggests that the processes by which ROF and Exos operate entail the reduction of the ROS and inflammation. As a result, this inhibition has the potential to hinder the progress of DOX. Therefore, the current investigation aimed to assess the impact of the concurrent administration of Rof and Exos, in comparison to their individual effects, on renoprotection and antioxidant activity in a CKD rat model induced by DOX.

1. Material and methods

1.1. Isolation and Characterization of Adipose mesenchymal stem cells (ADMSCs)

Adipose-derived mesenchymal stem cells (ADMSCs) were collected from the para-gonadal fat of male rats. Briefly, fats were cut into smaller pieces and washed. Fats were chemically digested and centrifuged. The supernatant was removed and the pellet was re-suspended in DMEM media supplemented with 10% FBS and 1% Penicillin-streptomycin antibiotic. Then it was transferred into 25 cm^2 tissue culture flask and incubated in humidified incubator at 37^{o}c and 5%CO_{2}.

1.2. Extraction of ADMSCs-derived exosomes

When ADMSCs reached 80% confluence, the medium was replaced with a serum-free alternative for 24 hour the centrifugation for 10 min at 300 xg. This was followed by a further centrifugation step at 2000 xg for 30 min. The resultant pellet was washed and subjected to centrifugation at 100,000 xg for 70 min. Finally, the pellet containing exosomes were resuspended in PBS [21].

1.3. Exosomes characterization

Transmission electron microscopy (TEM)

Exosome samples were fixed using 4% paraformaldehyde. The samples were applied onto grids that had been coated with Formvar carbon. The grids were subjected to two water rinses before staining with 1% phosphotungstic acid solution. The exosomes that were produced were analyzed after the drying process using a transmission electron microscope. (JEOL JEM-2100 at 160 KV, Electron Microscope Unit, Mansoura University, Egypt).

1.4. Experimental Animals

A total of seventy male Sprague-Dawley rats, with weights 200±20 gm, were accommodated in polycarbonate cages at a density of four rats per cage. The rats were subjected to a 12-hour light-dark cycle and kept at a temperature of 24ºC with a humidity range of 50-70%. The care and techniques implemented in the research adhered to the animal care recommendations set forth by the National Institutes of Health (NIH) and were approved by the Institutional Animal Ethics Committee of the Faculty of Science at Zagazig University, Egypt. [IRB No. ZU-IACUC/1/F/129/ 2020].

1.4.1. Animal Groups

The animals were randomly separated into seven groups, with each group consisting of 10 rats. (I) Control group: animals were injected with 0.9 % saline in tail vein; (II) Roflumilast (ROF) group: rats were administered orally with 1.2 mg/kg ROF [22]. (III) Exos group: rats injected with 100 µg Exos two times in tail vein [23]**,** (IV) Doxorubicin (DOX) group: rats injected with 4 mg/kg DOX twice at day1 and 14 from experiment [24], (V) DOX + ROF group: Rats were received ROF for 7 days after each DOX injection, (VI) DOX + Exos group: Rats injection with exosomes 5 days after the administration of both doses of DOX, and (VII) DOX + ROF +Exos group: Rats were received ROF and Exos after each DOX injection.

1.4.2. Collection of blood and tissue samples

Blood samples were collected from each rat via cardiac puncture after 30 days following the initial DOX administration. The samples were collected in vacationer blood collection containers without the use of anticoagulant. The blood samples underwent centrifugation at a speed of 4000 rpm for a duration of 10 min in order to obtain the serum. To induce euthanasia in rats, an overdose of thiopental was administered. The left kidney was surgically excised and then preserved at a temperature of -80 ^{o}C for oxidative analysis. Conversely, the right kidney was placed in a solution of 10% buffered formalin to enable subsequent histopathological examinations.

1.4.3. Biochemical analysis

The serum was utilized to identify the levels of serum creatinine (SCr), blood urea nitrogen (BUN), urea, and total protein. This was achieved by employing specific kits in accordance with the instructions provided by the manufacturer (Diamond Diagnostics, Cairo, Egypt) using Architect system (Abbott Diagnostics, Germany).

1.4.4. Oxidative stress measurements

In order to assess the level of oxidative stress in renal cells, the quantities of malondialdehyde (MDA) concentration and reduced glutathione (GSH) activity were measured across all experimental cohorts. The accomplishment was made by using commercially accessible kits and adhering to the protocols outlined by the producer (Biodiagnostic Co., Giza, Egypt).

1.4.5. Histopathological Examination

Hematoxylin and eosin (H&E) staining was employed for the histological examination of the kidney. In brief, the tissues were subjected to formalin fixation, dehydration, and paraffin embedding. The kidney tissues were sliced into 5-µm sections. Following this, the aforementioned slices underwent H&E staining. The assessment of tubulointerstitial injury, chronicity, and regeneration was conducted utilizing a semi-quantitative pathological scoring system as outlined by Shi et al. [25].

1.5. Statistical analysis

The mean ± standard deviation (SD) values across the different groups were compared using a one-way analysis of variance (ANOVA) and a post hoc test. The parameter correlation analysis was conducted using the SPSS software program (IBM Corp., USA). A significance level of $p \leq 0.05$ was deemed to indicate statistical significance. The graphs were generated using Prism 8 (GraphPad Software, California, USA).

2. Results

2.1. Culturing of ADMSCs

The cells underwent subculturing until they reached passage 3, at which point a homogeneous fibroblastic cell monolayer was observed (Figure 1A). The phenotypic characterization of ADMSCs was conducted using flow cytometry analysis, as depicted in Figure 1B. The results demonstrated that ADMSCs exhibit a positive expression of CD105 at a rate of 91.2%, but the expression of CD45 is positively observed at a rate of 3.6%. This observation demonstrated the successful isolation of ADMSCs that displayed the characteristics of MSCs.

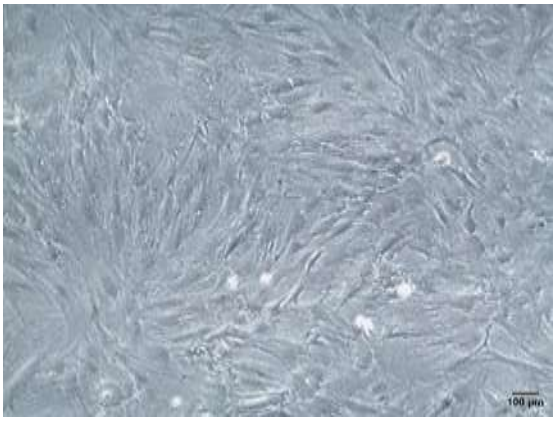

Figure (1): ADMSCs in passage 3

2.2. Characterization of ADMSCs-Exos

Exosomes were extracted from the culture media of ADMSCs and then characterized using TEM. The Exos were seen to possess a cup-shaped morphology, with a diameter ranging from 30 to 150 nm, as depicted in Figure 2A. Furthermore, the flow cytometry technique was employed to identify the specific surface antigens associated with Exos, namely CD9, CD83, and CD63. The findings indicated that the positive rates for CD9, CD63, and CD83 were 97.21%, 98.1%, and 98.44% correspondingly, as shown in Figure 2B. The confirmation of exosomal features was achieved through the identification of CD9, CD83, and CD63.

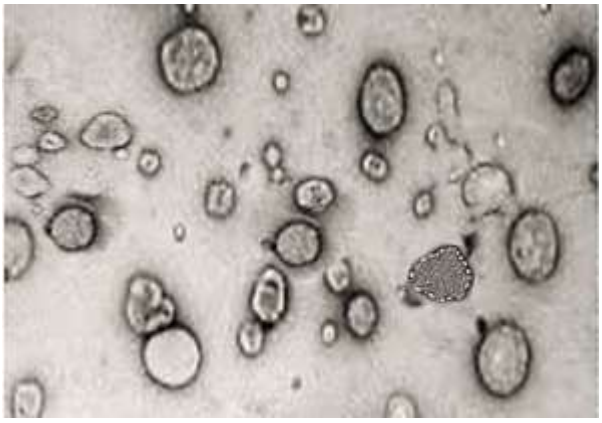

Figure (2): TEM examination of exosomes (Arrow). Scale bar = 100 nm

2.3. Detection of kidney functions

In order to assess the impact of ROF and Exos on renal function in the presence of DOX, various parameters including serum creatinine (SCr), blood urea nitrogen (BUN), urea, and total protein were measured and their results are presented in Table 1. The groups treated with ROF and Exos exhibited levels of SCr, BUN, urea, and total protein that were within the normal range. In contrast, the DOX group exhibited a statistically significant elevation in all assessed renal function when compared to the control group ($p < 0.05$). The administration of ROF in rats treated with DOX resulted in a notable reduction in serum creatinine SCr, BUN, urea, and total protein levels as compared to the DOX group ($p <0.05$). In a similar vein. The DOX+Exos group had a notable decline in renal function in comparison to the DOX group ($p < 0.05$). Furthermore, the administration of both REF and Exos demonstrated the greatest enhancement in renal function levels as compared to the DOX, DOX+ROF, and DOX+Exos groups ($p < 0.05$).

Table (1): kidney functions in different treated groups

Groups	Creatinine (mg/dL)	BUN (mg/dL)	Urea (mg/dL)	Total protein (mg/dL)
Control	0.45±0.02	18.4±0.9	24.8±1.21	5.91±0.28
ROF	0.48±0.13	19.1±1.02	26.1±1.84	6.12±0.78
Exos	0.46±0.11	18.9±1.1	25.9±2.34	6.41±0.59
DOX	3.14±0.21*	81.6±4.12*	98.3±3.54*	20.5±0.97*
DOX+ROF	1.41±0.19$^{*\#}$	58.3± 2.31$^{*\#}$	79.4±3.04$^{*\#}$	14.11±0.82$^{*\#}$
DOX+ Exos	0.95± 0.15$^{*\#\$}$	46.1±2.11$^{*\#\$}$	49.3± 2.61$^{*\#\$}$	9.32±0.77$^{*\#\$}$
DOX+ROF+Exos	0.63±0.13$^{*\#\$€}$	28.3± 1.34$^{*\#\$€}$	32.7±2.31$^{*\#\$€}$	7.33± 0.42$^{*\#\$}$

*Significant vs Control, #vs DOX, $vs DOX+ROF and €vs DOX+Exos. Statistical significance was determined where $P <0.05$

2.4. Measurements of antioxidant status

The activity of Glutathione reduced (GSH) and the levels of Malondialdehyde (MDA) were measured in the control group, the group treated with DOX, and various other treated groups (Figure 3). There were no statistically significant variations seen in the levels of MDA and the activity of GSH between the groups of rats exposed to ROF, Exos, and the control group ($p > 0.05$). The DOX group demonstrated a notable increase in levels of MDA and a decrease in GSH activity when compared to the control group ($p < 0.05$). In contrast, the administration of ROF or Exos resulted in a noteworthy reduction in MDA levels and an elevation in GSH activity when compared to the DOX group ($p < 0.05$). In addition, it was observed that the combined administration of ROF and Exos resulted in the most significant reduction in MDA levels and the highest GSH activity, when compared to the groups treated with DOX alone, DOX+ROF, and DOX+Exos ($p < 0.05$).

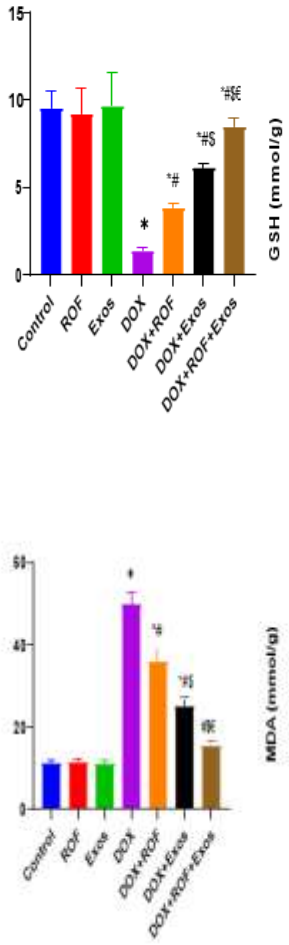

Figure (3): kidney Malondialdehyde (MDA) and Glutathione reduced (GSH) levels

2.5. Histopathological studies on kidney Tissues

Morphological alterations were detected in renal specimens of the all-treated groups. The DOX group exhibited a significantly greater degeneration score compared to the control group ($P< 0.05$). Conversely, treatment with ROF, Exos, and the combination of both resulted in lower degeneration scores and higher regeneration scores compared to the DOX group ($P< 0.05$; Figure 4A). The Control, ROF, and Exos groups had typical renal morphology, as depicted in Figure 4 B-D. On the other hand, the DOX group exhibited significant necrosis, glomerular and tubular atrophy, tubular dilatation as well as protein accumulation in the tubular lumen (Figure 4E). In contrast, the groups treated with ROF or Exos in the DOX experiment exhibited a notable decrease in degeneration and an increase in regeneration indices, as evidenced by the presence of mitotic figures and conspicuous nucleoli (Figure 4F, G). Furthermore, the DOX+ROF+Exos group had stronger regeneration indices compared to the DOX, ROF, or Exos groups individually (Figure 4H).

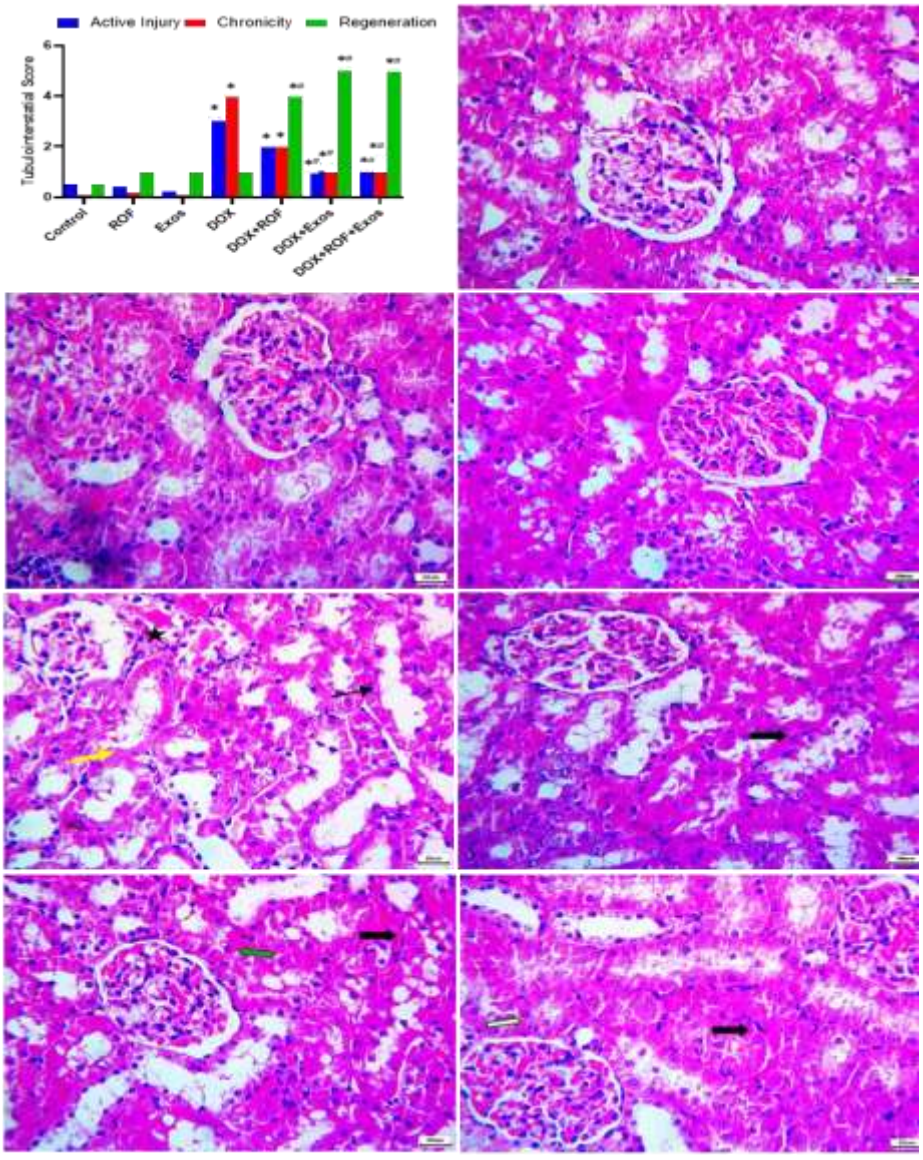

Figure (4): Photomicrographs of renal sections showing; Degeneration score (A), Normal morphology in control, ROF & Exos groups (B-D). Marked dilated tubules (black arrow), necrotic tubules (yellow arrow), and hyaline cast (star) in DOX group (E). Mitotic figure (bold black arrow) in ROF group (F). Prominent nucleui (green bold arrow) and Mitotic figure (bold black arrow) in DOX+Exos group (G). Mitotic figure (bold black arrow) and Prominent nucleui (green bold arrow) in DOX+ROF+Exos group (H). H&E, X: 400

3. Discussion

Doxorubicin is widely acknowledged as a prevalent chemotherapy medication. However, excessive or prolonged use of DOX can result in drug-induced renal impairment and potentially kidney failure [26]. Hence, it is imperative to safeguard against renal failure caused by DOX. This study primarily investigated the protective properties of Exosomes and Roflumilast, as well as their probable processes, in the treatment of DOX-induced CKD.

Exosomes-based therapy has become more widely used in recent years as a promising treatment method for several clinical conditions. The supplied exosomes have the capacity to be taken up by recipient cells, resulting in alterations in several biological processes at the sub-cellular and molecular levels [27]. Exosome-based therapy, being a cell-free approach, presents several advantages when compared to cell-based therapies. The benefits include improved stability and ability to be stored for longer periods, convenient accessibility to wound sites, less risk of immune rejection, and lack of toxicity [28].

The current study focused on extracting exosomes from the conditioned cell culture medium of ADMSCs, which were identified as CD105-positive and CD45-negative based on their phenotype. The ADMSCs exhibited the essential traits of MSCs in accordance with the parameters put forward by the Society of Cellular Therapy [29]. The technique of transmission electron microscopy (TEM) was used to analyze isolated exosomes in a general manner. Exo-MSCs was reported to display a consistent range of sizes, with diameters ranging from 30 to 100 nm with a unique cup-shaped structure [30]. Our TEM study confirmed the presence of nano-vesicles, which displayed a diverse range of average dimensions ranging from 30 to 150 nm. In addition, the flow cytometry method was used to determine the particular surface antigens linked to Exos, specifically CD9, CD83, and CD63. The tetraspanin family includes CD9, CD63, and CD81, which are surface markers usually present in exosomes [31]. The confirmation of exosome features was achieved through the identification of CD9, CD83, and CD63.

CKD is distinguished by many symptoms, such as alterations in urine composition, structural modifications in the kidneys, and diminished renal function. Proteinuria has been recognized as a significant risk factor for the progression of CKD [32]. Previous studies have shown that DOX leads to notable hypoalbuminemia and hyperlipidemia, as seen by increased levels of serum creatinine, BUN, and total urine protein [33, 34]. The present study revealed that the implementation of DOX led to a statistically significant increase in Scr, BUN, and total protein levels. Based on a prior investigation, the use of Exos was determined to have a beneficial effect on kidney function and structure, resulting in improved rates of survival [35]. Wan et al. [36] presented findings indicating that exosomes derived from bone marrow possess the capability to decrease levels of Scr and BUN in the context of renal injury. During our experiment, we found that administering many injections of Exos led to a significant drop in death rates with normal renal function.

Moreover, it was reported that the administration of ROF, either on its own or in conjunction with other drugs, has a positive impact on renal function by improving renal functions. As a result, the renal function is enhanced, leading to decreasing the levels of Scr, BUN, and urea [37]. The results of our study showed a significant decrease in Scr, BUN, urea, and total protein levels as compared to the CKD group. This finding aligns with the research conducted by Patel et al. [38], which emphasized the positive effect of ROF in improving renal function in cases of adenine-induced CKD. Additionally, Tikoo et al. [39], demonstrated that ROF reduces Scr and BUN levels in a rat model of diabetic nephropathy. Moreover, rats that received both Exos and ROF had the greatest enhancement in kidney function, indicating the renoprotective effect of this combined therapy.

Oxidative stress is a condition characterized by an imbalance between the generation of reactive oxygen species (ROS) and the capacity of cells or tissues to efficiently remove them. Extensive research suggests that the imbalance of the redox state in the kidneys has a major impact on the activation of fibrogenic pathways, leading to the development of renal failure [40].

Multiple studies indicate that the toxicity caused by DOX may be a result of oxidative stress, leading to the oxidation and cross-linking of cellular thiols and the peroxidation of membrane lipids [41]. Malondialdehyde (MDA) serves as a dependable biomarker for quantifying levels of ROS, as it is the final outcome of lipid peroxidation [42]. Glutathione reduced (GSH) plays a crucial part in the process of eliminating harmful foreign substances and in protecting against the damaging effects of ROS and free radicals through its antioxidant properties [43]. Our analysis revealed elevated levels of MDA and Low levels of GSH in the group treated with DOX. This finding is consistent with earlier studies indicating that DOX-induced oxidative stress

in renal tissues is characterized by increased MDA and decreased GSH levels [44].

The exosomes' capacity to diminish renal oxidative stress was in line with the observed enhancements in renal function, as evidenced by reduced levels of Scr, BUN, and MDA, as well as increased GSH levels and improved renal structure subsequent to the induction of CKD. Prior studies have shown that exosomes derived from MSCs have a simultaneous inhibitory effect on ROS in the context of renal ischemia [45] as well as exosomes that have been pre-treated with Melatonin [46]. Furthermore, it was observed that ROF decreased the levels of MDA and increased the activity of GSH, which aligns with previous research indicating a significant increase in SOD and GSH levels, as well as a significant decrease in MDA levels. This suggests that enhancing the antioxidant capacity may be one of the strategies employed by ROF to protect renal function [22, 38, 39]. Therefore, the administration of both ROF and Exos to rats can provide more protection to the kidneys against the harmful effects of free radicals produced by DOX, compared to utilizing Exos or ROF alone.

In a harmony with renal function and oxidative stress findings, Dox group showed a necrosis, glomerular and tubular atrophy, tubular dilatation as well as protein accumulation in the tubular lumen. This results is in agreement with Zhao et al. [4], who found that mice treated with DOX exhibited notable kidney damage, including fibrosis, glomerulosclerosis, glomerular and renal tubular atrophy, infiltration of inflammatory cells, collagen deposition, and thickening of the basement membrane. Conversely, the administration of exosomes results in notable restoration of the kidneys, accompanied by reduced inflammation and tubular atrophy. Similarly, administration of ROF resulted in enhanced kidney morphology, particularly in the group that received both ROF and Exos. These observations align with earlier research that have identified the renoprotective effect of Exos and ROF on renal morphology [18, 23].

4. Conclusion

The present work reveals new evidence suggesting that the administration of exosomes derived from ADMSCs, in conjunction with Roflumilast, led to a more beneficial therapeutic effect for CKD generated by DOX, in comparison to treatment with exosomes alone or Roflumilast alone. The antioxidant properties of Roflumilast and Exos may be responsible for their protective effects. However, additional research is required to gain a comprehensive understanding of the probable mechanism of these combinations in CKD.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper**.**

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