Scientific Research & Studies Center-Faculty of Science- Zagazig University- Egypt

**Biochemistry Letters**

Journal home page:

# The renoprotective impact of adipose mesenchymal stem cell-derived exosomes coupled with Roflumilast

**Bassant Yahia [1] ،Mohamed H. Sherif [2] ،Nashwa Barakat [3] ،Ahmed A. Shokeir [3,4] ،and Mohamed Ali [1]**

**[1] Biochemistry Department, Faculty of Science, Zagazig University**
**[2] Chemistry Department, Faculty of Science, Zagazig University**
**[3] Urology and Nephrology Center, Mansoura University, Mansoura, 35516, Egypt**
**[4] Center of excellence for Genome and Cancer Research, Urology and Nephrology Center, Mansoura University, Egypt**

**ARTICLE INFO**

**Received : 18/6/2024**
**Accepted : 30/8/2024**
**Available online : 31/8/2024**

***Keywords:***
chronic kidney disease, Doxorubicin, Exosomes, Roflumilast , , inflammation, Fibrosis, Glomerular filtration rate , Mesenchymal stem cells , Small exosomes -derived adipose stem cells ,

**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.

*Correspondence author: **Mohamed Ali,** https://orcid.org/0000-0003-4105-776X, mohamedali_eg@zu.edu.eg

**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
.

## 1. Introduction:

It is commonly accepted that chronic kidney disease (CKD) is a worldwide epidemic. Proteinuria is the basis for the characterization and categorization of renal diseases and is an essential marker of renal dysfunction [1]. Chronic proteinuria is a major risk factor for the development of end-stage renal disease, as numerous clinical investigations have shown [2]. The glomerular filtration barrier being disrupted or the proximal tubule's reabsorption being compromised are the two primary causes of proteinuria [3]. Preclinical research has made considerable use of the Doxorubicin (DOX) model to mimic the course of chronic kidney disease. Renal function decrease and proteinuria are the main features of this paradigm [4]. The anthracycline antibiotic DOX is well known for being a highly effective chemotherapy drug. Treatment with this technique is widely used for a variety of human neoplasms, such as hepatocellular carcinoma, nephrotoxicity, and neurofibromatosis [5]. However, due to its deleterious effects on multiple organs and tissues, DOX use has been restricted [6]. The exact process that causes the nephrotoxicity brought on by DOX is still unclear. On the other hand, a number of researchers have suggested that DOX causes reactive oxygen species (ROS) to be produced [7].

When there is an imbalance between the generation of reactive oxygen species (ROS) and the capacity of cells or tissues to remove or neutralize them .

Mesenchymal stem cells (MSCs) have been shown to have an effect on the enhancement of tissue regeneration in recent studies [8]. Adipose-derived mesenchymal stem cells (ADMSCs) have been shown in these experiments to exhibit pro-regenerative properties when transplanted into injured kidney tissue [9]. However, a number of limitations still exist when it comes to using MSCs in treatment, including the requirement for invasive collection techniques, a finite supply of isolated cells, reliance on donor age, and a lengthy in vitro multiplication period [10]. Many studies have been carried out to investigate potential stem cell replacements, with an emphasis on repairing damaged tissues and promoting tissue regeneration. Recent studies have demonstrated that MSCs contribute to the milieu surrounding wounded tissues by releasing exosomes (Exos). Meanwhile, within wounded tissues, exos play a part in controlling cellular processes like proliferation and differentiation. As a result, these Exos aid in the lesions previously stated' indirect healing [11]. Exosomes produced from MSCs have the ability to trigger an immune response and enable a bidirectional regulation of immune tolerance [12]. Therefore, Exos presents itself as a viable non-cellular therapeutic strategy for the goal of tissue regeneration by reducing the risk of transplanted stem cell dysfunction and modification [13].

A class of hydrolytic enzymes known as phosphodiesterases (PDEs) is essential to the breakdown of cyclic nucleotides. There are eleven subfamilies in all within the PDE [14]. One member of the PDE subfamily, phosphodiesterase-4 (PDE4), has been linked to a number of inflammatory diseases and has been shown to be expressed in some leukocytes [15]. As a second-generation inhibitor of phosphodiesterase 4 (PDE4), rofmilast (ROF) has been shown to decrease PDE4-mediated inflammatory responses in humans [16]. Previous studies have shown that giving PDE4 inhibitors to rats and mice before they were exposed to dangerous drugs was an effective way to reduce kidney damage and impairment [17, 18]. However, it is yet unclear how ROF affects renal function and previous renal impairment.

There are limited pharmacological alternatives available at the moment to slow down the course of CKD. Therefore, developing a therapy approach that targets the fundamental mechanisms linked to chronic kidney disease (CKD) has significant promise to address the disease's etiology.

According to the idea, reducing ROS and inflammation is one of the ways that ROF and Exos function. This restriction therefore has the potential to impede DOX's advancement. Thus, the objective of the present study was to evaluate the combined effects of Rof and Exos on renoprotection and antioxidant activity in a DOX-induced CKD rat model, as opposed to the effects of either drug alone.

## 2. Material and methods

### 2.1.Extraction of ADMSCs-derived exosomes

Male rats were used to generate adipose-derived mesenchymal stem cells (ADMSCs) from their para-gonadal fat. In a nutshell, fats were cleaned and chopped into tiny bits. Fats were centrifuged after being chemically broken down. After removing the supernatant, the pellet was again suspended in DMEM medium that had 10% FBS and 1% antibiotic Penicillin-Streptomycin added to it. After that, it was put into a 25 cm2 tissue culture flask and kept at 37 °C with 5% CO2 in a humidified incubator. Following a 24-hour centrifugation at 300 xg for 10 min, the media was changed to a serum-free alternative once the ADMSCs had reached 80% confluence. A second centrifugation step at 2000 xg for 30 min came next. After being cleaned, the resulting pellet was centrifuged at 100,000 xg for 70 minutes. Lastly, PBS was used to resuspend the pellet containing exosomes [19].

### 2.2.Exosomes characterization

#### 2.2.1 Transmission electron microscopy (TEM)

Samples of exosomes were preserved with 4% paraformaldehyde. The grids were coated with Formvar carbon before the samples were attached. The grids were stained with a 1% phosphotungstic acid solution after two water rinses. After drying, the resultant exosomes were examined under a transmission electron microscope. (Electron

Microscope Unit, Mansoura University, Egypt; JEOL JEM-2100 at 160 KV).

**2.2.2 Nanoparticle tracking analysis**

The exosomes were diluted (1:10) in PBS for nanoparticle tracking analysis (NTA) by NanoSight LM20 (NanoSight, Malvern Panalytical Ltd, Malvern, UK). The Brownian motion of each particle was tracked between frames and the size was calculated by using the Stokes-Einstein equation .

**2.2.3. Exosome labeling with PKH26**

Following the manufacturer's instructions, an aliquot of frozen EV was resuspended in 1 milliliter of PBS and tagged using the PKH26 Fluorescent Cell Linker Kits (Sigma-Aldrich). PKH26 (Sigma-Aldrich, St. Louis, MO, USA) was done to confirm the exosome location within the renal tissue. After diluting the exosomes pellet with PKH-26 kit solution to 1 mL, 2 μL of fluorochrome was added to the suspension, and it was incubated for 15 minutes at 38.5°C. Subsequently, the suspension was mixed with 7 milliliters of serum-free HG-DMEM and centrifuged twice, at 100,000 g for one hour at 4°C. In order to inject the final pellet into an experimentally treated rat later, it was quickly resuspended in HG-DMEM and kept at −80°C.

**2.3.Experimental Animals**

Seventy male Sprague-Dawley rats, weighing 200±20 grams, were housed in cages made of polycarbonate, four rats per cage. The rats were housed in a 24-hour light-dark cycle with a humidity range of 50–70% and a temperature of 24ºC. The Institutional Animal Ethics Committee of the Faculty of Science at Zagazig University in Egypt authorized the methods and care used in the study, which complied with the National Institutes of Health's (NIH) guidelines. [IRB No. ZU-IACUC/1/F/129/ 2020 ].

**2.3.1. Animal Groups**

Five groups of ten rats each were created by randomly dividing the animals. In the first group, animals received an injection of 0.9% saline in the tail vein; in the second group, rats received injections of doxorubicin (DOX) at doses of 4 mg/kg twice on days 1 and 14 of the experiment [21], in the third group, ROF was given to rats for 7 days following each DOX injection; in the fourth group, exosomes were given to rats for 5 days following the administration of both doses of DOX; and in the fifth group, ROF and Exos were given to rats following each DOX injection..

**2.3.2. Collection of blood**

A heart puncture was used to obtain blood samples from each rat 30 days after the first dose of DOX. Traveler blood collection vials were used to get the samples, and anticoagulant was not used. The serum was extracted from the blood samples by centrifugation at 4000 rpm for 10 minutes.

**2.3.3. Biochemical analysis**

Serum creatinine (SCr), blood urea nitrogen (BUN), urea, and total protein levels were measured in the serum. This was accomplished by utilizing particular kits and the Architect system (Abbott Diagnostics, Germany) in compliance with the manufacturer's instructions (Diamond Diagnostics, Cairo, Egypt).

**2.3.4. 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).

## 3. Results

### 3.1.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 1

### 3.2.Nanoparticle tracking

Nanoparticle tracking analysis is a method for analyzing particles in liquids that relates the rate of Brownian motion to particle size. NTA allows the determination of a size distribution profile of small particles with a diameter of approximately 10-1000 nanometers (nm) in liquid suspension. Consistent with transmission electron microscopy, the nanoparticle tracking analysis data for exosomes showed that the sharpest size distribution curves were 122 ± 1.4 indicating more homogeneous preparations as shown in Figure [2].

### 3.3.Exosomes uptake

Exosomes uptake by cells was examined to show the ability of exosomes to enter the cells, Cells kept at 37 °C were incubated with PKH26-labeled EVs for 1.5 hours. Images of MSCs incubated at different temperatures, much more fluorescence was observed in cells incubated at 37°C those shown in Figure [4], demonstrating that EVs entered into the cells.

### 3.4.Evaluation 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), and total protein were measured and their results are presented in Figure 3. 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, 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 ($8p < 0.05$) in ( Fig .4,5,6,7).

### 3.5. Measurements of antioxidant and oxidative stress Markers:

#### 3.5.1. Detection of melanodialdehyde activity (MDA)

Malondialdehyde (MDA) levels were detected in control, CKD, and different treated groups (Table 1, Fig. 8). No statistical differences were detected between control, group ($p > 0.05$). Conversely, CKD group revealed a significant elevation in MDA levels compared to the control group ($p < 0.05$). On the other hand, treatment with ROF or Exos showed significant decrease in MDA levels compared to CKD group ($p < 0.05$). Furthermore, treatment with both ROF and Exos ./showed the lowest

level of MDA compared to CKD, CKD+ROF, CKD+Exos groups ($p < 0.05$) with no difference with control group ($p > 0.05$)

### 3.5.2. Detection of reduced glutathione (GSH) activity

The activity of GSH was determined in control and compared with the different treated groups (Table 2, Fig. 9). No statistical difference in GSH activity was detected control, groups ($p > 0.05$). Contrariwise, CKD group showed a significant low activity of GSH compared to the control group ($p < 0.05$). Treatment with ROF or Exos showed significant enhancement in GSH activity compared to CKD group ($p < 0.05$). Moreover, CKD+ ROF+Exos group showed the highest GSH activity compared to CKD, CKD+ROF, CKD+Exos groups ($p<0.05$).

### 3.6. Gene expression by real time PCR:

### 3.6.1. Apoptotic markers (BAX):

The expression of apoptotic marker BAX was detected in the all-treated groups

(Table 3; Fig. 10 ). Compared to the control group,did not show any statistical difference ($p > 0.05$). The expression of BAX was significantly high in CKD group compared to the control group ($p < 0.05$), whereas CKD group treated with ROF or Exos decreased the BAX expression compared to CKD group ($p < 0.05$). Moreover, CKD+ROF+Exos group manifested the lowest BAX expression compared to CKD, CKD+ROF, and CKD+Exos groups ($p < 0.05$).

## 4. Discussion

l.It is commonly known that doxorubicin is a commonly used chemotherapy drug. On the other hand, long-term or severe DOX use may cause renal damage brought on by the medicine and maybe even renal failure [22]. Therefore, prevention of renal failure due to DOX is essential. The main focus of this work was to examine the potential mechanisms of action of both roflumast and exosomes in the therapy of DOX-induced chronic kidney disease.

In recent years, exosome-based therapy has gained popularity as a possible means of treating a number of clinical disorders. Recipient cells have the ability to absorb the provided exosomes, which can change a number of biological processes at the molecular and subcellular levels [23]. Comparing exosome-based therapy to cell-based therapies reveals various advantages due to its cell-free nature. Benefits include less danger of immunological rejection, greater stability and longer storage life, ease of accessibility to wound sites, and lack of toxicity [24].

Exosome extraction from the conditioned cell culture medium of ADMSCs—which were found to be CD45-negative and CD105-positive based on their phenotype—was the main objective of the current investigation. The ADMSCs demonstrated the fundamental characteristics of MSCs in line with the guidelines established by the Society of Cellular Therapy [25]. Transmission electron microscopy (TEM) was employed as a universal analytical approach for isolated exosomes. According to reports, exo-MSCs have a distinct cup-shaped structure and exhibit a consistent range of sizes, with diameters between 30 and 100 nm [26]. The existence of nano-vesicles was verified by our TEM analysis, which showed a wide range of typical dimensions from 30 to 150 nm. Furthermore, the flow cytometry technique was employed to identify

the specific surface antigens—namely, CD9, CD83, and CD63—associated with Exos. The surface markers CD9, CD63, and CD81, which are typically found in exosomes, belong to the tetraspanin family [27]. The detection of CD9, CD83, and CD63 allowed for the confirmation of exosome characteristics.

Numerous symptoms, including changes in the content of urine, structural changes in the kidneys, and reduced renal function, are indicative of chronic kidney disease (CKD). It is now known that proteinuria poses a serious risk to the development of chronic kidney disease (CKD) [28]. Prior research has demonstrated that DOX causes significant hyperlipidemia and hypoalbuminemia, as seen by elevated BUN, total urine protein, and serum creatinine levels [29, 30]. The results of this investigation showed that the use of DOX increased the levels of total protein, BUN, and Scr in a statistically significant way. Exos was found to have a positive impact on kidney function and structure based on a previous study, which increased survival rates [31]. According to research by Wan et al. [32], bone marrow-derived exosomes have the ability to lower BUN and Scr levels when renal damage is present. In our trial, we discovered that giving several Exos injections resulted in a notable decrease in the death rates among patients with normal renal function.

Furthermore, it has been documented that the administration of ROF, either alone or in combination with other medications, improves renal functions and has a beneficial effect on renal function. Consequently, elevated renal function results in decreased levels of urea, BUN, and Scr [33]. Comparing the results of our study to the CKD group, we found a substantial drop in the levels of total protein, urea, BUN, and Scr. This result is consistent with a study by Patel et al. [34], which highlighted the beneficial role ROF plays in enhancing renal function in patients with adenine-induced chronic kidney disease. Furthermore, ROF lowers Scr and BUN levels in a rat model of diabetic nephropathy, as shown by Tikoo et al. [35]. Furthermore, the highest improvement in kidney function was seen in rats who received both Exos and ROF, suggesting that the combined therapy has a renoprotective impact.

## 5. Conclusion

In contrast to therapy with exosomes alone or Roflumilast alone, the current work provides additional evidence indicating that the injection of exosomes obtained from ADMSCs in conjunction with Roflumilast led to a more advantageous therapeutic outcome for CKD caused by DOX. The preventive effects of Exos and Roflumilast may be attributed to their antioxidant capabilities. Nevertheless, more investigation is needed to fully comprehend the likely mechanism of these combos in CKD.

## Conflict of interest

The authors declare that none of the work reported in this study could have been influenced by any known competing financial interests or personal relations.

## References

1. Sharma, S., B.J.K. Smyth, and B.P. Research, From proteinuria to fibrosis: An update on pathophysiology and treatment options. 2021. 46(4): p. 411-420.

2. Pugh, D., P.J. Gallacher, and N.J.D. Dhaun, Management of hypertension in chronic kidney disease. 2019. 79: p. 365-379.
3. Liu, D., L.-L.J.R.F.M. Lv, and Therapies, New understanding on the role of proteinuria in progression of chronic kidney disease. 2019: p. 487-500.
4. Zhao, L., S. Han, and C.J.J.o.E. Chai, Huangkui capsule alleviates doxorubicin-induced proteinuria via protecting against podocyte damage and inhibiting JAK/STAT signaling. 2023. 306: p. 116150.
5. Xiang, C., Y. Yan, and D.J.J.o.p.s. Zhang, Alleviation of the doxorubicin-induced nephrotoxicity by fasudil in vivo and in vitro. 2021. 145(1): p. 6-15.
6. Tacar, O., P. Sriamornsak, C.R.J.J.o.p. Dass, and pharmacology, Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems. 2013. 65(2): p. 157-170.
7. Renu, K., L.P. Pureti, B. Vellingiri, and A.J.T.R. Valsala Gopalakrishnan, Toxic effects and molecular mechanism of doxorubicin on different organs–an update. 2022. 41(2): p. 650-674.
8. Okamura, D.M. and S.J.R.b. Pennathur, The balance of powers: redox regulation of fibrogenic pathways in kidney injury. 2015. 6: p. 495-504.
9. Lohmor, S., R. Verma, V. Malik, and D.J.C.B.L. Kaushik, Current status of Regulatory perspectives on Stem Cell Therapy in India. 2020. 7(3): p. 176-182.
10. Awadalla, A., A.M. Hussein, M. Ali, N. Barakat, E.T. Hamam, R.W. Magar, et al., Possible mechanisms for the renoprotective action of adipose-derived mesenchymal stem cells with CD44-targeted hyaluronic acid against renal ischemia. 2021. 272: p. 119221.
11. Awadalla, A., A.M. Hussein, M. Ali, N. Barakat, E.T. Hamam, R.W. Magar, et al., Possible mechanisms for the renoprotective action of adipose-derived mesenchymal stem cells with CD44-targeted hyaluronic acid against renal ischemia. 2021. 272: p. 119221.
12. Komaki, M., Y. Numata, C. Morioka, I. Honda, M. Tooi, N. Yokoyama, et al., Exosomes of human placenta-derived mesenchymal stem cells stimulate angiogenesis. 2017. 8(1): p. 1-12.
13. Wang, L., L. Hu, X. Zhou, Z. Xiong, C. Zhang, H.M. Shehada, et al., Exosomes secreted by human adipose mesenchymal stem cells promote scarless cutaneous repair by regulating extracellular matrix remodelling. 2017. 7(1): p. 13321.
14. Bucan, V., D. Vaslaitis, C.-T. Peck, S. Strauß, P.M. Vogt, and C.J.M.N. Radtke, Effect of exosomes from rat adipose-derived mesenchymal stem cells on

neurite outgrowth and sciatic nerve regeneration after crush injury. 2019. 56: p. 1812-1824.
15. Kumar, N., A.M. Goldminz, N. Kim, and A.B.J.B.m. Gottlieb, Phosphodiesterase 4-targeted treatments for autoimmune diseases. 2013. 11: p. 1-8.
16. Costa, W.C., V.A. Beltrami, G.H. Campolina-Silva, C.M. Queiroz-Junior, R.M. Florentino, J.R. Machado, et al., Therapeutic treatment with phosphodiesterase-4 inhibitors alleviates kidney injury and renal fibrosis by increasing MMP-9 in a doxorubicin-induced nephrotoxicity mouse model. 2023. 115: p. 109583.
17. Patel, P., S. Patel, P. Chudasama, S. Soni, and M.J.E.J.o.P. Raval, Roflumilast alleviates adenine-induced chronic kidney disease by regulating inflammatory biomarkers. 2023. 949: p. 175731.
18. Ansari, M., R. Aloliet, M. Ganaie, T. Khan, Najeeb-ur-Rehman, F. Imam, et al., Roflumilast, a phosphodiesterase 4 inhibitor, attenuates cadmium-induced renal toxicity via modulation of NF-κB activation and induction of NQO1 in rats. 2019. 38(5): p. 588-597.
19. Xu, M., X. Yu, X. Meng, S. Huang, Y. Zhang, A. Zhang, et al., Inhibition of PDE4/PDE4B improves renal function and ameliorates inflammation in cisplatin-induced acute kidney injury. 2020. 318(3): p. F576-F588.
20. Franquesa, M., M.J. Hoogduijn, E. Ripoll, F. Luk, M. Salih, M.G. Betjes, et al., Update on controls for isolation and quantification methodology of extracellular vesicles derived from adipose tissue mesenchymal stem cells. 2014. 5: p. 525.
21. Yang, W., J. Wang, L. Shi, L. Yu, Y. Qian, Y. Liu, et al., Podocyte injury and overexpression of vascular endothelial growth factor and transforming growth factor-beta 1 in adriamycin-induced nephropathy in rats. Cytokine, 2012. 59(2): p. 370-6.
22. Shi, M., K.L. McMillan, J. Wu, N. Gillings, B. Flores, O.W. Moe, et al., Cisplatin nephrotoxicity as a model of chronic kidney disease. Lab Invest, 2018. 98(8): p. 1105-1121.
23. Gao, F., B. Zuo, Y. Wang, S. Li, J. Yang, and D.J.L.s. Sun, Protective function of exosomes from adipose tissue-derived mesenchymal stem cells in acute kidney injury through SIRT1 pathway. 2020. 255: p. 117719.
24. Rahmati, S., F. Shojaei, A. Shojaeian, L. Rezakhani, and M.B. Dehkordi, An overview of current knowledge in biological functions and potential theragnostic applications of exosomes. Chemistry physics of lipids, 2020. 226: p. 104836.
25. Basu, J. and J.W. Ludlow, Exosomes for repair, regeneration and rejuvenation. Expert Opinion on Biological Therapy, 2016. 16(4): p. 489-506.

26. Dominici, M., K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, et al., Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy, 2006. 8(4): p. 315-7.
27. Tang, Y., Y. Zhou, and H.J. Li, Advances in mesenchymal stem cell exosomes: a review. Stem Cell Res Ther, 2021. 12(1): p. 71.
28. Wu, S.C., P.J. Kuo, C.S. Rau, Y.C. Wu, C.J. Wu, T.H. Lu, et al., Subpopulations of exosomes purified via different exosomal markers carry different microRNA contents. Int J Med Sci, 2021. 18(4): p. 1058-1066.
29. Webster, A.C., E.V. Nagler, R.L. Morton, and P. Masson, Chronic kidney disease. The lancet, 2017. 389(10075): p. 1238-1252.
30. Amarasiri, S.S., A.P. Attanayake, L. Arawwawala, K. Jayatilaka, and L.K.B. Mudduwa, Protective effects of three selected standardized medicinal plant extracts used in Sri Lankan traditional medicine in adriamycin induced nephrotoxic Wistar rats. J Ethnopharmacol, 2020. 259: p. 112933.
31. Ding, Z.H., L.M. Xu, S.Z. Wang, J.Q. Kou, Y.L. Xu, C.X. Chen, et al., Ameliorating Adriamycin-Induced Chronic Kidney Disease in Rats by Orally Administrated Cardiotoxin from Naja naja atra Venom. Evid Based Complement Alternat Med, 2014. 2014: p. 621756.
32. Alasmari, W.A., A. Abdelfattah-Hassan, H.M. El-Ghazali, S.A. Abdo, D. Ibrahim, N.A. ElSawy, et al., Exosomes Derived from BM-MSCs Mitigate the Development of Chronic Kidney Damage Post-Menopause via Interfering with Fibrosis and Apoptosis. Biomolecules, 2022. 12(5).
33. Wan, F., R.C. Yang, Y.W. Tang, X.L. Tang, T. Ye, J. Zheng, et al., BMSC-derived exosomes protect against kidney injury through regulating klotho in 5/6 nephrectomy rats. Eur J Med Res, 2022. 27(1): p. 118.
34. Zhong, Y., Y. Wu, R. Liu, Y. Deng, S.K. Mallipattu, P.E. Klotman, et al., Roflumilast enhances the renal protective effects of retinoids in an HIV-1 transgenic mouse model of rapidly progressive renal failure. Kidney international, 2012. 81(9): p. 856-864.
35. Patel, P., S. Patel, P. Chudasama, S. Soni, and M. Raval, Roflumilast alleviates adenine-induced chronic kidney disease by regulating inflammatory biomarkers. Eur J Pharmacol, 2023. 949: p. 175731.

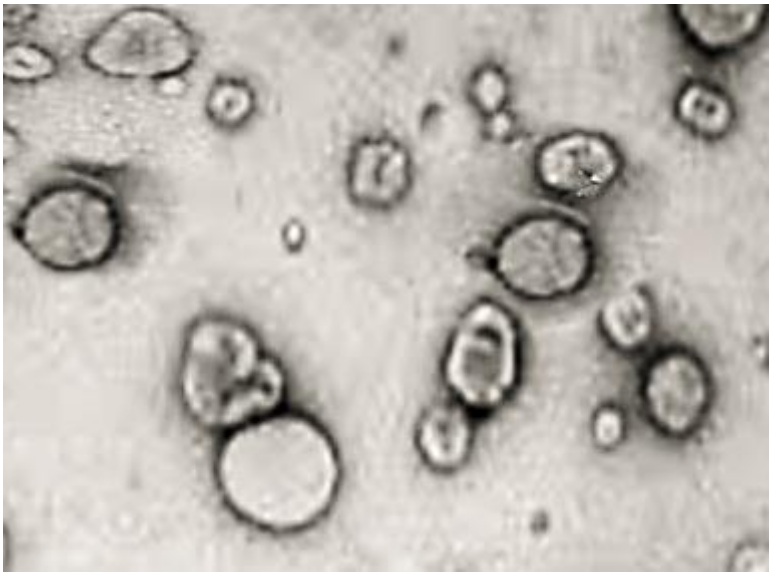

**Figure (1) :** Electron-microscopic observation of exosomes (Arrow). Scale bar = 100 nm.

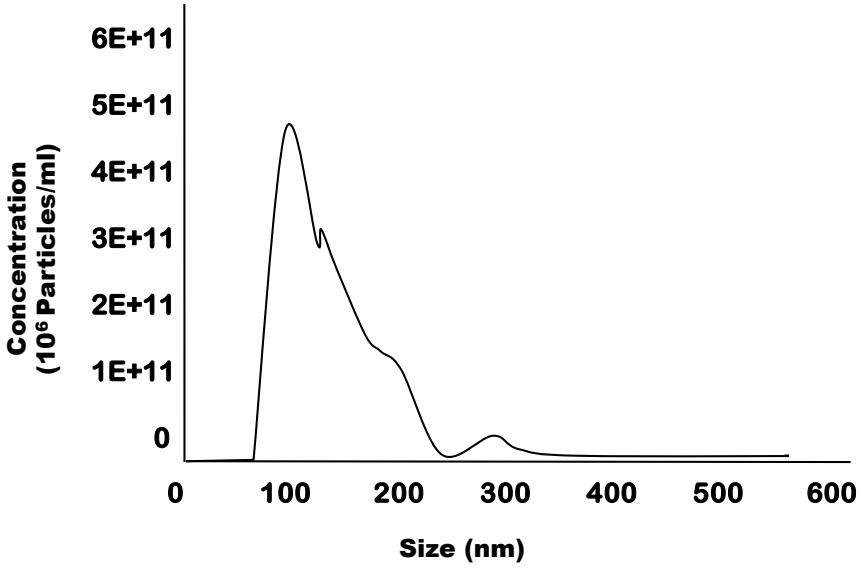


**Figure (2) the nanoparticle tracking analysis data showed that the sharpest size distribution curves were 122 ± 1.4 indicating more homogeneous preparations**

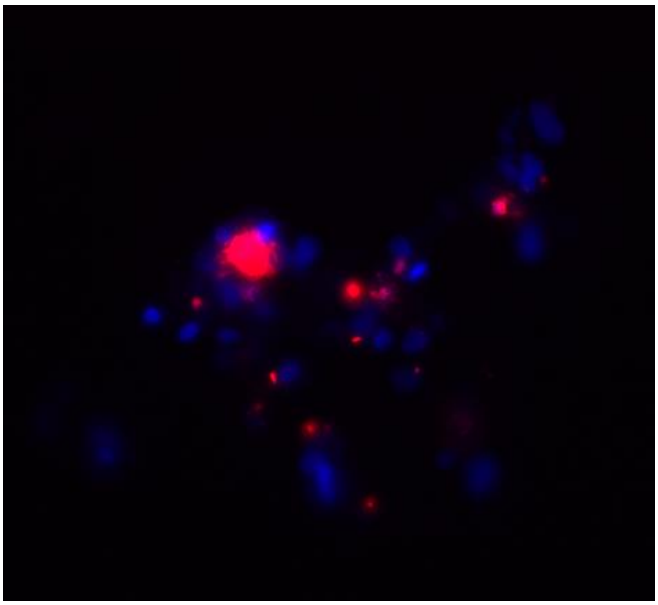

**Figure (3)** Exosomes uptake by cells was examined to show the ability of exosomes to enter the cells, Cells kept at 37 °C were incubated with PKH26-labeled EVs for 1.5 hours.

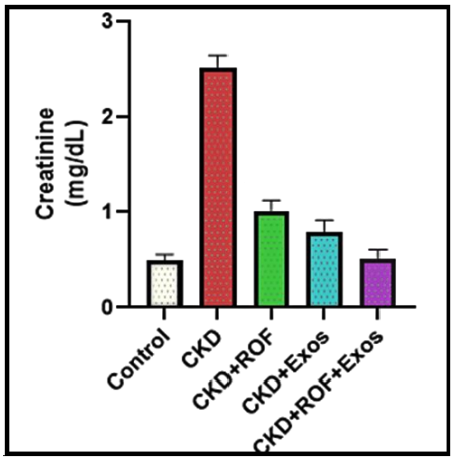


**Figure (4): Effect of ROF and Exos on SCr**

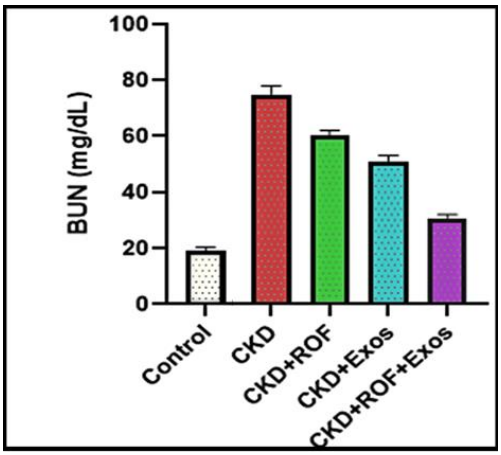


**Figure (5): Effect of ROF and Exos on BUN**

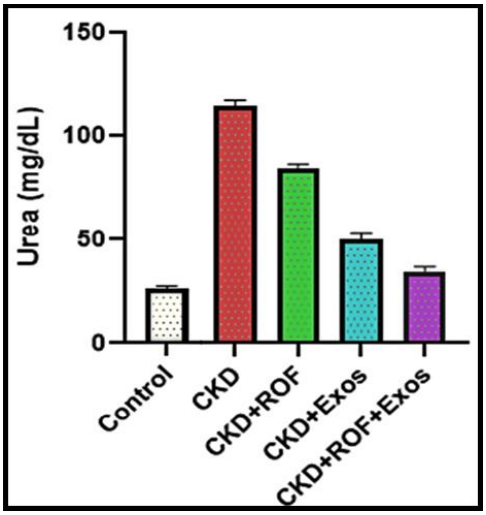


**Figure (6): Effect of ROF and Exos on Urea**

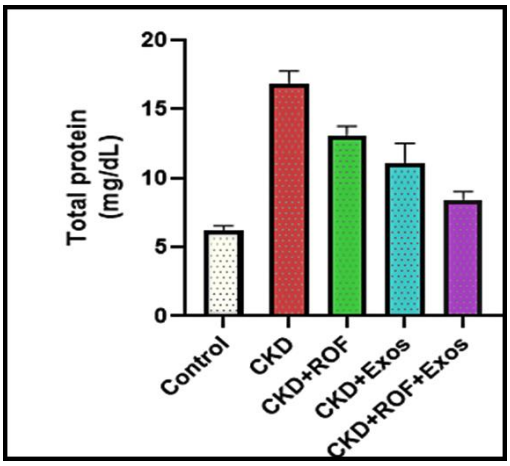


**Figure (7): Effect of ROF and Exos on total protein**

**Table (1): Effect of ROF and Exos on kidney Malondialdehyde (MDA) (nmol/ml) level among the studied groups:**

| | MDA (mmol/g) |
|---|---|
| **Control** | 11.22±0.6 |
| **CKD** | 51±2.7[abc] |
| **CKD+ROF** | 35±3.1[abc] |
| **CKD+ Exos** | 25.1±1.88 [abd] |
| **CKD+ROF+Exos** | 15.50±1.17[bcd] |

All data are expressed as mean ± SD. One-way ANOVA test with Scheffe's post hoc test. [a] Significant vs Control, [b] vs CKD, [c] vs CKD+ROF and [d] vs CKD+Exos. Statistical significance was determined where P <0.05.

**Table (2): Effect of ROF and Exos on kidney Glutathione reduced (GSH) (nmol/ml) level among the studied groups:**

| Groups | GSH (mmol/g) |
|---|---|
| **Control** | 9.56±0.97 |
| **ROF** | 9.23±1.48 |
| **CKD+ROF** | 3.86±0.24[abc] |
| **CKD+ Exos** | 6.15±0.26[abd] |
| **CKD+ROF+Exos** | 8.5±0.5[abcd] |

*All data are expressed as mean ± SD. One-way ANOVA test with Scheffe's post hoc test. [a] Significant vs Control, [b] vs CKD, [c]vs CKD+ROF and [d] vs CKD+Exos. Statistical significance was determined where P <0.05.*

**Table (3): Effect of ROF and Exos on BAX expression among the studied groups**

| Groups | BAX |
|---|---|
| Control | 1.07±0.08 |
| CKD | 4.95±0.21 [ab] |
| CKD+ROF | 3.89±0.14 [abc] |
| CKD+ Exos | 2.5±0.14 [abcd] |
| CKD+ROF+Exos | 1.55±0.14 [abcd] |

*All data are expressed as mean ± SD. One-way ANOVA test with Scheffe's post hoc test. [a] Significant vs Control[b] vs CKD, [c] vs CKD+ROF and [d] vs CKD+Exos. Statistical significance was determined where P <0.05.*

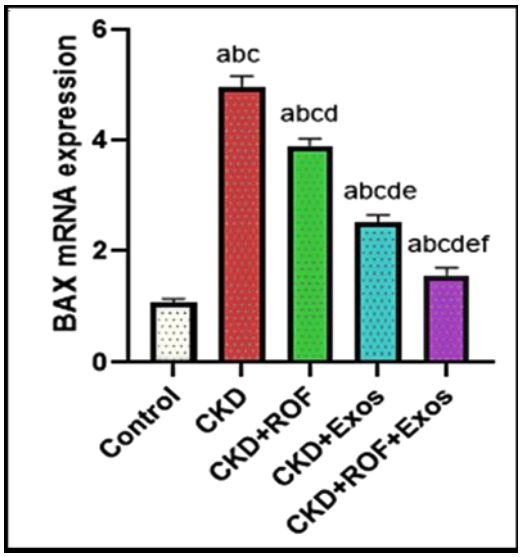


**Figure (8): Effect of ROF and Exos on kidney BAX expression**