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Original article

# Impact of preconditioning stem cells with all-trans retinoic acid signaling pathway on cisplatin-induced nephrotoxicity by down-regulation of TGFβ1, IL-6, and caspase-3 and up-regulation of HIF1α and VEGF

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## ABSTRACT

The survival reduction after transplantation limited the clinical uses of stem cells so the current study explored preconditioning adipose-derived stem cells (ADMSCs) and all-trans retinoic acid (ATRA) effects on cisplatin that caused acute kidney injury (AKI). One hundred and fifty Sprague–Dawley male rats were distributed into five groups: control group; Cisplatin (CIS) group; CIS and ATRA group; CIS and ADMSC group, and CIS, ATRA, and ADMSCs group. Ten rats were euthanized after 3rd, 7th, and 11th days from CIS injection. Renal function, molecular studies, and histopathological analysis were studied. The preconditioning of ADMSCs with ATRA increased the viability of the cells which was reflected in the amelioration of kidney functions after CIS injection by the significant reduction of serum creatinine, microalbuminuria, as well as NO, and the significant rise of creatinine clearance, as well as SOD compared to the group of cisplatin. ATRA also supported ADMSCs by a significant down-regulation of caspase-3, il-6 and TGFβ1, and a significant up-regulation of HIF1, VEGF and CD31 compared to group of cisplatin which reversed the cisplatin effect. ATRA increased renoprotective properties of ADMSCs against cisplatin-induced AKI by reducing the apoptosis, inflammation, and stimulating angiogenesis.

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## 1. Introduction

Cisplatin is a highly effective medicine for many human malignancies, and, despite its effectiveness, high dose from cisplatin limited by accumulative and acute kidney toxicity (Shen et al., 2012).

Acute kidney injury (AKI) is a serious and relatively side effect of CIS treatment, which can be seen in nearly 30% of patients after

a single dose. Research indicated that CIS can induce nephrotoxicity by injury pathway, that contains the cisplatin conversion to toxic metabolites, damage of mitochondrial, and nuclear DNA, as well as ionic homeostasis disruption, the reactive oxygen species (ROS) release (Wang et al., 2020), induction of inflammation (Volarevic et al., 2019), as well as activation of the apoptotic pathway (Dugbartey et al., 2016).

Depending on the previous mechanisms, various renoprotective approaches were established. These approaches are categorized according to their main target: pathway of cell death, CIS uptake by renal cell, metabolism of CIS, oxidative stress and inflammation, and Cell-cycle regulators, (Pabla & Dong, 2008).

Mesenchymal stem cells (MSCs) are hopeful tools of therapeutic applications for stem cells as they are found in different sources, like adipose tissue, bone marrow, as well as an umbilical cord (Wei et al., 2013). However, the transplanted stem cells death, the reduction of their migration and the tissue damage by ROS, inflammation, apoptotic cascade activation, and poor vascular supply, make a critical barrier to their advantage (Pourjafar et al.,

*Abbreviations:* ADMSCs, Adipose Mesenchymal Stem Cells; AKI, Acute Kidney Injury; ATRA, All-Trans Retinoic Acid; Cis, Cisplatin; HIF-1, Hypoxia-inducible factor-1; IL-6, Interleukin-6; NO, Nitric oxide; SOD, Super oxide dismutase; TGFβ1, Transforming growth factor beta; VEGF, Vascular endothelial growth factor.

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2017); therefore, modulating the culture conditions of stem cells can improve their therapeutic efficacy. All-trans retinoic acid (ATRA) is the biological vitamin A metabolite (all-trans-retinol). ATRA has important roles in cell growth and differentiation, immune function, survival, and reproduction by binding to its nuclear receptors (Pourjafar et al., 2017). The viability and proliferation of MSCs were reported to be increased via the preconditioning of MSCs with ATRA. This metabolite can also reduce apoptosis and induce the migration capacity of MSCs by enhancing specific transcription factors expression (Pourjafar et al., 2017). ATRA has other roles, such as anti-proliferative, anti-inflammatory, and anti-cancer effects; it can also catch and prevent free radicals without interacting with biological targets, through the impairment of lipid peroxidation inside the membrane of cell (Yucel et al., 2019).

Several lines of evidence demonstrated that ATRA is a critical factor for nephron formation due to its cooperation in the modulation of the expression of genes through nephron progenitors (Wingert and Davidson, 2011). Another study proved that ATRA participates vigorously to activate the expression of genes in tubular as well as podocyte lineages (Li et al., 2014). Thus, this present study aimed to examine ATRA pretreatment effect on the renoprotective effect of ADMSCs against cisplatin-induced AKI in rats.

## 2. Materials and methods

### 2.1. Preparation of complete primary culture media

Briefly, under laminar airflow, Dulbecco's modified Eagle's medium (DMEM) media was aliquoted in 50 ml falcon tubes and preserved at 2–8 °C. Fetal bovine serum (FBS) was well-kept at –20 °C, thawed, and deactivated for 20 min at 56 °C inside a water bath, then aliquoted in 15 ml falcon tubes. Penicillin/streptomycin antibiotic was preserved at –20 °C and was put in the water bath until thawed and then aliquoted in 15 ml falcon tubes at a volume of 5 ml/tube and was preserved at –20 °C. Finally, the tubes, which contained 50 ml medium, 5 ml FBS (10%), and 0.5 ml penicillin/streptomycin antibiotic were filtered using a filter system with 250 ml and warmed in the water bath at 37 °C before used for cultures.

### 2.2. Preparation of adipose-derived MSCs

ADMSCs were obtained from a male Sprague–Dawley (SD) rat. Briefly, the rat was subjected to anesthesia, the para gonadal fat was obtained and was put in phosphate-buffered saline (PBS), and small pieces of fat were cut and centrifuged 10 min at 2000 rpm. After that, collagenase I 0.075% (Sigma, USA) was used for putting the fat inside it at 37 °C for 1 h to complete digestion and was centrifuged for 10 min at 2000 rpm. The pellet was resuspended in PBS, while the formed supernatant was removed, and the pellet was centrifuged again 10 min at 2000 rpm.

The DMEM supplemented with 10% FBS (GIBCO, Thermo, USA) was used to resuspend the pellet, and it was transferred to a tissue culture flask and kept in a 5% CO<sub>2</sub> incubator. Two times weekly, the medium was replaced till the cells reached 70% – 80% confluence, and treated with 1 mM EDTA (Invitrogen, Thermo, USA) and 0.25% trypsin 3 min. At the same culture conditions, the ADMSCs were replaced in a 1:3 split ratio. After the third passage, cells were used for experiment.

### 2.3. Flow cytometric analysis

Adipose stem cells were trypsinized to release stem cells and investigated by flow cytometry, where the cells were washed

two times by PBS, and then aliquots of cells were prepared at (1×10<sup>6</sup>/ml) concentration and then incubated at 25 °C in the dark for 30 min with following antibodies: Phycoerythrin (PE)-conjugated CD45 and PE-conjugated CD105. After that, labeled cells were washed by PBS then detected by flow cytometer (Beckman Coulter, USA).

### 2.4. Pretreatment of ADMSCs with retinoic acid and a cell viability assay

The colorimetric MTT assay was used for cell viability assessment, where ADMSCs were first pretreated with different concentrations of ATRA as follows: the cells were put at 5×10<sup>3</sup>cell/well, ATRA was used at various concentrations (0, 0.5, 1, 5, and 10 μmol/L) for the treatment of the ADMSCs. After that, cells with ATRA were incubated for 24 and 48 h; after incubation, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Aldrich, USA) was incubated with the cells at 37 °C for 4 h and 100 μL DMSO (Sigma–Aldrich) was added. At a wavelength 570 nm, an automatic microplate reader is used to detect the optical density of the solubilized formazan.

### 2.5. Animals

150 male Sprague Dawley (SD) rats, weighing approximately 180–230 g were well-kept at standard conditions and a temperature fixed at 20 °C. All the experiments were conducted in conformity with the Urology and Nephrology Center, Mansoura University, guidelines for the care and use of laboratory animals (ILAR 1996), and were approved by Mansoura University, Mansoura, Egypt ethics committee (Code # MDP.19.12.35.R1).

### 2.6. Experimental design

Normal group (n = 30): This group was injected with 1 ml 0.9% saline intraperitoneally (IP). (Cisplatin 'CIS' group) (n = 30): This group was injected IP by 6 mg/kg CIS as a single dose. CIS and ATRA group (n = 30): This group was injected IP by cisplatin at 6, and, after one day of injection, the rats were injected intravenously with 10 μM ATRA. CIS and ADMSCs group (n = 30): Rats were injected with 0.5 ml of culture media containing 1×10<sup>6</sup> ADMSCs in the tail vein. CIS, ATRA, and adipose-ADMSCs group (n = 30): This group was injected IP by CIS at 6 mg/kg, and, after one day from the injection, the rats were injected intravenously with 1×10<sup>6</sup> ADMSCs preconditioned for 48 h with 10 μM ATRA. Before the sacrifice of each group, urine samples were collected randomly for the estimation of microalbuminuria (MAU), and then, the urine was collected during one day. The volume of urine was determined, and the urinary creatinine was calculated. Then, 10 rats in each group were sacrificed at days 3, 7, and 11 after treatment, and the kidney tissue and blood samples were obtained.

### 2.7. Biochemical measurements

The blood samples were obtained from hearts of all rats in blood vacutainer tubes without any anticoagulant. The tubes were centrifuged for 10 min at 4000 r.p.m at 25 °C, the serum creatinine was determined using the Architect c4000 system (Abbott Diagnostics, Wiesbaden, Germany). Microalbuminuria (MAU) was determined in the urine samples according to Cambiaso et al. (1988) (Cambiaso et al., 1988), by using the Albumin (Microalbuminuria) Kit (COD: 31924), supplied by (Biosystem S.A., 30. 08,030 Barcelona (Spain). The creatinine clearance was measured from the following equation (Mahmoud et al., 2007).

$$\text{CrCl (mL/min)} = \frac{\text{Urine creatinine (mg/dL)} * \text{urine volume (mL/24 h)}}{\text{(Serum creatinine (mg/dL)} * 1440 \text{ (min)})}$$

2.8. Assay of nitric oxide marker (NO) and antioxidant (SOD enzyme activity) in kidney tissues

Nitric oxide was determined in kidney tissue colorimetrically using (Biodiagnostic, Egypt). Nitrate was reduced to nitrite by adding Vanadium trichloride to the supernatant according to Miranda et al.'s method (Miranda et al., 2001), while superoxide dismutase in kidney tissue was estimated colorimetrically using (Biodiagnostic, Egypt).

2.9. Real-time PCR for TGFβ1, IL-6, VEGF, HIF1α, and Caspase-3 genes

The Trizol reagent (Invitrogen Corporation, New York) extracted the total RNA from kidney tissues. cDNA reverse transcription kit (Applied Biosystem, USA) used to synthesize cDNA from 1 μg RNA. RT-PCR investigation was done by SYBER Green PCR Master Mix (Biosystem, USA) and performed using an Applied Biosystem (Step One™, USA).

The PCR reaction was 1 cycle for 2 min at 50 °C for amplification, then 40 cycles, each cycle containing denaturation for 15 s at 95 °C, annealing for 1 min at 60 °C, and elongation for 1 min at 72 °C. Relative expression of VEGF, IL-6, Caspase-3, TGFβ1 and HIF1α was calculated using the 2<sup>-ΔΔCt</sup> method. Selective primer sequences of genes are listed in Table 1.

2.10. Histopathological examination

Kidney specimens were fixed in paraffin; specimens were cut into 4-μm thick slices by a microtome (Leica RM 2155, England) then stained with hematoxylin and eosin to examine under light microscopic at 400x magnification. The scoring system (Nasir et al., 2011) regarded active injury changes as those that comprise interstitial infiltration and necrotic tubules by inflammatory cells; it also regarded regenerative changes that included the presence of mitotic figures and solid sheets between the tubules.

2.11. Study of renal caspase-3 by immunohistochemical examination

The immunohistochemistry staining assay was done to assess the renal caspase-3, where the paraffin-embedded slices of kidney tissues (3 μm thickness) were first deparaffinized, then incubated for 30 min with goat serum 5% (Sigma–Aldrich), and then with hydrogen peroxide 3% (Fisher Scientific) for 15 min for the activation of endogenous enzymes.

After that, the slides were incubated with activated caspase-3 antibodies (RB-1197-P1; Fremont Blvd, Fremont, CA, USA) at 4 °C

Table 1 Sequences of primers for real- time RT-PCR.

Gene	Sequence	Accession no
TGFβ1	F- 5'-CTGCTGACCCCACTGATAC-3'	NM_021578.2
	R- 5'-AGCCCTGTATTCCGTCTCT-3'	
IL-6	F- 5'-GCCCTTCAGGAACAGCTATGA-3'	NM_012589.2
	R- 5'-TGTCACAACATCAGTCCCAAGA-3'	
VEGF	F- 5'-ACGAAAGCGCAAGAAATCCC-3'	NM_031836.3
	R- 5'-TTAACTCAAGCTGCCTCGCC-3'	
HIF1α	F- 5'-TGCTTGGTGTGATTTGTGA-3'	NM_024359.1
	R- 5'-GGTCAGATGATCAGATCCCA-3'	
Caspase-3	F- 5'-GGACCTGTGGACCTGAAAAA-3'	NM_012922.2
	R- 5'-GCATGCCATATCATCTCAG-3'	
GAPDH	F- 5'-TATCGGACGCTGGTTAC-3'	NM_017008.4
	R- 5'-CTGTGCCGTGAACCTTGC-3'	

overnight, then incubation 30 min with biotinylated secondary antibody (Power - Stain 1.0 Poly HRP DAB Kit), and then, the slides were incubated with Vectastain Elite ABC reagent for 30 min, followed by diaminobenzidine (DAB) incubation for 2 min, and hematoxylin counterstaining for 1 min; the sections examined with an Olympus BX51 light microscope.

2.12. Immunofluorescence examination

The slices of kidney tissues were prepared using a cryostat and mounted on super frost slides, and then we performed the blocking step. The slides were incubated in 5% normal serum for 1 h, the blocking solution was aspirated followed by incubation with CD31 (BBA7) at 25 °C for 1 h, and then the slides were incubated with anti-rat IgG for 1 h at RT in the dark. The slides were developed with diaminobenzidine (DAB) (California, USA) staining. Kidney tissue sections were examined using fluorescent microscope leica for positive nuclei. The expression of CD31 was measured as follows: the number of CD31 positive nuclei was calculated in 10 fields at 200 X, and then we calculated the mean number of positive nuclei (Xue et al., 2018).

2.13. Statistical analysis

The results are expressed as the mean ± standard deviation, and statistical differences were determined using one-way analysis of variance (ANOVA) taking p < 0.05 as the confidence interval. All statistical analyses were performed using SPSS version 20 (IBM Corp., USA).

3. Results

3.1. Isolation and characterization of ADMSCs

Using flow cytometric analysis, the cultured cells expressing a CD105 positive rate were 84.5%, and the negative rate of CD105 was 15.5%, while the positive rate of CD45 was only 8.7%, and the negative rate was 91.3%. These results are in accordance with the expression characteristics of surface antigens of ADMSCs (Fig. 1 a, b).

3.2. ATRA increased ADMSC viability

The third passage of ADMSCs (Fig. 2a) pretreated with ATRA showed fibroblast-like morphology and increased regeneration (Fig. 2b). As shown in Fig. 3, ATRA significantly improved the proliferation of ADMSC at 24 and 48 h. The cellular viability was

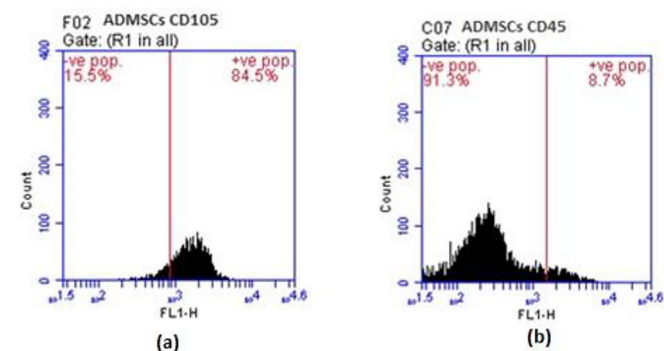
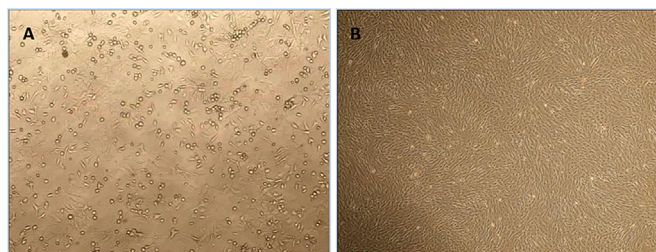
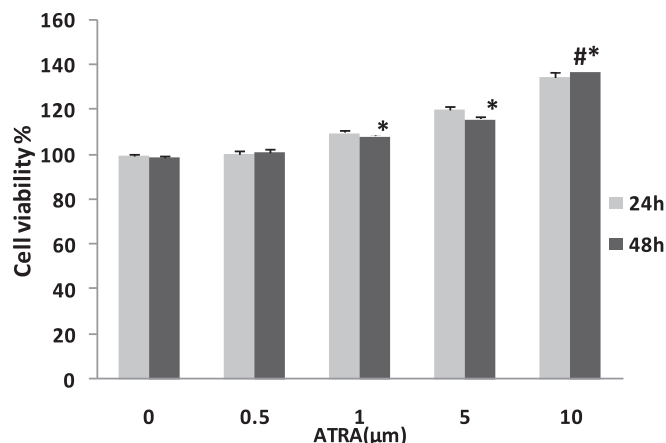


Fig. 1. Flow cytometric analysis of adipose-derived stem cells (ADMSCs) disclosed that their expression level of surface antigens, such as CD105, was positive (a); but CD45 was negative (b) (at Passage 3).



**Fig. 2.** Photomicrograph showing a) Untreated ADMSCs, ADMSCs treated with all-trans retinoic acid (ATRA) for 48 h ( $\times 40$ ).



**Fig. 3.** Effects of ATRA on ADMSCs proliferation. \* compared to control, # compared to 10 μm ATRA at 24 h.

significantly increased in all treated ADMSCs except for 0.5 μmol/L ATRA for 24 and 48 h compared to control ( $P < 0.05$ ). The viability reached the maximum increase in concentration 10 μmol/L at 48 h compared to 10 μmol/L at 24 h ( $P < 0.05$ ).

### 3.3. Biochemical parameters

The serum creatinine (SCr), microalbuminuria (MAU) in mg/dl, and creatinine clearance (CrCl) in ml/min of all the studied groups

**Table 2**  
Effects of ADMSCs pretreated with ATRA on the serum creatinine, microalbuminuria (MAU), and creatinine clearance.

Groups	3d		7d		11d	
	Basal	Test	Basal	Test	Basal	Test
<b>SCr (mg/dl)</b>						
Control	0.51 ± 0.03	0.55 ± 0.04	0.45 ± 0.02	0.57 ± 0.04	0.46 ± 0.03	0.56 ± 0.05
CIS	0.42 ± 0.11	5.27 ± 0.76 <sup>a</sup>	0.48 ± 0.11	3.52 ± 0.45 <sup>a</sup>	0.45 ± 0.12	1.79 ± 0.62 <sup>a</sup>
ATRA	0.43 ± 0.12	3.11 ± 0.60 <sup>ab</sup>	0.43 ± 0.01	2.35 ± 0.64 <sup>ab</sup>	0.43 ± 0.10	1.44 ± 0.44 <sup>a</sup>
ADMSCs	0.44 ± 0.14	2.71 ± 0.55 <sup>abc</sup>	0.42 ± 0.12	1.74 ± 0.34 <sup>ab</sup>	0.42 ± 0.11	1.15 ± 0.21 <sup>abc</sup>
ATRA + ADMSCs	0.43 ± 0.10	2.23 ± 0.41 <sup>abc</sup>	0.43 ± 0.12	1.52 ± 0.55 <sup>abcd</sup>	0.41 ± 0.09	0.84 ± 0.05 <sup>b</sup>
<b>MAU (mg/dl)</b>						
Control	1.06 ± 0.16	1.02 ± 0.11	1.09 ± 0.22	1.03 ± 0.22	1.03 ± 0.2	0.96 ± 0.15
CIS	1.09 ± 0.28	9.75 ± 1.60 <sup>a</sup>	1.02 ± 0.38	5.41 ± 1.09 <sup>a</sup>	0.92 ± 0.2	2.83 ± 0.75 <sup>a</sup>
ATRA	0.92 ± 0.2	5.78 ± 0.77 <sup>ab</sup>	0.92 ± 0.18	4.13 ± 0.55 <sup>ab</sup>	0.92 ± 0.18	2.15 ± 0.41 <sup>ad</sup>
ADMSCs	0.95 ± 0.1	4.38 ± 0.73 <sup>abc</sup>	0.93 ± 0.17	3.21 ± 0.57 <sup>ab</sup>	0.93 ± 0.17	1.78 ± 0.62 <sup>ab</sup>
ATRA + ADMSCs	0.93 ± 0.16	3.19 ± 0.43 <sup>abcd</sup>	0.94 ± 0.12	2.53 ± 0.58 <sup>abcd</sup>	0.89 ± 0.17	1.33 ± 0.44 <sup>b</sup>
<b>CrCl (ml/min)</b>						
Control	1.64 ± 0.51	1.69 ± 0.43	1.59 ± 0.31	1.65 ± 0.52	1.67 ± 0.25	1.72 ± 0.61
CIS	1.48 ± 0.58	0.21 ± 0.04 <sup>d</sup>	1.32 ± 0.28	0.48 ± 0.08 <sup>a</sup>	1.56 ± 0.75	0.63 ± 0.09 <sup>a</sup>
ATRA	1.64 ± 0.49	0.53 ± 0.04 <sup>ab</sup>	1.63 ± 0.51	0.71 ± 0.11 <sup>ab</sup>	1.89 ± 0.66	0.91 ± 0.15 <sup>ab</sup>
ADMSCs	1.75 ± 0.51	0.67 ± 0.14 <sup>abc</sup>	1.97 ± 0.48	0.82 ± 0.16 <sup>abc</sup>	2.04 ± 0.58	1.04 ± 0.11 <sup>abc</sup>
ATRA + ADMSCs	1.81 ± 0.71	0.98 ± 0.009 <sup>abcd</sup>	1.99 ± 0.92	1.24 ± 0.23 <sup>abcd</sup>	2.12 ± 0.77	1.58 ± 0.37 <sup>bcd</sup>

Data are expressed as the means ± SD of thirty rats per group. Significant difference from the <sup>a</sup>control group, <sup>b</sup>cisplatin group, <sup>c</sup>retinoic acid group, and <sup>d</sup>stem cell group using one-way analysis of variance (ANOVA) followed by the post hoc test for multiple comparison (Scheffé test) at ( $P \leq 0.05$ ).

are shown in Table 2. The mean basal SCr, MAU, and CrCl were compared among the different groups. SCr and MAU were significantly increased in the CIS group compared with the control group ( $P \leq 0.05$ ) at 3, 7, and 11 days. CrCl showed a significant decrease in the CIS group compared with the control group. Groups treated with ADMSCs alone or ADMSCs pretreated with ATRA had a significant decrease in SCr and MAU levels and a significant rise in CrCl compared to the CIS group ( $P \leq 0.05$ ) at different endpoints.

### 3.4. Oxidative stress and antioxidants parameters

As shown in Table 3, CIS increased the renal NO compared to the control group. The administration of ADMSCs alone or pretreated with ATRA to CIS rats reduced the renal NO concentrations compared to the CIS group. SOD activity was significantly decreased in CIS group compared with the control group ( $P \leq 0.05$ ). ADMSCs significantly increased renal SOD levels. ADMSCs pretreated with ATRA showed the most increase in SOD compared to the control group ( $P \leq 0.05$ ) at the three times intervals.

### 3.5. Effects of ADMSCs on renal TGFβ1, IL-6, VEGF, HIF1α, and Caspase-3 expression

The inflammatory marker TGFβ1 and IL-6, angiogenesis markers VEGF and HIF1α, and apoptotic marker Caspase-3 revealed a significant up-regulation in CIS group compared to the control group. The ADMSCs alone or pretreated with ATRA groups showed a significant down-regulation in TGFβ1, IL-6 and caspase-3 compared to the CIS group, and they showed a significant up-regulation in HIF1α and VEGF compared to the CIS group at all-time intervals (Fig. 4).

### 3.6. Histopathological examination

Injury of the kidney tubules was quantified using a scoring system, the effects of retinoic acid, ADMSCs, and a combination of both on the tubulointerstitial damage score at different time intervals (Fig. 5A). The normal group presented normal kidney architecture (Fig. 5B). In the cisplatin-induced injury group, apoptosis, dilated irregular tubules, and interstitial inflammatory infiltrate were detected at the different time intervals (Fig. 5C, D).

**Table 3**  
Effects of ADMSCs pretreated with ATRA on the kidney nitric oxide (NO) ( $\mu\text{mol/l}$ ) level and super oxide dismutase (SOD) concentration.

Groups	3 d	7 d	11 d
<b>NO (<math>\mu\text{mol/l}</math>)</b>			
Control	10.21 $\pm$ 0.1	10.27 $\pm$ 0.15	10.2 $\pm$ 0.2
CIS	55.38 $\pm$ 1.1 <sup>a</sup>	51.98 $\pm$ 1.14 <sup>a</sup>	43.94 $\pm$ 2.35 <sup>a</sup>
ATRA	40.7 $\pm$ 1.007 <sup>ab</sup>	33.7 $\pm$ 1.45 <sup>ab</sup>	31.7 $\pm$ 1.18 <sup>ab</sup>
ADMSCs	28.5 $\pm$ 1.81 <sup>abc</sup>	24.5 $\pm$ 1.6 <sup>abc</sup>	20.1 $\pm$ 1.12 <sup>abc</sup>
ATRA + ADMSCs	21.26 $\pm$ 0.6 <sup>abcd</sup>	17.7 $\pm$ 1.12 <sup>abcd</sup>	14.11 $\pm$ 0.47 <sup>abcd</sup>
<b>SOD (U/gm tissue)</b>			
Control	5.46 $\pm$ 0.5	5.48 $\pm$ 0.24	5.53 $\pm$ 0.2
CIS	1.17 $\pm$ 0.18 <sup>a</sup>	1.2 $\pm$ 0.11 <sup>a</sup>	1.4 $\pm$ 0.08 <sup>a</sup>
ATRA	1.98 $\pm$ 0.1 <sup>ab</sup>	2.27 $\pm$ 0.17 <sup>ab</sup>	2.6 $\pm$ 0.15 <sup>ab</sup>
ADMSCs	2.5 $\pm$ 0.13 <sup>ab</sup>	3.00 $\pm$ 0.13 <sup>abc</sup>	4.6 $\pm$ 0.13 <sup>abc</sup>
ATRA + ADMSCs	3.4 $\pm$ 0.07 <sup>abcd</sup>	3.9 $\pm$ 0.1 <sup>abcd</sup>	4.71 $\pm$ 0.15 <sup>abc</sup>

Data are expressed as the means  $\pm$  SD of thirty rats per group. Significant difference from the <sup>a</sup>control group, <sup>b</sup>cisplatin group, <sup>c</sup>retinoic acid group, and <sup>d</sup>stem cell group using one-way analysis of variance (ANOVA) followed by the post hoc test for multiple comparison (Scheffé test) at ( $P \leq 0.05$ ).

However, when the rats were treated with ATRA (Fig. 5E) and/or ADMSCs (Fig. 5F), less inflammation with prominent nuclei compared to the CIS group was observed. At the same time, the ADMSCs pretreated with the ATRA group showed the lowest inflammation and little apoptosis with prominent nuclei and mitotic figures compared to the CIS group (Fig. 5G).

3.7. Immunohistochemical observation

The caspase-3 immunopositivity was presented in the renal tubules in different treated groups at different times (Fig. 6A). The immunohistochemistry staining revealed a negative caspase-3 expression in the control group (Fig. 6B), while the CIS group exposed a significant increase in caspase-3 compared to the control group (Fig. 6C). The ATRA and ADMSC groups indicated a significant reduction in caspase-3 (Fig. 6D, E), with maximum significant attenuation in ADMSCs pretreated with ATRA group compared with the CIS group (Fig. 6F).

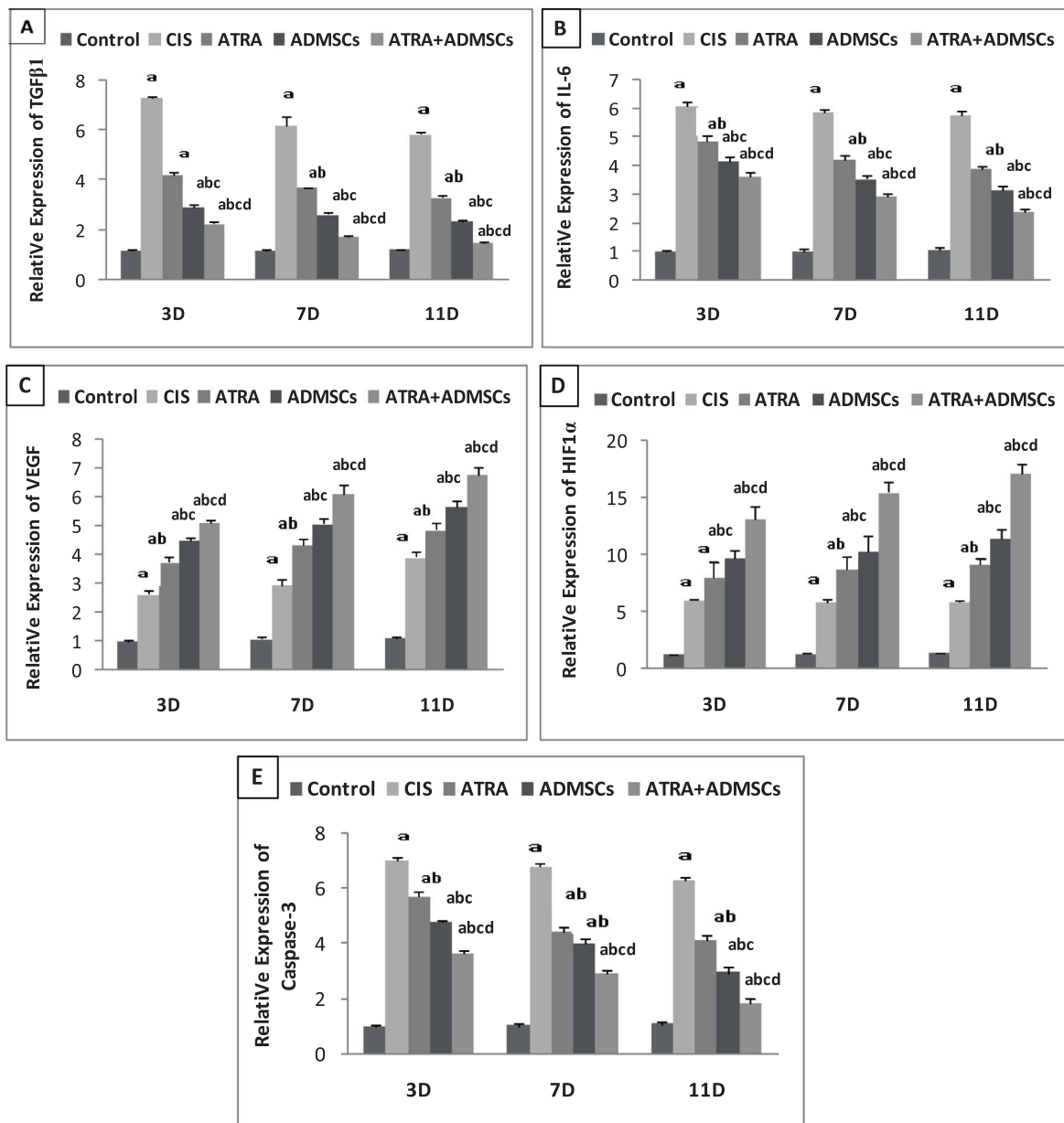
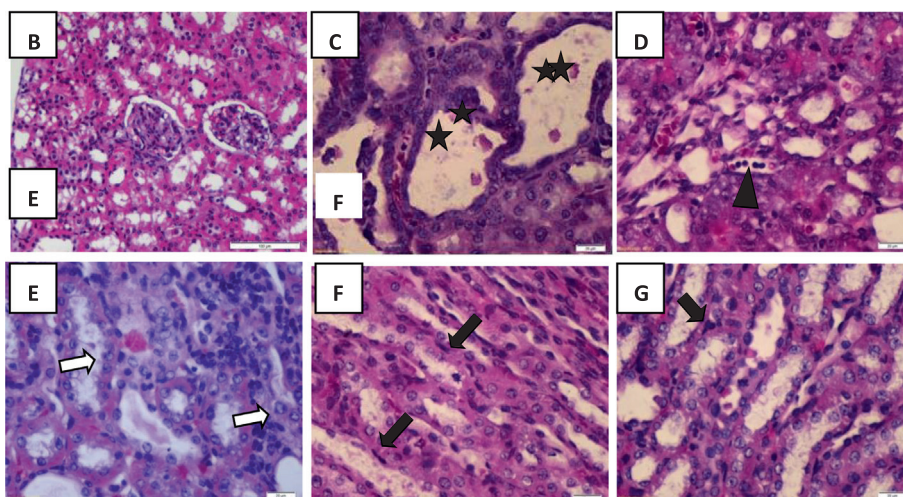
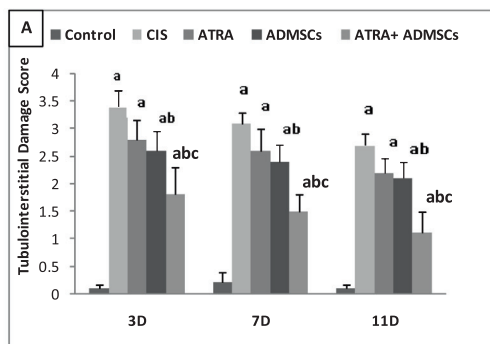


Fig. 4. Effect of ADMSCs pretreated with ATRA on a) TGFβ1, b) IL-6, c) VEGF, d) HIF1α, and e) Caspase-3 gene expression.



**Fig. 5. A ~ G:** Histological changes of the outer medulla in the rat kidney after 3 days of cisplatin injection showing; a) Effects of retinoic acid, ADMSCs, and the combination of both on the tubulointerstitial damage score at different time intervals in rat kidneys B) the control group with normal kidney structure of the outer medulla, C) the cisplatin group with apoptosis, dilated irregular tubules (star), loss of brush borders, and casts in the medulla at 3 days, D) the CIS group with interstitial inflammatory infiltrate (arrow head) in the medulla at 3 days, E) the ATRA group with marked prominent nucleoli (white arrow) in the medulla at 3 days, F) the ADMSCs group with mitotic figures (bold arrow) in the medulla at 3 days, and G) the ADMSCs pretreated with ATRA group with mitotic figures (bold arrow) in the medulla at 3 days. HE reduced from X 400.

### 3.8. Immunofluorescent examination of CD31

The immunofluorescent score is shown in Fig. 7A, and the CD31 staining of the cytoplasmic membranes for endothelial cells of the peritubular capillaries showed no significant change between the cisplatin and control groups (Fig. 7B, C), while the treated groups with either ATRA (Fig. 7D) or ADMSCs (Fig. 7E) caused a significant rise in CD31 compared with CIS group, with the maximum significant increase in ADMSCs pretreated with ATRA group (Fig. 7F).

## 4. Discussion

Recently, therapeutic applications of stem cells have arisen as an attractive approach for supporting therapy for numerous diseases. However, implantation of stem cells has talented therapeutic application for the treatment of renal syndromes; it has not reached achievements of drug delivery. Thus, approaches of pretreatment are needed to rise stem cells efficacy before transplantation (Yu et al., 2013). The aim of this study was to evaluate the effect of ATRA on the proliferation and survival of ADMSCs and to study the effects on the improvement of their activity in acute kidney injury induced by cisplatin.

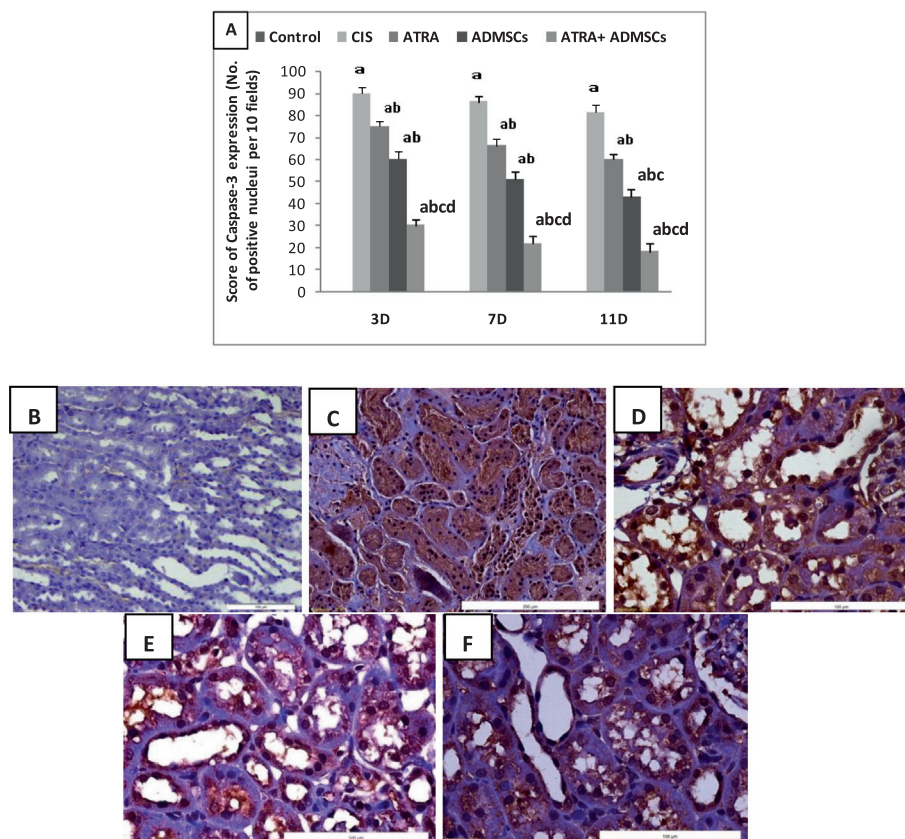
ATRA is an important signaling molecule, as it regulates many transcription factors. Many studies stated that ATRA plays a role to protect kidney against renal syndromes (Li et al., 2013). In many

experimental types of the kidney syndrome, ATRA was exposed to protect against renal damage. Research showed that rats with experimental mesangioproliferative glomerulonephritis treated with ATRA demonstrated inhibition of albuminuria (Lazzeri et al., 2014).

In our study, CIS caused damage to the kidneys, which was proven by the levels of serum creatinine and microalbuminuria, which markedly increased following CIS injection, and creatinine clearance markedly reduced compared to the control group. This is due to direct toxicity made by ROS after cisplatin injection as previously reported (Elhousseini et al., 2016).

For the rats treated with ATRA and treated with ADMSCs, the level of serum creatinine and microalbuminuria significantly decreased compared with CIS group. Similarly, creatinine clearance significantly increased in contrast to that in the CIS group. The ADMSCs treated with ATRA group showed stronger decreases in the serum creatinine and microalbuminuria and increases in the creatinine clearance almost to the normal level and pretreatment of ADMSCs with ATRA was capable of ameliorating renal dysfunction by increasing the paracrine effect of the ADMSCs (Pourjafar et al., 2017).

Cisplatin produces reactive oxygen species (ROS) by causing mitochondrial dysfunction. ROS in renal cells reduce the antioxidant enzyme activity and intracellular concentrations of antioxidants (Ozkok & Edelstein, 2014). CIS increased NO concentration while decreased SOD activity compared to control, which



**Fig. 6. A ~ F:** Immunohistochemical staining of caspase-3 in rat kidneys (magnification, 200 X) in different groups, a) Effects of retinoic acid, ADMSCs, and the combination of both on the expression of caspase-3 at different time intervals, (B) negative expression for caspase-3 of kidney sections in the control group at 11 days, (C) marked expression (81 positive nuclei in 10 randomly chosen field at 200 X) in the CIS group at 11 days, (D) moderate expression (60 positive nuclei in 10 randomly chosen field at 200 X) in the ATRA group at 11 days, (E) moderate expression (43 positive nuclei in 10 randomly chosen fields at 200 X) at 11 days in the ADMSCs group, and (F) mild expression (18 positive nuclei in 10 randomly chosen field at 200 X in the ADMSCs pretreated with ATRA group at 11 days.

was discussed in the previous studies (Gad et al., 2017; Kara et al., 2019). ADMSCs administration reduced AKI by suppressing oxidative stress (Kinnaird et al., 2004). ATRA was also reported to have a free radical scavenger effect (Shi et al., 2017), and that explained our results, which showed that the pretreatment of ADMSCs with ATRA improved SOD activity and decreased NO concentration compared to ADMSCs alone.

Recently, studies stated that ATRA has an anti-inflammatory effect, and this effect was exposed in a mouse model of lupus nephritis and in anti-glomerular basement membrane glomerulonephritis in rats (Lazzeri et al., 2014). Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) induces the release of fibroblasts and inflammatory cells into the injury part (Elsherbiny et al., 2016).

Our results showed that CIS caused elevation in the TGF $\beta$ 1 and IL-6 expression, and this was in agreement with previous studies (Elseweidy et al., 2018; Aboul-Naga et al., 2020). The ADMSCs injection pretreated with ATRA to rats treated with CIS reduced the expression of TGF $\beta$ 1, IL-6, and this was compatible with Li et al. (2013).

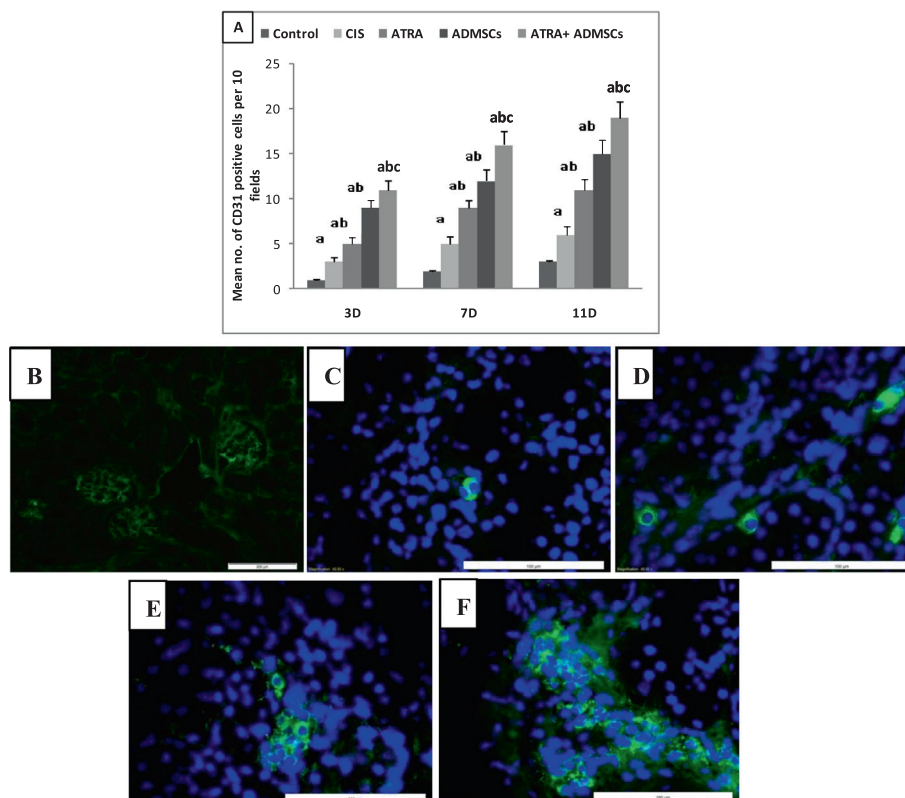
HIF-1 has a particular role in regulating oxygen homeostasis, mitochondrial respiration, and protecting the kidneys against CIS-induced tubular cell apoptosis, and any interference with it leads to increased ROS levels and apoptosis. The protein expression of HIF-1 $\alpha$  in their CIS group was significantly increased when compared with the control group (Liu et al., 2015).

CIS with ADMSCs group presented significantly increase in HIF-1 $\alpha$  expression. Hussein et al. (2016) established that ADMSCs protect the kidneys against ischemic-reperfusion damage by endoplasmic reticulum stresses and oxidative reduction of the

HIF-1  $\alpha$  activation. The expression became more marked in the CIS treated with both ATRA and ADMSCs group, which was in line with Fernandez Martinez et al. (2013), who established that ATRA induced HIF-1 $\alpha$  expression in human renal proximal tubular (HK2) cells and Pourjafar et al. (2016), who presented that ATRA improved HIF-1 mRNA level compared with untreated ADMSCs.

Kidney damage, including the infiltration of leukocytes, tubules dilation, and degenerative changes in the epithelium of the tubule lumens, was shown after CIS therapy by microscopic examination. These results were compatible with Elhusseini et al. (2016) findings (Elhusseini et al., 2016). Treatment with ADMSCs prior to CIS reversed oxidative damage, inflammation, and apoptosis in kidneys and, consequently, regenerated kidney cells, improved the structure, and prevented acute kidney injury and histopathological damage starting from day 3, which was compatible with Sakr et al. (2017). ATRA treatment was found to suppress interstitial mononuclear cell infiltration, and ATRA treatment for puromycin aminonucleoside nephrosis decreased proteinuria and reduced leukocyte infiltration (Lazzeri et al., 2014).

Previous studies indicated that CIS led, principally, to the apoptosis of renal tubular cells (Iwayama and Ueda, 2013). According to the gene and protein expression of caspase-3, we realized the ability of pretreated ADMSCs with ATRA to reduce the activity of caspase-3 and, thus, to reduce apoptosis. This is in agreement with Pourjafar et al. (2017), who documented that caspase-3 significantly inhibited in ATRA-treated ADMSCs so ATRA has ability to rise the viability of stem cells and stimulate the ADMSCs proliferation by reducing caspase-3 activity (Pourjafar et al., 2017).



**Fig. 7. A ~ F:** Immunofluorescent staining of CD31, (a) Effects of retinoic acid, ADMSCs, and the combination of both on the expression of CD31 at different time intervals and kidney specimens, (B) low expression for CD31 of kidney sections in the control group at 11 days (two positive nuclei in 10 randomly chosen fields at 200 X), (C) minimal expression (six positive nuclei in 10 randomly chosen fields at 200 X) in the cisplatin group at 11 days, (D) mild expression (11 positive nuclei in 10 randomly chosen fields at 200 X) in the retinoic acid group at 11 days, (E) moderate expression (average 15 positive nuclei in 10 randomly chosen fields at 200 X) at 11 days in the MSCs group, and (F) marked expression (19 positive nuclei in 10 randomly chosen field at 200 X in the ADMSCs pretreated with ATRA group at 11 days).

The role of angiogenesis potentiality as a cytoprotective effect of stem cell therapy was discussed in several studies. Vascular endothelial growth factor (VEGF) involved in vasculogenesis and angiogenesis, while CD31 is an endothelial cell marker that is upregulated during renal injury (Awadalla et al., 2021). This study found that the VEGF gene and CD31 protein increased in the CIS group. Also has shown that ATRA has high effects on vasculogenesis and angiogenesis.

ADMSCs pretreated with ATRA caused the CD31 overexpression in the endothelial cells of blood vessels of kidney tissues compared to ADMSC alone, and this may be due to the vascular inflammation and leakage of leukocytes from the blood vessels into the tissues, which was in accordance with El-Beltagy and Elghawet (2016), who documented that endothelial cells have an important role in tissue repair, inflammation, and angiogenesis.

One of our study limitations is in understanding the mechanism of ATRA in increasing ADMSCs' efficacy in leading to the improvement of the kidney structure after CIS therapy. ATRA may act as an inducer for several chemokine receptors and angiogenic factors. Further studies are necessary to confirm the role of preconditioning ADMSCs with ATRA in the nephrotoxicity area.

## 5. Conclusion

The current study revealed the effect of ATRA on the viability and proliferation of ADMSCs. Moreover, the renoprotective potentiality of ADMSCs was enhanced by the pretreatment with ATRA. This improvement was detected in restoring the antioxidant and free radical balance, decreasing the inflammation and the apoptosis induced by cisplatin. Therefore, ADMSCs- ATRA pretreatment

before transplantation could be essential for the development of clinically applicable interventions.

## Declaration of Competing 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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