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Paracrine action of human placental trophoblast cells attenuates cisplatin-induced acute kidney injury

Pengfei Liu[a,b], Yetong Feng[c], Xin Chen[d], Guangsuo Wang[e], Imran Nawaz[e,f], Lifu Hu[f], Lei Zhao[a,*]

[a] Department of Anesthesiology, The 2nd Clinical medical College (Shenzhen People's Hospital) of Jinan University, The 1st Affiliated Hospitals of Southern University of Science and Technology, Shenzhen 518020, China
[b] Integrated Chinese and Western Medicine Postdoctoral research station, Jinan University, Guangzhou 510632, China
[c] Department of Medicine, College of Medicine, University of Arizona, Tucson, AZ 85721, USA
[d] Department of Laboratory Medicine, The 2nd Clinical medical College (Shenzhen People's Hospital) of Jinan University, The 1st Affiliated Hospitals of Southern University of Science and Technology, Shenzhen 518020, China
[e] Department of Thoracic Surgery, The 2nd Clinical medical College (Shenzhen People's Hospital) of Jinan University, The 1st Affiliated Hospitals of Southern University of Science and Technology, Shenzhen 518020, China
[f] Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm 17177, Sweden

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ABSTRACT

Aims: The action of cell-based therapy against acute kidney injury (AKI) has been demonstrated by different groups for years. However, which kind of cells hold best therapeutic effect remains unclear. In this study, we mainly explored whether human placental trophoblast cells hold the potential to be applied in AKI therapy.

Main methods: To study the renoprotective effect, the trophoblast cells were isolated from human placenta and characterized by flow cytometry first. The AKI model was induced using cisplatin in NOD-SCID mice. The therapeutic effect of human placental trophoblast cells on renal function, apoptosis and inflammation were analyzed respectively.

Key findings: The administration of trophoblast cells isolated from human placenta improved the pathological changes of kidney tissues and renal dysfunction induced by cisplatin. In addition, the placental trophoblast cell-based treatment also showed anti-apoptotic effect and decreased the level of apoptotic genes (Bax and Caspase 3) expression in damaged kidney tissues obviously. All of the inflammatory components (MCP-1, IL-10 and RANTES) in kidney tissues were down-regulated with the therapy of placental trophoblast cells. Further analysis indicated that the paracrine effects of human placental trophoblast cells may hold a key position in the AKI therapy process.

Significance: In this study, we mainly developed a novel therapeutic strategy to treat cisplatin-induced AKI with human placental trophoblast cells. Even though the detailed mechanism and the optimizations of this cell-based therapy still need further investigation, the application of placental trophoblast cell holds special potential in the treatment of patients with AKI.

1. Introduction

Acute kidney injury (AKI) is caused by a variety of factors, such as drugs, hypoxia and inflammation. This disease has been considered as one of the most frequent clinical syndrome in recent years. AKI is characterized by a sudden loss of the renal function, which remains a substantial problem all over the world [1,2]. Even though remarkable progress has been made in AKI diagnosis and therapy (e.g. dialysis and renal replacement), the high morbidity and mortality of patients with AKI have not significantly been improved so far. At present, the overall mortality rates of AKI patients range between 3 and 5%, and the patients treated at the ICU hold a much higher risk (30–50%) [3]. Therefore, a novel and effective therapy strategy is necessary for the treatment of patients with AKI.

In recent years, cell-based therapy mode has been applied in kinds of diseases treatment in the clinic gradually. In the respect, the most attractive area is stem cell-based treatment [4,5]. For example, our previous studies have indicated that both bone marrow-derived mesenchymal stem cells and umbilical cord-derived mesenchymal stem cells could show renoprotective effect in gentamicin-induced AKI model [6–9]. In addition, some groups also developed other kinds of cell-based treatment, such as hematopoietic stem cells [10], spermatogonial stem

* Corresponding author.
E-mail address: zhao.lei@szhospital.com (L. Zhao).

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cells [11], even embryonic stem cells [12] and induced pluripotent stem cells [13]. The main mechanisms of stem cell-based therapy against AKI include differentiation-dependent mechanism and differentiation-independent mechanism. In detail, stem cells are able to localize to renal compartments and further contribute to kidney regeneration though cell differentiation directly (differentiation-dependent mechanism). Besides, the cells also hold the ability to secrete various of cell factors, which are renoprotective in AKI model (differentiation-independent mechanism). However, the position of differentiation-dependent mechanism in stem cell-based therapy against AKI is still in debate. Because some researchers found that the percentage of cells inside the tubular epithelium was < 5–8% of the total engrafted cells, and the renoprotective effect should be attributable to the paracrine effects of infused cells [14,15]. Therefore, the differentiation-independent mechanism may hold major action during the process of cell-based therapy against AKI, and the cell factors secreted by the infused cells exert anti-inflammatory and anti-apoptotic effects on damaged kidney tissue. This differentiation-independent mechanism is also approved by most scientists [7,14,16,17], indicating the key position of paracrine effects in cell-based therapy in AKI.

The placenta, a transient organ, forms during pregnancy, which supports the development of the fetus. During human placental development, placental trophoblast cells, which are located in the outermost layer of the maternal-fetal barrier, secrete lots of hormones and cell factors, and hold a significant position in the progress of embryonic development, so the dysfunction of placental trophoblast cells is related to kinds of pregnancy diseases [18,19]. More importantly, several factors secreted by placental trophoblast cells have been demonstrated to hold renoprotective effect in different AKI models, such as relaxin 1 (RLN1) [20], parathyroid hormone-related protein (PTHLH) [21], and epidermal growth factor (EGF) [22]. However, whether human placental trophoblast cells could be applied in AKI treatment directly remains unclear so far.

In this study, primary trophoblast cells were isolated from human placenta, and the renoprotective effect of human placental trophoblast cells on cisplatin-induced AKI was evaluated in NOD-SCID mice. Our results indicated that placental trophoblast cells-based therapy was a feasible strategy to repair kidney tissues in the mouse model of AKI via anti-inflammatory and anti-apoptosis effect. In addition, further analysis showed that the paracrine effects hold an important role in the cell-based therapy against AKI.

2. Materials and methods

This study has been approved by the Committee on the Ethics of Animal Experiments and Human Subject Research of The 2nd Clinical medical College (Shenzhen People's Hospital) of Jinan University. The work described was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments. In addition, all animal experiments was carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.1. Isolation of human placental trophoblast cells and cell culture

The isolation of human placental trophoblast cells was performed as the references [23, 24]. In brief, human term placenta was harvested from caesarean deliveries. Then, the villous tissue was separated from connective tissue and blood vessels. After rinsed for 3 times with PBS, the villous tissue was cut into small pieces, and digested using 0.25% trypsin plus 0.1 mg/ml DNase I at 37 °C for 20 min. The cell suspension was collected and centrifuged at 350*g* for 10 min. The cell pellets were resuspended and layered on the top of a preformed Percoll gradient (65%, 55%, 50%, 45%, 35%, 30% and 25%), then centrifuged at 730*g* for 30 min at 4 °C. The trophoblast cells was collected between the 45% and 35% Percoll solution. Finally, the cells were washed with PBS, and plated on a Matrigel-coated culture surface, and cultured using DMEM/F12 (Gibco) supplemented with 10% FBS (Gibco), 100 U/ml penicillin and 0.1 g/ml streptomycin (Sigma) in a humidified 37 °C incubator with 5% CO_2. The media were changed every 2 days.

Human renal proximal tubular cell line, HK-2, was purchased from the American Type Culture Collection (ATCC). The cells were cultured using DMEM (Gibco) supplemented with 10% FBS (Gibco), 100 U/ml penicillin and 0.1 g/ml streptomycin (Sigma) in a humidified 37 °C incubator with 5% CO_2. In the cell viability assay, the cells were exposed to cisplatin (20 μM) for 12 or 24 h in the presence or absence of different placental trophoblast cell-based conditioned media.

2.2. Flow cytometry

Human placental trophoblast cells were dissociated into single cells using 0.25% trypsin. The cells were further fixed with Fixation Buffer (BD Biosciences) and permeated with Perm/Wash Buffer (BD Biosciences). Then, the cells were prepared at a concentration of 1.0×10^5 cells in 0.1 ml of PBS. The antibodies, including VIMENTIN conjugated to FITC (Abcam), CK-7 conjugated to PE (LSBio), CD31 conjugated to PE (BioLegend), CD45 conjugated to APC (BD Biosciences), and CD163 conjugated to FITC (BioLegend), were added and incubated at 4 °C for 30 min. After three washes in PBS, the cells were collected and analyzed using FACScalibur (BD Bioscience).

2.3. Cell proliferation assay

To evaluate cell proliferation ability in different group, the proliferation index was measured with CCK-8 method (Dojindo) as the references [6, 25, 26]. In brief, 20 μl of CCK-8 solution was added into different wells, which contained 200 μl medium, and incubated for 4 h at 37 °C. The Abs of different groups at 450 nm were detected respectively ($n = 3$). The wells which only contained medium were used as the blank group. The proliferation index = Ab of experimental group-Ab of blank group, was applied to evaluated cell proliferation ability.

2.4. Preparation and treatment of AKI model

In this study, NOD-SCID mice (Beijing Vital River Laboratory Animal Technology) were used in the establishment of AKI model to avoid xenogenic immune rejection. Eight-week-old male NOD-SCID mice were randomly divided into 3 groups and treated as follows (n = 6 in each group): (1) Control group: normal healthy mice; (2) Cisplatin group: the mice were given a single intraperitoneal injection of cisplatin (20 mg/kg) to induce nephrotoxicity; (3) cisplatin + trophoblast cells group: the mice were given a single intraperitoneal injection of cisplatin (20 mg/kg), and after 24 h, human placental trophoblast cells were administered by a single intravenous injection of 5×10^6 cells/kg. All of animals were sacrificed at 96 h after cisplatin injection. Urine, blood, and tissue samples were collected respectively to evaluate the renal function and tissue damage in each group. In our study, blood was collected from the retrobulbar venous plexus and heparin was used as anticoagulant. After centrifugation, the plasma was collected for the following detection. In addition, urine samples of each group were collected using metabolic cages, and further used to evaluate renal function.

2.5. Evaluation of kidney function

Blood and Urine samples were collected from mice in each groups. Blood urea nitrogen (BUN) and serum creatinine (Scr) was assessed by BUN Colorimetric Detection Kit and Creatinine Colorimetric Detection Kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. In addition, the activity of *N*-acetyl-beta-D-glucosaminidase (NAG) and lysozyme (LZM) in urine were also detected using NAG Detection Kit and LZM Detection Kit (Nanjing

Jiancheng Bioengineering Institute) according to the manufacturer's instructions respectively.

To evaluate the effect of human placental trophoblast cells on the enlargement and accrementition of renal tissue damage induced by cisplatin, the percentage of bilateral kidney weight/body weight (kidney/body weight (%)) was measured in each group.

For histological and immunohistochemical assay, the kidney tissue in each group were fixed with 4% formalin, embedded using paraffin, and sectioned to 5 mm-thick slice. The tissue slices were further processed by hematoxylin and eosin (H&E) staining and immunostaining as our previous studies [8,9]. The tubular injury score in each group was calculated as the reference [2].

2.6. Cell apoptosis assay

The expression levels of apoptotic genes (Bax and Caspase 3) in the kidney tissues were detected using real-time qPCR and immunohistochemistry respectively. The activated Caspase3 in kidney tissues was further detected using Caspase 3 colorimetric assay kit (KeyGEN) according to its manufacturer's instructions.

2.7. Detection of inflammatory components

In our study, the inflammatory components (MCP-1, IL-10 and RANTES) in the kidney tissues of each group were measured using RT-qPCR and ELISA. For the ELISA assay, kidney tissues (200 mg) in different groups were homogenized with PBS solution, and further centrifuged at 10000*g* for 20 min at 4 °C. Then the supernatant was used for ELISA as the manufacturer's instructions of MCP-1 colorimetric assay kit (eBioscience), IL-10 colorimetric assay kit (Abcam) and RANTES colorimetric assay kit (Abcam).

2.8. Real-time quantitative PCR (RT-qPCR)

40 mg of kidney tissues or 4×10^6 cells were homogenized using 1 mL of Trizol reagent (Invitrogen), and total RNA was extracted according to the manufacturer's instructions. Then cDNA was synthesized using 2 μg of mRNA and the Transcriptor first-strand cDNA synthesis kit (Promega). RT-qPCR was performed using a Thermal Cycler Dice TM Real-Time System and SYBR Green Premix EX TaqTM (Takara). The β-actin gene (ACTB) was used for RT-qPCR normalization and all experiments were performed in triplicate. Primer sequences are as follows:

Bax Forward 5′-AGACAGGGGCCTTTTTGCTAC-3′
Reverse 5′-AATTCGCCGGAGACACTCG-3′
Caspase 3 Forward 5′-CTCGCTCTGGTACGGATGTG-3′
Reverse 5′-TCCCATAAATGACCCCTTCATCA-3′
MCP1 Forward 5′-TTAAAAACCTGGATCGGAACCAA-3′
Reverse 5′-GCATTAGCTTCAGATTTACGGGT-3′
IL-10 Forward 5′-CTTACTGACTGGCATGAGGATCA-3′
Reverse 5′-GCAGCTCTAGGAGCATGTGG-3′
RANTES Forward 5′-GCTGCTTTGCCTACCTCTCC-3′
Reverse 5′-TCGAGTGACAAACACGACTGC-3′
RLN1 Forward 5′-CCTGGGGTTCTGGCTATTGC-3′
Reverse 5′-AATTCACGGGCATATTCACGG-3′
PTHLH Forward 5′-CATCAGCTACTGCATGACAAGG-3′
Reverse 5′-GGTGGTTTTTGGTGTTGGGAG-3′
EGF Forward 5′-AGCATCTCTCGGATTGACCCA-3′
Reverse 5′-CCTGTCCCGTTAAGGAAAACTCT-3′
VEGF Forward 5′-GCACATAGAGAGAATGAGCTTCC-3′
Reverse 5′-CTCCGCTCTGAACAAGGCT-3′
ACTB Forward 5′-CCCAGAGCAAGAGAGG-3′
Reverse 5′-GTCCAGACGCAGGATG-3′

2.9. Western blot

In this study, cells in different groups were harvested using RIPA lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% sodium deoxycholate, 1% TritonX-100 and 0.1% SDS), plus the phosphatase inhibitors (2 mM sodium orthovanadate) and the protease inhibitor (0.1 mg/ml aprotinin, 0.5 mg/ml leupeptin, and 0.6 mg/ml pepstatin A). The protein concentration was further determined using a BCA protein estimation kit (Pierce, USA). Equal amounts (10 μg) of protein were loaded per lane in a 12% acrylamide gel. The primary antibodies used were anti-Bax (1:3000; Abcam, ab53154), anti-Caspase 3 (1:3000; Abcam, ab13847) and anti-GAPDH (1:3000; Santa, sc-47724). Anti-mouse or rabbit HRP and an Amersham ECL kit (GE Healthcare, USA) were applied to detect the protein. Finally, the band densities in each group were quantified by densitometry (Quantity One v4.62).

2.10. Small interfering RNAs (siRNAs) transfection

To knockdown RLN1, PTHLH, EGF and VEGF, siRNAs for different genes (siRNA for RLN1: SI04168941; siRNA for PTHLH: SI03031854; siRNA for EGF: SI00030674; siRNA for VEGF: SI04130749) were purchased from Qiagen. The scrambled control siRNA from QIAGEN (1022076) were used in the control group. All of the siRNAs were transfected into human placental trophoblast cells using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. The effect of siRNA transfection was evaluated by RT-qPCR.

2.11. Evaluation of paracrine effects

When human placental trophoblast cells reach about 80% confluence, the cells were first transfected with different siRNAs. 48 h later, the medium was changed and the cell were cultured for another 24 h. Then, the medium (human placental trophoblast cell-based conditioned medium) was collected to evaluate paracrine effects using different ELISA kits. Human RLN1 ELISA Kit, Human EGF ELISA Kit and Human VEGF ELISA Kit were purchased from Abcam, and Human PTHLH ELISA Kit was purchased from KAMIYA BIOMEDICAL COMPANY. Human PTHLH was purchased from Sigma-Aldrich (SRP4651).

2.12. Statistical analysis

Results are presented as mean ± SD. Statistical analysis was performed using SPSS 17.0. Skewness and kurtosis were analyzed to assure normal distribution. If *Z*-score for both skewness and kurtosis was within the range of −1.96 to 1.96, the data were considered to follow normal distribution. Unpaired Student's *t*-tests were applied to compare the means of two groups. One-way ANOVA with Bonferroni's correction was used to compare the means of three or more groups. $p<0.05$ was considered statistically significant.

3. Results

3.1. Characterization of placental trophoblast cells

The trophoblast cells isolated from human placenta grew in pave stone-like structure (Fig. 1A). The cells were characterized using Flow cytometry, and the results indicated that human placental trophoblast cells used in our study were positive for CK-7 (95.44%) and nearly negative for VIMENTIN (0.89%), CD31 (0.66%), CD45 (0.53%), and CD163 (0.72%) (Fig. 1B–F). In addition, we also evaluated the proliferation ability of placental trophoblast cells in different passages (Passage 1 (P1), Passage 2 (P2) and Passage 3 (P3)). The results showed that the cells were hard to proliferate after P2 (Fig. 1G). Therefore, the cells in P1 were mainly collected for the following detection.

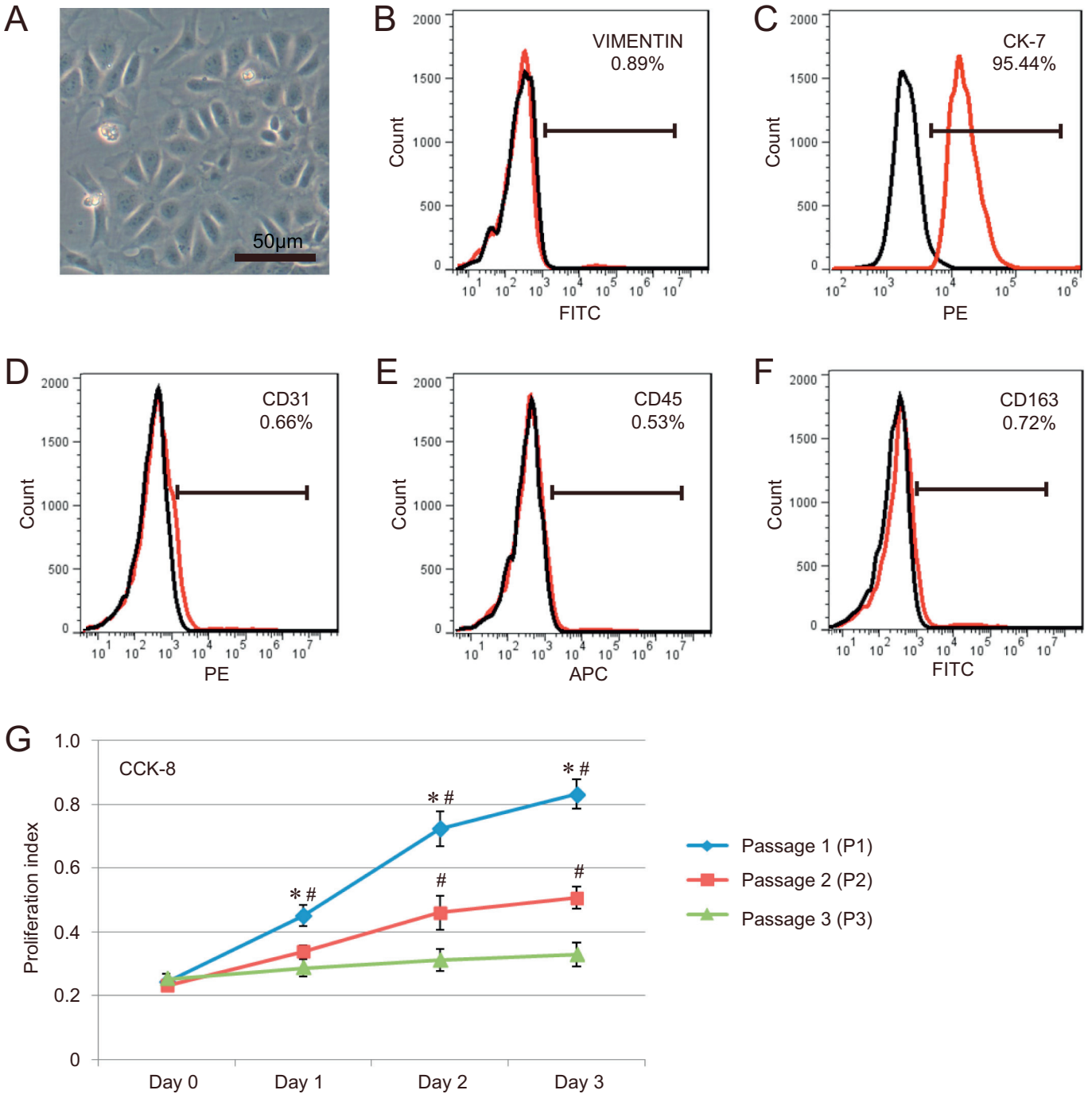


Fig. 1. Characterization of human placental trophoblast cells. A. The phase contrast of human placental trophoblast cells. B–F. Immunophenotype of isolated placental trophoblast cells. The isolated human placental trophoblast cells were characterized by flow cytometry. Herein, the human placental trophoblast cells were positive for CK-7, and nearly negative for VIMENTIN, CD31, CD45 and CD163. G. Proliferation index of human placental trophoblast cells in different passages. Results are expressed as mean ± SEM. *: $P < 0.05$ compared with P2. #: $P < 0.05$ compared with P3.

3.2. Therapeutic action of human placental trophoblast cells against AKI

To evaluate the therapeutic effect of human placental trophoblast cells in cisplatin-induced AKI, the pathological changes were observed via H&E staining in mouse kidney tubules, kidney glomeruli, and collecting tubules respectively. The results indicated that typical pathological changes were induced by cisplatin treatment, including the tubular dilatation, necrosis, and effusion in mouse kidney tubules and mouse collecting tubules, as well as the widening of Bowman's space in kidney glomeruli. The treatment of placental trophoblast cells showed obvious renoprotective effect and the treated group exhibited fewer dilated tubules, as well as less effusion in kidney tubules and collecting tubules, together with the reduced Bowman's space in kidney glomeruli (Fig. 2A–B).

In addition, the percentage of bilateral kidney weight/body weight (kidney/body weight (%)) was measured in each group. We found that the kidney/body weight (%) was below 2% in the Control group, whereas that of the Cisplatin group exceeded 3%. The administration of placental trophoblast cells could release the enlargement of mouse kidney and reduced the kidney/body weight (%) effectively (Fig. 2C).

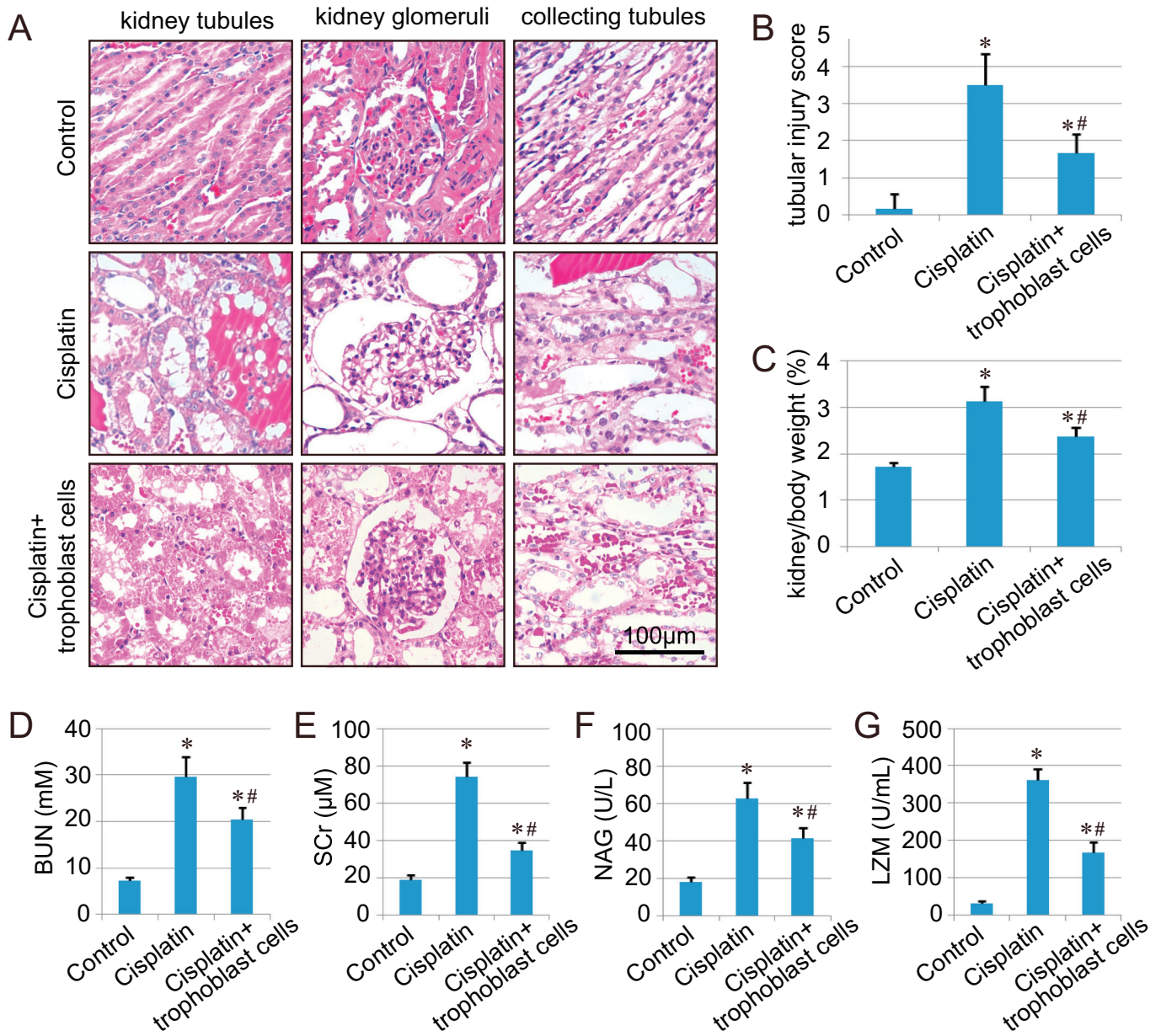


Fig. 2. Evaluation of human placental trophoblast cell-based therapy against cisplatin-induced AKI. A. Detection of pathological changes of kidney tissues in each group using HE staining. B. Histological score of each group. C. kidney/body weight (%) of each group. D–E. The level of BUN (D) and Scr (E) in blood. F–G. The activity of NAG (F) and LZM (G) in urine. Results are expressed as mean ± SEM. *: $P < 0.05$ compared with the Control group. #: $P < 0.05$ compared with the Cisplatin group.

Besides, the level of BUN and Scr in blood, and the activity of NAG and LZM in urine were detected to evaluate kidney function. The results showed that cisplatin affected kidney function and increased level of BUN and Scr in blood, and the activity of NAG and LZM in urine. The injection of human placental trophoblast cells significantly decreased BUN and Scr level as well as NAG and LZM activity, indicating the the therapeutic action of human placental trophoblast cells against AKI (Fig. 2D–G).

3.3. Anti-apoptotic effect of placental trophoblast cell-based treatment

In our study, the expression of different apoptotic genes (Caspase 3 and Bax) in the kidney tissues were first measured using RT-qPCR, and the results showed that the mRNA levels of both apoptotic genes were much higher in the cisplatin-induce AKI model group than those in the control group. The human placental trophoblast cell-based treatments were efficient to induce anti-apoptosis effect, and the treated group showed lower expression of both Bax and Caspase 3 compared with the Cisplatin group (Fig. 3A). In addition, the activated Caspase 3 in kidney tissues of different groups was also determined herein, and The result was similar to that of qPCR, indicating the anti-apoptosis effect of placental trophoblast cell-based treatments (Fig. 3B). The apoptotic gene expression in the kidney tubules, kidney glomeruli, and collecting tubules were further analyzed using immunohischemistry respectively. We found that cisplatin increased the expression of both Bax and Caspase 3 in kidney tubules and collecting tubules, but not kidney glomeruli, and administration of placental trophoblast cells decreased the expression of the apoptosis genes obviously, which was consistent with RT-qPCR and Caspase 3 activity assay results (Fig. 3C-3E).

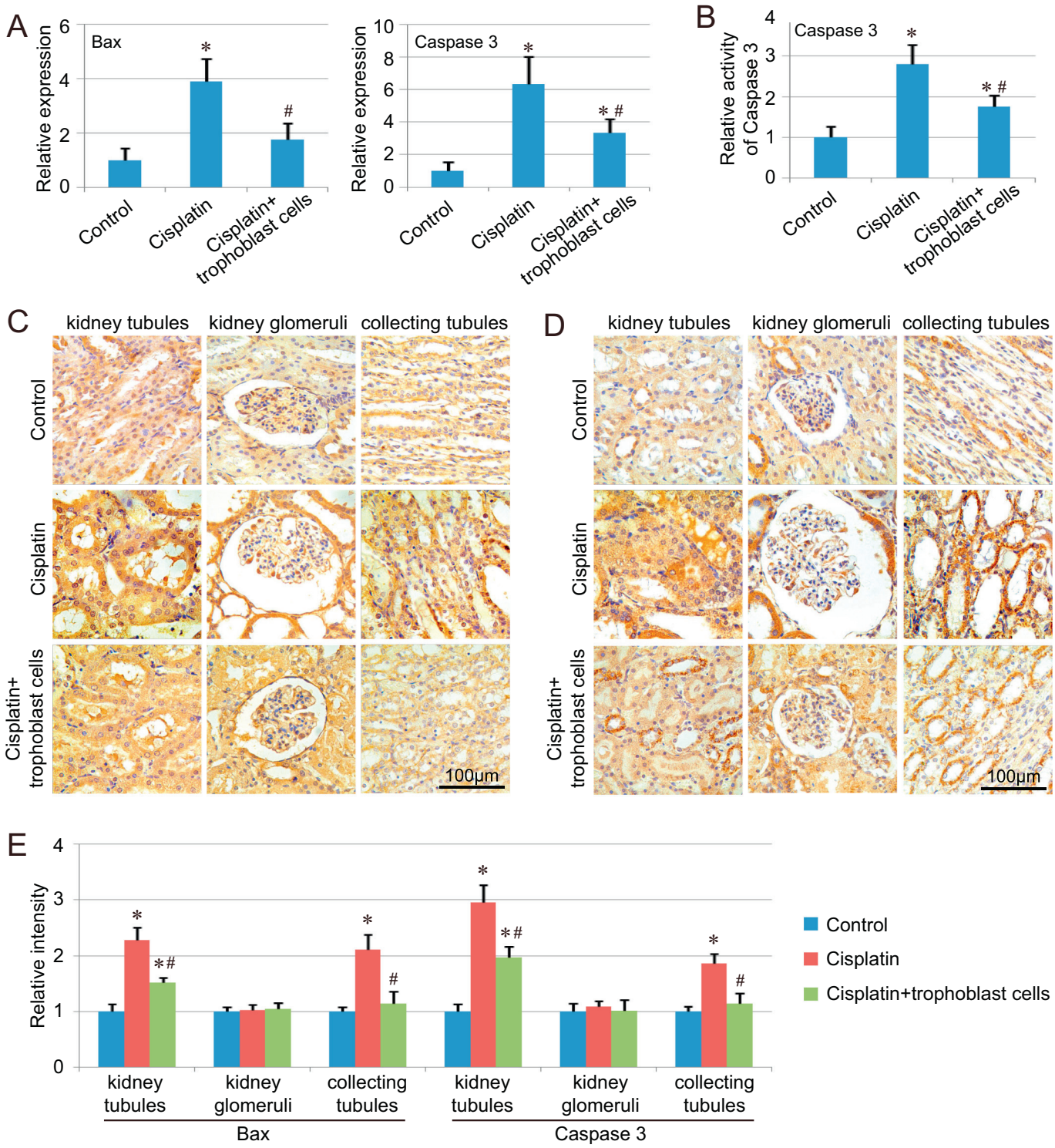


Fig. 3. Expression of apoptotic genes expression in the kidney tissues of each group. A. Detection of apoptotic genes (Bax and Caspase 3) expression using RT-qPCR. B. Evaluation of activated Caspase 3 in different groups. C-E. Detection of apoptotic genes (Bax and Caspase 3) expression using immunohistochemistry. The gene expression level or Caspase 3 activity in the Control group was considered as "1.0", and the relative gene expression level or Caspase 3 activity of other group was further evaluated. Results are expressed as mean ± SEM. *: $P < 0.05$ compared with the Control group. #: $P < 0.05$ compared with the Cisplatin group.

3.4. Inflammatory components assay

The relative level of inflammatory components (MCP-1, IL-10 and RANTES) in kidney tissues of were detected using RT-qPCR and ELISA respectively. All of the inflammatory components were up-regulated in the Cisplatin group, compared with the Control group. The administration of human placental trophoblast cell decreased the level of MCP-1, IL-10 and RANTES in kidney tissues significantly, suggesting the anti-inflammatory effect of human placental trophoblast cell-based treatment in the cisplatin-induce AKI model (Fig. 4A–B).

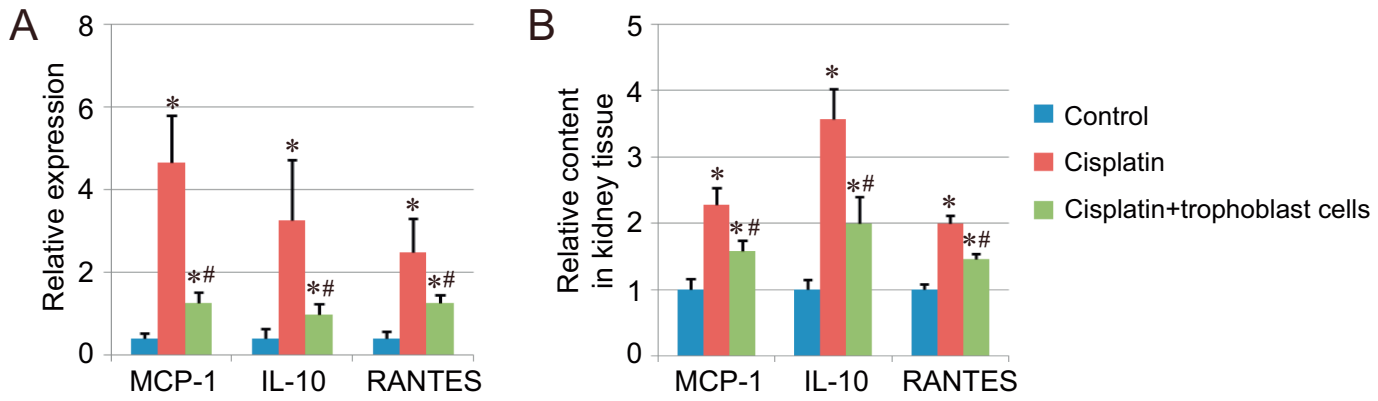


Fig. 4. Evaluation of inflammatory reaction in the kidney tissues of different group. A. Detection of different inflammatory components (MCP-1, IL-10 and RANTES) using RT-qPCR. B. Detection of different inflammatory components (MCP-1, IL-10 and RANTES) using ELISA. The level of different inflammatory components in the Control group was considered as "1.0", and the relative gene expression level of other group was further evaluated. Results are expressed as mean ± SEM. *: $P < 0.05$ compared with the Control group. #: $P < 0.05$ compared with the Cisplatin group.

3.5. Paracrine effects of human placental trophoblast cells in AKI treatment

The differentiation-independent mechanism in cell-based AKI treatment has been approved by most scientists, indicating the key position of paracrine effects. Therefore, the paracrine effects of human placental trophoblast cells were analyzed in vitro in our study. To induce the renal tubular cell injury model in vitro, human HK-2 cells were treated with cisplatin (20 μM). The placental trophoblast cell-based conditioned medium was harvested and applied in HK-2 cell culture at the same time. After treated with cisplatin for 12 or 24 h, the proliferation index of each group was detected, and the results showed that cisplatin treatment decreased the proliferation ability of HK-2 cells cultured using normal medium and conditioned medium. However, compared with the cells cultured with normal medium, placental trophoblast cell-based conditioned medium increased cell viability and protected HK-2 cells from cisplatin-induced damage (Fig. 5A). To further explore which kind of factor secreted by placental trophoblast cells hold key position in the protection, the knockdown of several factors (RLN1, PTHLH, EGF and VEGF) was induced by siRNA transfection, followed by qPCR and ELISA detection to confirm the knockdown efficiency. The results indicated that all of the siRNA transfection could induce the knockdown effect in both mRNA and protein level (Fig. 5B). The conditioned medium from siRNA-transfected placental trophoblast cells were harvested and used to culture HK-2 cells treated with cisplatin. Cell viability of each groups was evaluated after 24-hour treatment. Compared with the Cisplatin group, all of conditioned medium groups show better cell viability. However, the knockdown of RLN1, PTHLH and EGF decreased the protective function of conditioned medium (Fig. 5C). The anti-apoptosis effect of placental trophoblast cell-based conditioned medium was evaluated in different groups. The western blot results indicated that cisplatin treatment induced the up-regulation of apoptotic genes (Bax and Caspase 3), which could be rescued by different conditioned media. Compared with normal conditioned medium group, the knockdown of RLN1, PTHLH and EGF increased the level of Bax and Caspase 3 in cisplatin treated HK-2 cells. Besides, the conditioned medium from PTHLH-knockdown cells showed less renoprotective effect than other three knockdown groups, indicating the key position of PTHLH in paracrine effects of human placental trophoblast cells against AKI (Fig. 5D–E).

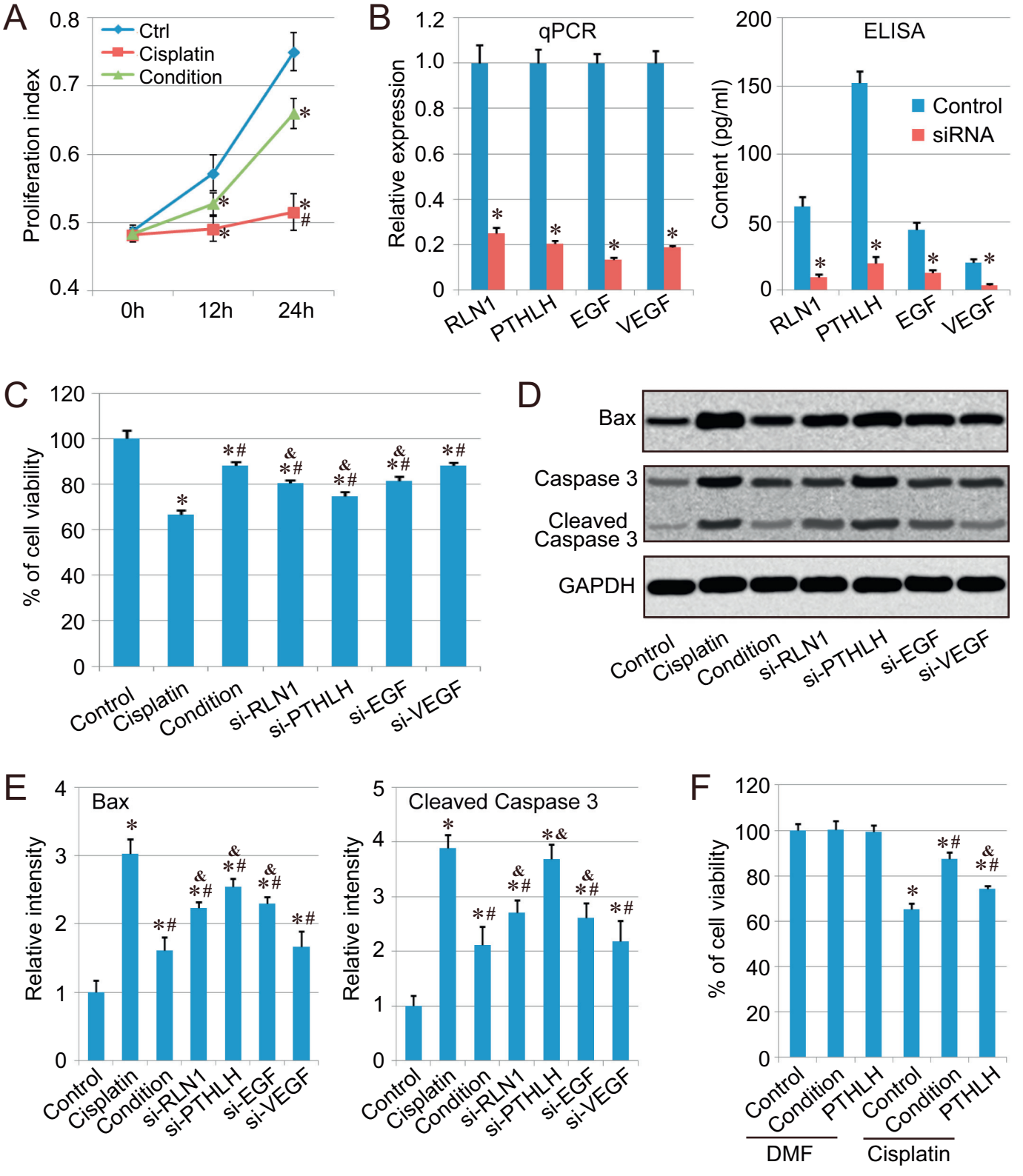
The protective action of PTHLH against cisplatin-induced injury was further evaluated using PTHLH protein treatment on HK-2 cell model directly. The results indicated that the treatment with PTHLH didn't affect cell proliferation ability, and showed some protection from cisplatin-induced injury. However, the protective effect was still weaker than the conditioned medium, indicating that other components in the conditioned medium hold some therapeutic action against cisplatin-induced injury (Fig. 5F).

4. Discussion

Even though the action of cell-based therapy against AKI has been demonstrated for years, which kind of cells hold best therapeutic effect remains unclear. In this study, our results indicated that human placental trophoblast cells hold the potential to be applied in AKI therapy. Different from other cell sources, placental trophoblast cells hold lower expression of most human leukocyte antigen, which makes the cells promising in allogeneic transplantation [27–29]. More importantly, the cells secrete some special factors related to pregnancy and embryonic development, such as RLN1 and PTHLH, which have been demonstrated to hold renoprotective effect in different AKI models [20,21]. Therefore, the paracrine effects of human placental trophoblast cells could keep a key position in the AKI therapy process.

In addition, the mechanisms for cell-based therapy against AKI include both differentiation-dependent mechanism and differentiation-independent mechanism, and some studies indicated that trophoblast cells also hold some differentiation ability [30–32]. Therefore, hNA (Human Nuclear Antigen, a specific marker for human cells)-positive cells were tried to be detected in kidney tissue slice via Immunohischemistry in our study. However, the trophoblast cells treated group didn't show any positive staining (data not shown), indicating that few placental trophoblast cells localized to renal compartments or contributed to kidney regeneration via cell differentiation. Therefore, the differentiation-independent mechanism still hold major action during the process of human placental trophoblast cell-based therapy against AKI.

Several factors (RLN1, PTHLH, EGF and VEGF) secreted by placental trophoblast cells were further analyzed in our study. Among those factors, PTHLH hold the highest level in the conditioned medium, while the level of VEGF was lowest. In the knockdown assay, we also noticed that the PTHLH knockdown abolished the protective effect of placental trophoblast cell-based conditioned medium significantly, but the knockdown of VEGF didn't show obvious effect on the protective action of conditioned medium, indicating that PTHLH might hold more important position in placental trophoblast cell-based therapy against AKI than other cell factors. PTHLH is an important member of the parathyroid hormone family. It could be secreted by kinds of cancer cells and regulate cancer development and progress. However, it also could be secreted by normal cells and regulate the endochondral bone development via maintaining the endochondral growth plate at a constant width, as well as the epithelial-mesenchymal interactions during the formation of mammary glands [33–35]. In addition, the renoprotective effect of PTHLH has been demonstrated by several groups



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[21,36,37]. Our study also indicated the important position of PTHLH in the renoprotective effect of placental trophoblast cell-based therapy. However, only four factors were evaluated in our study, and more cell factors need be compared with each other to conform the position of PTHLH. More importantly, the role of PTHLH still needs to be evaluated in vivo, which could help us to understand the biological function of PTHLH or other factors in placental trophoblast cell-based AKI therapy intensively.

In addition, even though the treatment with single PTHLH protein showed some protection from cisplatin in HK-2 cell model, but the effect was still weaker than the conditioned medium, indicating that other factors secreted by trophoblasts also hold an important position in

Fig. 5. Paracrine effects of human placental trophoblast cells in AKI treatment. A. Protective effect of placental trophoblast cell-based conditioned medium against cisplatin-induced damage in HK-2 cells. B. Evaluation of different siRNAs knockdown efficiency. Herein, the scrambled control siRNA were used in the control group, and specific siRNA targeting different genes were used in other experimental groups. C. Evaluation of cell viability in different groups. HK-2 cells were exposed to cisplatin (20 μM) for 24 h. The cells treated with cisplatin in the presence of conditioned medium was used as Cisplatin group. The cell viability of Control group (HK-2 cells cultured using normal medium) was considered as "100%". The conditioned media were harvested from different siRNA-knockdown cells (condition: Cells transfected with control siRNA; si-RLN1: Cells transfected with human RLN1-siRNA; si-PTHLH: Cells transfected with human PTHLH-siRNA; si-EGF: Cells transfected with human EGF-siRNA; si-VEGF: Cells transfected with human VEGF-siRNA). D–E. Evaluation of different apoptotic genes expression. The relative intensity of Control group was considered as "1.0", and the relative gene expression level of other group was further evaluated. Results are expressed as mean ± SEM. *: $P < 0.05$ compared with the Control group. #: $P < 0.05$ compared with the Cisplatin group. &: $P < 0.05$ compared with the Condition group. F. Evaluation of PTHLH protective effect on cisplatin-induced injury. HK-2 cells were treated with PTHLH (0.152 ng/ml, the same concentration as PTHLH in the conditioned medium) or conditioned medium in presence of cisplatin or DMF (the solvent for cisplatin). *: $P < 0.05$ compared with the same group in the presence of DMF. #: $P < 0.05$ compared with the Control group in presence of cisplatin. &: $P < 0.05$ compared with the Condition group in presence of cisplatin.

the protection. Those factors might release the cisplatin-induced injury directly, or protect the cells via enhancing the therapeutic action of PTHLH. Even though the detailed mechanism is still unclear, the administration of trophoblasts may be more effective than single PTHLH in AKI treatment.

In the evaluation in vitro, the conditioned medium was added to HK-2 culture at the same time as cisplatin treatment as the references [2, 38]. However, the trophoblasts were injected after the administration of cisplatin in vivo, which is similar to other stem cell-based treatment against cisplatin-induced AKI [1,39,40]. This is mainly because the cisplatin could affect the viability of trophoblasts directly, if we inject trophoblasts and cisplatin at same time. But the conditioned medium doesn't have the "cell viability" problem, so it can be used together with cisplatin in the treatment in vitro. We also tried to pre-treat HK-2 using cisplatin for 12 h, and then culture the cell with the conditioned medium, which was similar to in vivo experiment. However, the conditioned medium was hard to show any improvement of cell viability. It could be possible that 12-hour cisplatin treatment had already led to irreversible injury to HK-2 cells. Therefore, the suitable dose and time in cisplatin pre-treatment cell model still need further exploration.

In this study, human placental trophoblast cells only can be passaged twice, and are hard to be maintained in vitro. Compared with some adult stem cells, which have been applied in AKI treatment widely, the proliferation ability of placental trophoblast cells is limited. Even though immortalized placental trophoblast cell line, HTR-8/SVneo, has been established by some researchers [41], whether such immortalized trophoblast cell line could be used in AKI treatment is unclear. Therefore, more suitable culture condition in vitro should be established to maintain the primary placental trophoblast cells. If the primary placental trophoblast cells was maintained well in vitro, gene-modified stable cell lines could be established and the function of different factors secreted by placental trophoblast cells in AKI model could be evaluated in vivo.

Besides, our previous studies have established several methods to improve cell migratory ability in stem cell-based therapy against AKI, such as administration of stem cells with Muscone, and IGF-1 overexpression in stem cells [8,9]. In this study, the paracrine effects of human placental trophoblast cells may hold a key position in the AKI therapy process, and we didn't find the trophoblast cells in kidney tissues. Therefore, maybe the enhancement of the migratory ability of human placental trophoblast cells could further improve the therapeutic action against AKI. However, whether the placental trophoblast cells could localize to renal compartments and further contribute to kidney regeneration via cell differentiation, is still far away from our understanding. Recently, Zhao et al. demonstrated that BMP-2 could promote the invasion ability of human trophoblast cells via the up-regulation of N-cadherin expression and the activation of non-canonical SMAD2/3 signaling and Activin A production [42,43]. Therefore, maybe the administration of placental trophoblast cells with BMP-2 could receive better therapeutic effect against AKI than single trophoblast cell-based treatment.

5. Conclusion

In conclusion, our study developed a novel strategy to treat cisplatin-induced AKI using human placental trophoblast cells. The administration of human placental trophoblast cells improved the pathological changes in kidney tissues as well as kidney function. In addition, the placental trophoblast cell-based treatment also showed anti-apoptotic effect and anti-inflammatory effect in the AKI model. Further analysis indicated that the paracrine effects of human placental trophoblast cells may hold a key position in the AKI therapy process. Even though the detailed mechanism and the optimizations of this therapy mode still need further investigation, the application of placental trophoblast cell may hold special potential in the treatment of patients with AKI.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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