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Stem cell therapy in renal diseases

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Abstract

Objective: to evaluate the stem cell in treating renal diseases. To evaluate the effect of stem cells on renal cell apoptosis and necroptosis. **Background:** The use of stem cells is the hope for all patients with end-stage renal diseases. We isolate stem cells from umbilical cord blood to treat renal diseases by using the Vero cell line as a renal cell which is treated by hydrogen peroxide as renal failure. P53, RIPK1, and EGFR genes were detected by RT PCR to show the improvement by using stem cell therapy for renal diseases. Mesenchymal stem cells are the future hope for the treatment of renal diseases associated with renal cell apoptosis and necroptosis.

Methodology: Mesenchymal CD105 Stem Cells Separation from Umbilical Cord Blood from 20 pregnant females. By forming buffy coat mononuclear leukocytes. Magnetic labeling of CD105 and Separation to stem cells. stem cell proliferation by using Dulbecco Modified Eagle medium (DMEM). After proliferation passages 1, 2, and 3 freeze cells at -20c. For one week then thawing at room temperature to make stem cell extraction. Vero cell line treated by H2O2 1.6 mm for 5 hours to reach sub-lethal. The cytotoxicity of the cell was caused by hydrogen peroxide measured by MTT assay and Spectrophotometry. The Vero cell line is divided into 4 groups group1: normal control, group 2: sub-lethal, group 3: sub-lethal treated by stem cell extraction, and group 4: sub-lethal treated by stem cells. P53, RIPK1, and EGFR genes were detected in 4 groups by RT PCR. **Results:** P53, RIPK1, and EGFR levels showed a highly significant difference among studied groups with elevated levels of P53 and RIPK1 and reduced levels of EGFR in the sub-lethal renal cell group with an improvement of cells treated with mesenchymal stem cells. Mesenchymal stem cells showed better results when compared with mesenchymal stem cell extract. **Conclusion:** Mesenchymal stem cells demonstrated a good effect on renal cell line injury, apoptosis, and necroptosis.

Keywords: stem cell, renal, therapy, necroptosis

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Introduction

Kidney diseases have become a global public health problem due to their rapidly growing incidence. These diseases affect over 10% of the global population, because of a global rise in the aging population, as well as an increase in the frequency of their main etiologies, such as diabetes, cardiovascular diseases, and hypertension [1].

As a routine treatment for kidney diseases, multidrug therapy cannot reverse the process of entering end-stage renal disease (ESRD) in most patients, and those with ESRD require renal replacement therapy, i.e., maintenance dialysis or kidney transplantation [2].

Owing to high medical costs and adverse impacts on the patient's quality of life, dialysis is not considered an ideal treatment strategy [3].

Stem cells are defined as cells that have clonogenic and self-renewing capabilities and differentiate into multiple cell lineages. Stem cells are found in all of us, from the early stages of human development to the end of life. Stem cells are basic cells of all multicellular organisms having the potency to differentiate into a wide range of adult cells [4].

Self-renewal and totipotency are characteristic of stem cells. Though totipotency is shown by very early embryonic stem cells, the adult stem cells possess multipotency and differential plasticity which can be exploited for a future generation of therapeutic options [5].

All stem cells may prove useful for medical research, but each of the different types has both promise and limitations [6].

For decades, researchers have been studying the biology of stem cells to figure out how development works and to find new ways of treating health problems [7].

The scientific researchers and medical doctors of today hope to make the legendary concept of regeneration into reality by developing therapies to restore lost, damaged, or aging cells and tissues in the human body [8].

This research has opened new horizons for stem cell research.

The use of SCs is a promising therapeutic strategy for kidney diseases as well. Increasing results obtained in models of acute kidney injury (AKI) and chronic kidney disease (CKD) document that SCs have therapeutic potential in the repair of renal injury, preserving renal function and structure thus prolonging animal survival. The effects were initially attributed to SCs implanting damaged tissue, differentiating, and replacing damaged cells [9].

Materials and Methods

This study was carried out in the Stem Cell Laboratory at Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Menoufia Governorate, Egypt from January 2016 to June 2018.

This work follows ethical standards and was approved by the ethical committee of GEBRI.

Informed written consent was obtained from all participants to give human blood cords for stem cell collection.

Umbilical cord blood collection

Pregnant female aging 28-35 years with gestational age 37-42 weeks.

50 ml of umbilical cord blood were collected through a syringe needle and kept in a sterile 50 ml costar falcon tube containing acid citrate dextrose anticoagulant (4ml/50 ml UCB), blood samples were transferred to the stem cell lab for processing after collection to preserve cell viability.

Females with chronic diseases or chronic infections were excluded

I. Preparation of CD^{105}Mesenchymal Stem Cells

1. Mesenchymal CD^{105}Stem Cells Separation from Umbilical Cord Blood.

Umbilical cord blood samples were collected from human healthy pregnant women after giving birth from the department of Obstetrics and gynecology faculty of medicine of Menofia

University. The volunteers' age ranged from twenty-eight to thirty-five years. The blood Samples were collected from twenty cases. The collection takes place according to the esthetical committee roles of the faculty of medicine after taking consent from them.

1- Blood samples were collected on EDTA (Ethylene Di Amine Tetrachloro-l acid tube in a cooling container then transferred within 2 hr. to Stem Cells Laboratory at Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City.

2- Umbilical cord blood was diluted with Phosphate buffer saline 3:1, and 9 ml of diluted blood cells suspension was carefully layered over 3 ml Ficollhypaque (Gibco, Grand Island, NY, USA,1.077 density) in a 15ml falcon tube.

3- Centrifuged at 1500 rpm for 20 minutes at +4°C in a swinging-bucket rotor without a break.

4- The obtained Buffy coat containing mononuclear cells was gently collected and aspirated off and transferred into a new 15 ml falcon tube and filled with PBS containing 2 mM EDTA.

5- Gently suspended using vortex and centrifuged at 1000 rpm for 10 minutes at +4°C. This procedure is repeated twice under the same conditions.

6- The cells proceeded for CD^{105}Mesenchymal stem cell separation. The final volume of 300 µl / 10^8total cells, was then subjected to magnetic labeling.

2. Magnetic labeling of CD105 and Separation.

1. Cells were disaggregated by gently pipetting several times
2. Passed through 30µl nylon mesh (Pre - Separation Filters# 130-041-407) to remove cell clumps.
3. The cell pellet was resuspended in 90 µl running buffer (MACS separation buffer containing 0.5% bovine serum albumin, phosphate buffered saline, pH 7.2, and 2 mM EDTA and 0.09% sodium azide), 10 µl of CD^{105}+ Progenitor Cell Isolation Kit was added to the cell suspension and mix well.
4. Incubated for 30 minutes in the refrigerator at +4°C, washed by adding 1 mL of buffer, and centrifuged again in a +4°C cooling centrifuge at 1000 rpm for 10 minutes.
5. The magnetic separation column was placed in the magnetic field of the MACS Separator. The cell suspension was then applied to the column, where CD^{105+} cells were attached to the column and non-attached cells were eluted.
6. After complete separation, the separation column was separated from the magnetic field and CD^{105+} was eluted by using a running buffer to undergo proliferation in vitro.

3. CD^{105+}Mesenchymal Stem Cells Proliferation.

CD^{105}+ Mesenchymal stem cells were seeded in a 75 cm3 cell culture flask with a vented cap (Nunc A\S, Roskilde, Denmark) at cell density3×10^6/ ml in (DMEM) Dulbecco Modified Eagle medium (Sigma, St Louis, Mo, USA): supplemented with 2.4 g sodium bicarbonate/lit. 10% Fetal Bovine Serum (Sigma, St Louis, Mo, USA): inactivated at 56◦G water bath for 30 min before use.) and incubated at 37° C in 5% CO_2incubator and 95% humidity for three days.

4. Extraction of CD105+ Cells.

After proliferation passages 1, 2, and 3 freeze cells at -20c. For one week then thawing at room temperature, collection in a falcon tube, centrifugation at 10.000 rpm for 30 min. then collecting supernatant in Eppendorf tubes for use.

II. Maintenance of VERO Cell Line

Vero cell line (African green monkey kidney cells)

Cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) growth medium (Invitrogen-Gibco), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen-Gibco) in 75 cm3 cell culture flask flasks

were incubated in 5% CO_2incubator with 95% humidity at 37ºC

III. Trypsinization of Cells

1. Confluent monolayer of Vero cells in 75-cm2 flasks was examined under an inverted microscope to examine, the viability of cells and to sure free of contamination.
2. The old medium was aspirated off and 8 ml trypsin (1:250) pre-warmed at 37°C, added to each flask then incubated at 37°C for 10 min.
3. Cell culture was examined via an inverted microscope; if cells were detached; the action of trypsin was deactivated by the addition of equal amounts of growth medium supplemented with 10% FBS.
4. Transfer the cell suspension to the centrifuge tube with the medium and cells from step 1, and centrifuge at approximately 125 xg for 5 to 10 minutes. Discard the supernatant.
5. Resuspend the cell pellet in a fresh growth medium. Add appropriate aliquots of the cell suspension cells density (1.8×10^4 cells / well) in a volume of 100µl complete growth medium. Then Incubate cultures at 37°C for 24 hrs.

IV. Cytotoxicity Study and sub-lethal dose of H2O2 Determination.

Wells growth medium added by 0.1 mM, 0.2 mM, 0.4 mM, 0.8 mM, and 1.6 mM of the H2O2 per well in a 2×3 well plate for 0.5 hr., 1 hr., 2 hr,3 hr.,4 hr., and 5 hr. to induce Vero cell destruction then evaluation by MTT assay to determine the sub-lethal dose

We use one well as normal control not treated by H2O2.

The MTT method of monitoring in vitro cytotoxicity is well suited for use with multiwall plates. For best results, cells in the log phase of growth should be employed and the final cell number should not exceed 106 cells /well. Each test should include a blank containing a complete medium without cells.

1. Reconstitute each vial of MTT [M-5655] to use with 3 ml of medium or balanced salt solution without phenol red and serum. Add reconstituted MTT in an amount equal to 10% of the culture medium volume.
2. Return cultures to an incubator for 2-4 hours depending on cell type and maximum cell density. (An incubation period of 2 hours is generally adequate but may be lengthened for low cell densities or cells with lower metabolic activity.) Incubation times should be consistent when making comparisons.
3. After the incubation period, remove cultures from the incubator and dissolve the resulting formazan crystals by adding an amount of MTT Solubilization Solution [M-8910] equal to the original culture medium volume.
4. Gently mixing in a gyratory shaker will enhance dissolution. Occasionally, especially in dense cultures, pipetting up and down [trituration] may be required to completely dissolve the MTT formazan crystals.
5. Spectrophotometrically measure the absorbance at a wavelength of 570 nm. Measure the background absorbance of multi-well plates at 690 nm and subtract from the 450 nm measurement. Tests performed in multi-well plates can be read using the appropriate type of plate reader or the contents of individual wells may be transferred to the appropriate size cuvette for spectrophotometric measurement.

From the MTT assay, the well treated with 1.6 mM by H2O2 for 5 hours is the sub-lethal dose.

Vero Cells have been cultured for 24hr. then exposed to a sub-lethal dose of H2O2 for 5 Hr.

Then treated with CD105+ mesenchymal stem cells and CD105+ MSCs extraction stem cells for 24 hr. Untreated cells served as a control. Sublethal dose added.

Gene expression analysis P53 gene, RIPK1 gene, and EGFR gene (RT-PCR)

II.4.1. RNA isolation

In gene expression analysis, it is very important to prepare high-quality RNA because RNA can be

contaminated by genomic DNA. To reduce contamination of genomic DNA from RNA, proper RNA isolation and cDNA synthesis procedures need to follow. RNA isolation was carried out with the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.

1- Cells were transferred to a centrifuge tube, and pellet by centrifugation at 300 x g for 5 min. Completely aspirate supernatant.

2- Buffer RLT was added to monolayer cells. the cell lysate was collected with a rubber policeman. Vortex to mix until no cell clumps should be visible

3- One volume (350 µl) of 70% ethanol was added to the homogenized lysate and mixed well by pipetting without centrifugation.

4- The volume applied up to 700 µl of a sample, including any precipitate which may have formed, to a RNeasy mini spin column sitting in a 2-ml collection tube, and centrifuged for 15 secat ≥8000 x g (≥10,000 rpm)

5- 700 µl Buffer RW1was pipetted onto the RNeasy column and centrifuged for 15 sec at ≥8000 x g (≥10,000 rpm) to wash.

6- RNeasy column was transferred into a new 2-ml collection tube (supplied). 500 µl Buffer RPE was pipetted onto the RNeasy column and centrifuged for 15 sec at ≥8000 x g (≥10,000 rpm) to wash.

7- 500 µl Buffer RPE was pipetted onto the RNeasy column, then centrifuged for 2 min at maximum speed to dry the RNeasy membrane**.**

8- RNeasy column was transferred into a new 1.5-ml collection tube (supplied) and pipetted 30–50 µl of RNase-free water directly onto the RNeasy membrane. Finally centrifuged for 1 min at ≥8000 x g (≥10,000 rpm) to elute.

II.4.2. cDNA synthesis

1- 1 µg of total RNA was used to synthesize cDNA using iScriptTM One-Step RT-PCR Kit with SYBR® Greenis a convenient and highly sensitive solution for real-time quantitative PCR of RNA templates.

2- cDNA synthesis and PCR amplification are carried out in the same tube. This kit is optimized to deliver maximum RT-PCR efficiency, sensitivity, and specificity without compromising fluorescent signals.

3- The obtained cDNA was used to determine the mRNA expression levels of Bcl-2, and P53 by RT-PCR analysis.

4- GAPDHwas used as an internal control. (**Stagliano et al., 2003; Zhao et al., 2017).**

The reaction included 1 µl cDNA, 2 µl 10X Taq Buffer, 1.2 µl 25 mM $MgCl_2$, 0.4 µl 10 mM dNTP, 0.8 µl 1U/µL Taq polymerase, 1 µl each primer and DEPC water up to 20 µl. The PCR conditions were as follows: 95°C for 3 min and 30 cycles at 95°C for 30 sec, 56°C for 40 sec, and 72°C for 40 sec. **(Bustin, 2005 and kubista et al., 2006).**

The primers sequences used for the amplification of RIP, P53, EGFR, and GAPDH were as follows:

Target Gene	Primer Sequence
RIP	F 5′-GAGAGCTGGTAGTTAGTAGCATGA -3′ R5′- AATTCCAATAATGAACCCAATAGATTAGTT -3′
P53	F 5′-CCCCTCCTGGCCCCTGTCATCTTC-3′ R 5′-GCGCGCCTCACAACCTCCGTCAT-3′
EGFR	F 5′-GCGTCTTGCCGGAATGT-3′ R 5′-GGCTCACCCTCCAGAAGGTT-3′
GAPDH	F 5′- GTCTCCTCTGACTTCAACACGC -3′ R5′- ACCACCCTGTTGCTGTAGCCAA-3′

All primers were synthesized by (Sangon Biotech, Shanghai, China).

Statistical analysis:

Results were statistically analyzed by SPSS version 22(SPSS Inc., Chicago, IL, USA). Tests of normality were performed.

One way ANOVA test was used for comparison between more than 2 means of normally distributed variables.

Test of Homogeneity of Variances was performed, and Tukey test post hoc analysis was used for assumed equal variance.

P value was considered significant if <0.05

Results

This study was carried out at the Genetic Engineering and Biotechnology Research Institute, (GEBRI) University of Sadat city.

The research work evaluates the effect of mesenchymal stem cells on renal cell lines treated with hydrogen peroxide to induce apoptosis

Mesenchymal stem cells were obtained from umbilical cord blood of 20 pregnant females during delivery at Menoufia University hospital

It was found that hydrogen peroxide at concentration 1.6 mM at 5 hours showed maximum necroptosis on renal cell line

The renal cell line was divided into four groups; Group 1: control, Group 2: Sub-lethal group which is renal cell line treated with H_2O_2, Group 3: Renal cell line treated with H_2O_2 and mesenchymal stem cell line extraction, Group 4: Renal cell line treated with H_2O_2 and mesenchymal stem cell line.

P53, RIP-K, and EGFR genes were measured in four groups of renal cell line

Our results demonstrated that P53 and RIPK1 genes were elevated while EGFR was reduced when renal cells were treated with H_2O_2 (Table 1 and Figure 1). Renal cells treated with H_2O_2 showed improvement when stem cells were added as demonstrated by studied genes. The mesenchymal stem cell line showed better results compared to mesenchymal stem cell extract as demonstrated by post hoc analysis.

Table (1): Comparison of studied genes level among the studied groups:

	Group 1 Control (n=20)	**Group 2 Sub-lethal (n=20)**	**Group 3 Sub-lethal treatment with MSCs extract (n=20)**	**Group 4 Sub-lethal treatment with MSCs (n=20)**	**ANOVA (P value)**	**Post hoc test**
	Mean ±SD (Range)	Mean ±SD (Range)	Mean ±SD (Range)	Mean ±SD (Range)		
P53	4.54 ±0.64 R=2.73-6.37	10.84±1.53 6.49-15.16	6.55±0.92 3.93-9.18	4.37±0.61 2.62-6.13	F=180.86 P<0.001*	$P_{1,2,4,5,6}$<0.001* P_3=0.949
RIPK1	2.77 ±0.39 R=1.66-3.89	6.02±0.85 3.61-8.44	4.26±0.60 2.53-5.90	3.58±0.50 2.15-5.02	F=101.64 P<0.001*	$P_{1,2,3,4,5}$ <0.001* P_6=0.0.004*
EGFR	0.66 ±0.09 R=0.40-0.93	0.17±0.02 0.11-0.25	0.33±0.04 0.20-0.47	0.55±0.07 0.33-0.78	F=212.73 P<0.001*	$P_{1,2,3,4,5,6}$ <0.001*

*: significant P1: **Controls vs. Lethal, P2: Controls vs. Extract, P3: Controls vs. MSCs, P4: Sub-lethal vs. Extract, P5: Sub-lethal vs. MSCs, P6: Extract vs. MSCs. MSc: Mesenchymal stem cell**

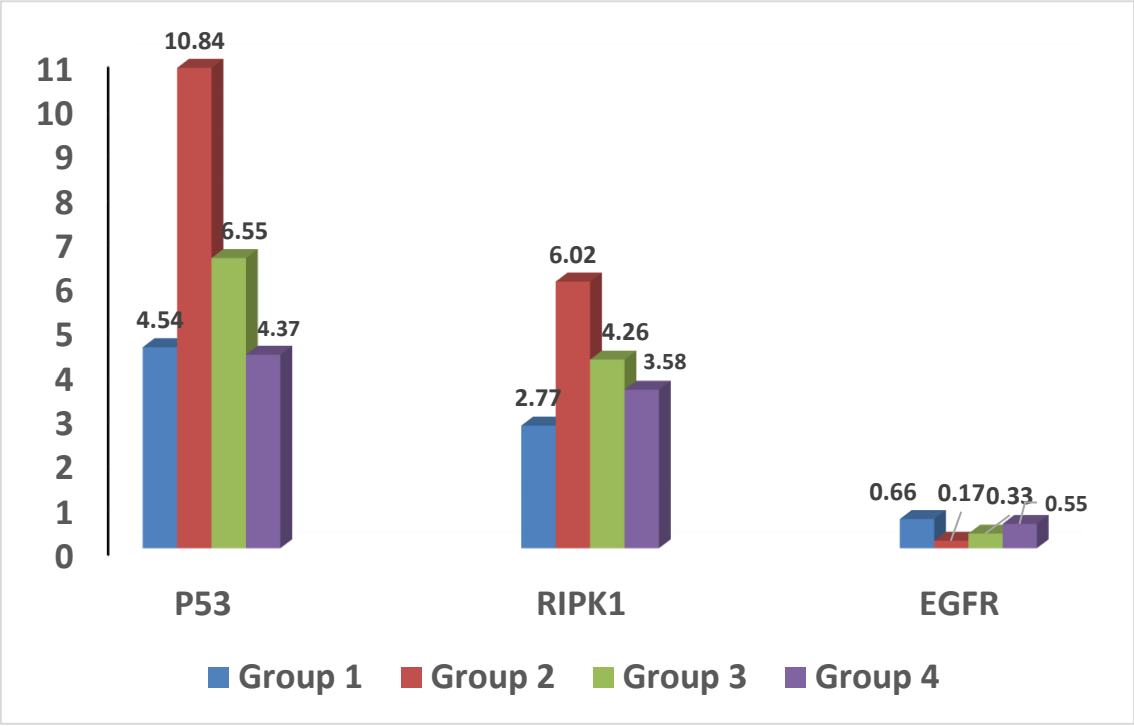

Figure 1: Comparison of the studied groups regarding P 53, RIPK1, and EGFR genes

Discussion

To date, the medical treatment of kidney diseases, by multidrug therapies can only delay the disease's progression. These drugs cannot reverse the progression into end-stage kidney disease (ESKD)and the need for dialysis or kidney transplantation [10].

Due to the high medical cost involved in dialysis therapy and dialysis does not restore or substitute all kidney functions [11].

the severe shortage of organ donors and potential organ rejection risks limit the practice of kidney transplantations {12].

In our research, we studied the effect of stem cells in treating renal diseases by using a Vero cell line as a renal cell, as a normal cell.

Renal cell diseases are induced by adding hydrogen peroxide to the renal cell line which leads to apoptosis and necroptosis.

In our study, we detect apoptosis and necroptosis and improvement in renal cell line by detecting 3 genes (P53, RIPK1, and EGFR)

p53 plays a role in regulation or progression through the cell cycle, apoptosis, and genomic stability It can activate DNA repair proteins when DNA has sustained damage. Thus, it may be an important factor in aging [13].

RIPK1 gene functions in a variety of cellular pathways related to both cell survival and death. In terms of cell death, RIPK1 plays a role in apoptosis and necroptosis. Some of the cell survival pathways RIPK1 participates in include NF-κB, Akt, and JNK [14].

EGFR is a transmembrane receptor tyrosine kinase that plays a critical role in cell growth, tissue development, and overall cellular homeostasis [15].

Our results demonstrated that high levels of P53 and RIPK genes and low level of EGFR gene was associated with renal cell line damage and necroptosis. Both mesenchymal stem cell extract and mesenchymal stem cells showed improvement in the three studied genes with better effects related to mesenchymal stem cells compared to extract.

The research showed that there is improvement in the renal cell by the stem cell therapy by detecting the changes in the 3 genes

The efficacy of MSC treatment on renal failure found that the elevated serum creatinine level was reduced in the animal models with renal failure following MSC therapy. Wang et al [16].

Further studies demonstrated that the subcellular mechanisms of MSC-mediated protection did not act directly at the sites of damage. One study indicated that the protective effects of MSCs are due to the secretion of factors that have paracrine effects [17].

Another study suggested that MSCs produce and secrete extracellular vesicles, which then lead to renal-protective effects [18].

The transplantation of MSCs enhances renal function and increases the expression and activity of ATPase in a rat model of CKD with reno-vascular hypertension. Moreover, MSCs improve renal morphology and reduce fibrosis in the kidney [19].

Conclusion

Mesenchymal stem cells demonstrated a good effect on renal cell line injury and necroptosis that opening the door for a possible therapeutic effect of mesenchymal stem cells in different renal diseases associated with renal cell apoptosis and necroptosis

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