Original Article

Therapeutic Effect of Kidney Tubular Cells-Derived Conditioned Medium on the Expression of MicroRNA-377, MicroRNA-29a, Aquapurin-1, Biochemical, and Histopathological Parameters Following Diabetic Nephropathy Injury in Rats

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Abstract

Background: Diabetic nephropathy (DN) is a critical complication of diabetes mellitus. This study evaluates whether administration of conditioned medium from kidney tubular cells (KTCs-CM) has the ability to be efficacious as an alternative to cell-based therapy for DN.

Materials and Methods: CM of rabbit kidney tubular cells (RK13; KTCs) has been collected and after centrifugation, filtered with 0.2 filters. Four groups of rats have been utilized, including control, DN, DN treated with CM, and sham group. After diabetes induction by streptozotocin (50 mg/kg body weight) in rats, 0.8 ml of the CM was injected to each rat three times per day for 3 consecutive days. Then, 24-h urine protein, blood urea nitrogen (BUN), and serum creatinine (Scr) have been measured through detection kits. The histopathological effects of CM on kidneys were evaluated by periodic acid–Schiff staining and the expression of microRNAs (miRNAs) 29a and 377 by using the real-time polymerase chain reaction. The expression of aquapurin-1 (AQP1) protein was also examined by Western blotting.

Results: Intravenous injections of KTCs-CM significantly reduced the urine volume, protein 24-h, BUN, and Scr, decreased the miRNA-377, and increased miRNA-29a and AQP1 in DN treated with CM rats.

Conclusion: KTCs-CM may have the potential to prevent kidney injury from diabetes by regulating the microRNAs related to DN and improving the expression of AQP1.

Keywords: Aquapurin-1, conditioned medium, diabetic nephropathy, kidney, microRNAs

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Introduction

Diabetic nephropathy (DN) as a severe and prevalent complication of type 1 and type 2 diabetes causes end-stage renal disease in up to diabetic patients.[1] Increased urinary albumin excretion is considered the first clinical manifestation of DN in humans. These symptoms gradually progress to proteinuria in the nephrotic range.[2] Glomerular hypertrophy, thickening of the glomerular basement membrane, and widening of the mesangial extracellular matrix are morphological substrates for these functional anomalies of the glomerular filter apparatus.[3]

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Glomerulosclerosis, glomerular capillary death, and fibrosis are all symptoms of advanced DN, which is marked by a rapid decrease in glomerular filtration rate.[4] Renin–angiotensin system obstruction, blood pressure control, and blood glucose levels are interventions that have been reported in order to reduce the DN rate so far.[5,6] None of the existing treatment options, however, will improve or avoid DN.[7] Some studies have shown that treatment impacts of the cells strongly are associated with released cellular factors. These factors consisted of signaling molecules and extracellular vesicles, which include exosomes, microvesicles, and apoptotic bodies.[8] In recent years, cells have been characterized for their secretory ability of various bioactive molecules in their conditioned media (CM) which are known as secretome. These secreted molecules are easily separated and demonstrated significant effects on tissue regeneration.[9] CM has advantages over cell therapy, which include the following the simplicity of preservation, sterilization, packaging, and storage for prolonged periods without losing its characteristics. The cell lines can be precisely measured for proper dosages and manufactured in large amounts without requiring the patient to undergo invasive extraction that causes saving both time and money.[10] On the other hand, membrane vesicles within the CM consist of microRNAs, proteins, and mRNAs with the ability to transfer to the targeted cells such as the epigenetic and genetic changes in the target cells.[11]

Based on some studies, the microRNAs have been considered to be one of the groups of the noncoding RNAs that are expressed in all tissues and play important roles in various diseases including DN.[12] Increased glucose leads to the suppression of superoxide dismutase (SOD) and activated p21 kinase. This suppression increases miR-377 expression and increases fibronectin production.[13] MiR-377 targets homoxygenase (HO-1). HO-1 is an essential antioxidant that prevents DN.[14] Researches have shown that the miR-377/HO-1 pathway may be a new pathway in which miR-377 inhibits HO-1 and ultimately leads to DN.[15,16] Moreover, three members of the miR-29 family are suppressed under high glucose conditions in proximal tubular cells, mesangial cells, and podocytes.[17] The members of miR-29 family are responsible for antifibrotic effects in DN. miR-29a targets directly 3′UTR of COL4α1 and COL4α2, leading to decreased expression of these two fibrotic genes.[18,19] We, therefore, hypothesized that these soluble factors in CM, which are present in serum-free kidney tubular cells (KTCs)-CM, may be responsible for the therapeutic effects of KTCs. KTCs-CM can be therapeutically administered. Thus, here, we tested the therapeutic potential of KTCs-CM in the DN model.

Materials and Methods

In this experimental study, 40 healthy and adult male Sprague–Dawley rats (8–10 weeks old, 220–270 gm) were used. The animals were obtained from the Animal House Center of Ahvaz Jundishapur University of the Medical Science (Ahvaz, Iran) and have been kept in cages with 12–12-h light/dark cycle at a temperature of 21°C–24°C. Moreover, each experiment has been done with regard to directions provided by the Ethical Committee of Ahvaz Jundishapur University of Medical Sciences (IR. AJUMS. ABHC. REC.1398.008) in Ahvaz, Iran.

Culture of kidney tubular cell line

The rabbit kidney tubular cell line (RK13; NCBICode: C523) has been purchased from the Pasteur Institute of Iran and then immediately transferred to the Dulbecco Modified Eagle Medium (DMEM) high glucose (Gibco, UK) that is composed of 2 mM L-glutamine and 100 U penicillin/streptomycin (all from Invitrogen, Waltham, MA, USA) and 10% fetal bovine serum (Gibco, UK) and stored at 37°C incubators and 5% CO_2 for proliferation. Then, the medium was replaced after 5 days and used phosphate-buffered saline (PBS, Sigma, USA) to wash it in order to remove the nonadherent cells. When the cells reached 90% confluence, they were passaged with 0.25 percent trypsin.

Preparation of conditioned medium

To CM collection from KTCs culture, when the cellular confluence reached 90%–95%, the medium was replaced with a serum-free DMEM medium and the cells were grown for 2 days.[20] After that, the CM was collected and centrifuged for 5 min at 440×g. The solution was re-centrifuged for 2 min at 17,400×g for purifying and debris elimination.[21] Then, the conditioned medium was concentrated with a centrifugal filter device (5 kDa cutoff, Amicon Ultra, Millipore, USA). The resulting CM was preserved in aliquots at −80º C until use. We used the KTCs-CM without either enrichment or dilution.[22]

Diabetes induction

The rats have been fasted for 12 h before the diabetes induction. Then, a single intraperitoneal injection of streptozotocin (STZ) (50 mg/kg body weight, Sigma, USA),[23] which has been freshly dissolved in the citrate buffer (0.1 M at pH 4.5), was used to induce diabetes. The control animals were injected with the vehicle buffer. Then, 72 h following the STZ injection, blood samples were taken from the tail veins of the animals and the level of the fasting blood glucose has been measured by a glucose strip test in a glucometer (EasyGluco Blood Glucose Monitoring System, Infopia, South Korea). Finally, rats with a fasting blood glucose level of more than 250 mg/dl were described as diabetic models.[24]

Experimental design

Forty animals were divided into four groups and each of them included ten rats: Group 1: control group that was given saline three times per day for 3 consecutive days; Group 2: sham group, received 0.8 ml of CM through the tails' veins three times per day for 3 consecutive days; Group 3: diabetic group that was received saline three times per day for 3 consecutive days; and Group 4: diabetic + KTCs-CM group that received 0.8 ml of CM through the tails' veins three times per day for 3 consecutive days.[25] Finally, the animals have been separately placed in the metabolic cages for 24 h to measure the 24-h urine total protein upon the completion of the experiment course.

Measurement of 24-h urinary protein, blood urea nitrogen, and serum creatinine to confirm diabetic nephropathy in animals

After the 6th week, 24 urine was collected and the BCA kit (A bicinchoninic acid kit, Sigma, USA) was used to measure urinary protein. At the end of experimental period, rats were sacrificed under ketamine and zylin anesthesia. The blood of the animals was collected from the left ventricle. Then it was centrifuged at 3500 rpm for 5 minutes. Finally, serum creatinine (Scr) and blood urea nitrogen (BUN) levels were measured (Scr, C011-1, BUN, C013-2, both purchased from Jiangcheng Bio, Nanjing, China). Then, kidneys were separated, their weight was measured. The left kidneys were washed by PBS and fixed with 10% buffered formalin for paraffin sections and periodic acid–Schiff (PAS) staining. The right kidneys were rinsed and snap-frozen in liquid nitrogen, and stored at −80°C for miR-29a and 377 assays.

Light microscope examination

We observed the PAS stained sections under a light microscope. Summarily, to calculate the quantitative results, three slides from each group were randomly observed and examined under a light microscope. Twenty consecutive glomeruli were evaluated in each slide and the ratio of damaged glomeruli to healthy glomeruli was calculated as a percentage.[26] Finally, the relative mesangial expansion was determined as the fold change from the normal controls.

Extraction of the micro-RNAs and synthesis of the complementary DNA

The miRNeasy/plasma kit (Roche, Cat. No; 05080576001, Germany) was used to extract the microRNAs from the frozen specimens. Then, a spectrophotometry at the wavelength equal to 260 and 280 nm (NanodropThermo Scientific S. N: D015) was utilized to determine RNA purity and concentration. After that, complementary DNA (cDNA) was synthesized from 1 µg of total RNA with the miScript II RT Kit (BONmiR, BON209001, Iran) based on the company's directions.

Quantitative real-time polymerase chain reaction

The quantitative real-time polymerase chain reaction (qRT-PCR) was applied with a light cycler 96 real-time PCR system (Roche Diagnostics; Indianapolis; IN: USA) to measure microRNA expression. Each PCR amplification was been accomplished in the triplicate reactions and into the resultant volume of 13 µL consisting of 6.5 µL 2x QuantiTect SYBR Green PCR Master Mix, 1 µL cDNA, 4.5 µL RNAase free water, and 0.5 µL microRNA-specific forward primer assay (miR-29a [ACT GAT TTC TTT TGG TGT], or miR377 [CGA TCA CAC AAA GGC A]; BONyakhteh: IRAN), 0.5 µL universal reverse primer ([ACT TAT GTT TTT GCC GTT T]; [BONyakhteh]), the initial activation phase at 95°C for 2 min for activating the HotStarTaq DNA polymerase and then 40 cycles at 95° for 5s and 60°C for 30 s. In addition, the nontemplate control (H_2O) was regularly in each PCR. Consequently, RNU87 (housekeeping), as the internal control, has been used to normalized micro-RNAs expression level, and finally, the fold change has been computed with $2^{-\Delta\Delta Ct}$. Each sample was assayed three times.

Western blot analysis

In the sample buffer, cell extracts were prepared from tissue samples collected from groups of rats. Table 1 lists the contents of the sample buffer.

In order to protein quantitative measurement in cell extract, 7.5% sodium dodecyl sulfate (SDS) page was prepared. In the first step, the cell extract was diluted in the sample buffer and boiled at 95°C for 5 min. After gel polymerization, cell extract samples were loaded onto the SDS page and the electrophoresis flowed at a voltage under 100 V. The separated proteins were transferred to the membrane by semidry transfer method at 100 V for an hour. In order to block the transferred proteins, the protein-containing membrane was washed with the blocking solution (containing PBS + 5% milk powder + 0.1% tween 20) for one hour. At the end of this step, the membrane was incubated in a solution containing the primary rabbit polyclonal anti-aquapurin-1 (AQP1) and the β-actin antibody (Abcam, UK) for an overnight. In the next step, the membrane was washed (washing buffer: PBS + 0.1% tween 20) three times for 5 min each time, then secondary antibodies were added. The membrane and the anti-rabbit secondary antibody (Abcam, UK) were incubated for one hour and then the washing step was repeated (three times). ECL solution was applied for detection and imaging of the proteins in the membrane. Lumigan PS-3 substrate is catalyzed in ECL solution by a horseradish peroxidase enzyme connected to the secondary antibody. The radiation produced by luminol was released as a result of this reaction and was detected using special films. The protein bands that resulted were compared between groups and statistically analyzed.

Statistical analysis

The data were analyzed using one-way ANOVA followed by *post hoc* least significant difference test and were presented as the mean ± standard deviation (SD). $P < 0.05$ was considered significant.

Results

Kidney tubular cells phenotype

Kidney tubular cell lines were observed daily in cell culture flasks. The morphology and growth rate of tubular cells were examined [Figure 1a]. The results of morphological

Table 1: Contents of sample buffer

Compounds	Quantity	pH
Tris-HCl	50 mM	7.9
PMSF	1 mM	-
DTT	1 mM	-
Aprotinin	5 µg/mL	-
Leupeptin	5 µg/mL	-
Pepstatin A	5 µg/mL	-

observations of cells with reverse microscopy showed morphology similar to epithelial cells. Epithelial-like cells showed rapid growth and cells with a density of 95% were observed after 3-4 days.

Weight of diabetic nephropathy kidney after treatment

The kidney weight decreased in the DN group treated with CM, which was significant compared to the untreated DN group ($P < 0.01$). In the sham group, kidney weight was not significantly different from control [Figure 1b].

24-h volume of urine in diabetic nephropathy rats after injection of kidney tubular cells-conditioned medium

Intravenous injection of the KTCs-CM significantly declined urine volume of DN + CM rats in comparison to the DN group ($P < 0.05$) [Figure 2a]. In the sham group, 24-h volume of urine was not significantly different from the control [Figure 2].

Biochemical analysis

The results showed that Scr, BUN, and 24-h protein remarkably elevated in the DN group in comparison to the control group. The KTCs-CM group significant decreases in Scr ($P < 0.05$), BUN ($P < 0.001$), and 24-h urine protein levels ($P < 0.001$) [Figure 2b-d]. In the sham group, biochemical markers were not significantly different from control.

Periodic acid–Schiff staining analysis

At the end of the 6th week following the creation of the DN model, focal mesangial matrix expansion in the DN group in comparison with the control group was observed. Moreover, the section obtained from kidneys of rats with DN demonstrated enhanced intraglomerular cells mainly in the mesangial region with the mesangial expansion [Figure 3 DN]. However, there were only minor increases in the mesangial matrix and intraglomerular cells in DN rats with CM, with no evidence of mesangial matrix proliferation [Figure 3 DN+CM]. The results of quantitative analysis in Figure 3, treatment with the KTCs-CM, remarkably ($P < 0.001$) ameliorated the mesangial expansion. The histological changes of sham group were similar to control group [Figure 3b].

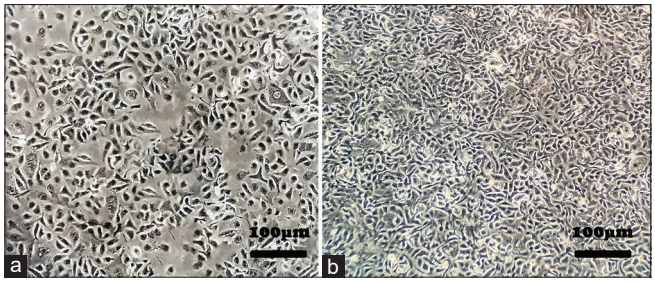

Figure 1: (a) Morphology and growth of kidney tubular cells. Growth cells after. (1) Two days and (2) Four days at × 100 magnification (scale bar: 100 μm) (b) Changes in kidney weight of animals in different groups. DN (diabetic nephropathy), treatment group (DN + CM). The values are expressed as the mean ± standard deviation. $^{**}P<0.0001$, $^{\#}P<0.01$; * and # symbols indicate comparison to control and DN groups, respectively

Effect of kidney tubular cells- conditioned medium on expression of miR-377 and miR-29a

As demonstrated in Figure 4, based on the qRT-PCR results, the miR-377 expression considerably increased ($P < 0.01$) [Figure 4a], but miR-29a expression significantly decreased following DN ($P < 0.01$) [Figure 4b]. In DN + CM group, expression of miR-377 significantly decreased ($P < 0.05$) but of miR-29a expression was enhanced significantly ($P < 0.001$). The levels of miR-377 and miR-29a in the sham group were similar to the control group [Figure 4a and b].

Evaluation of aquapurin-1 protein expression

Western blot analysis showed that AQP1 protein expression [Figure 5a] was significantly reduced in the DN group compared to the control group ($P < 0.01$) [Figure 5b]. In the diabetic group treated with CM, AQP1 was significantly expressed than the untreated group ($P < 0.05$) [Figure 5b]. It was also shown that there were no differences in AQP1 expression level in the sham group and control group.

Discussion

We found a large increase in kidney weight in the DN rats in this experiment. The kidney excessive growth is assigned to specific factors such as glucose overutilization and consecutive boost in rise uptake, lipogenesis, protein synthesis in the kidney tissue, and glycogen accumulation.[27] However, increased mesangial might also be a cause for kidney enlargement.[28] The KTCs-CM that was given to the DN group rats effectively inhibited kidney expansion. This finding is consistent with Ebrahim *et al*. research.[29] In our research, we discovered that diabetics with nephropathy have a higher 24-h protein excretion rate. Furthermore, serum BUN and Scr increased significantly in DN group. Renal glomeruli filtration barrier impairment can cause a rise in protein excretion in urine.[30,31] In addition, based on the Tagawa study,[32] proteinuria in DN rats is associated with ultrastructural and molecular variations in podocytes and slit diaphragm. The rate of protein excretion in the DN group treated with exosome was significantly reduced compared to the untreated DN group. Our findings are consistent with previous researchs.[33] In this study, an accumulation of mesangial matrix in rats with DN was observed. Some mesangial proteins, such as collagen I and III, have been shown to be expressed just in the terminal stage of glomerulosclerosis.[34] In addition, many mechanisms, such as cellular hemodynamics and overproduction of advanced glycation end products due to hyperglycemia, are responsible for increasing the mesangial matrix.[35] KTCs-CM reduces this accumulation of the mesangial matrix. Our findings are close to those of the previous report.[36]

The present study showed that miR-377 expression was significantly increased in kidney cells of DN rats. Increased miR-377 expression leads to suppression of SOD and p21 activated kinase, which increases fibronectin expression. Research has shown that the activity of some target genes such as PAK1 and SOD1/2 is reduced by miR-377. According to

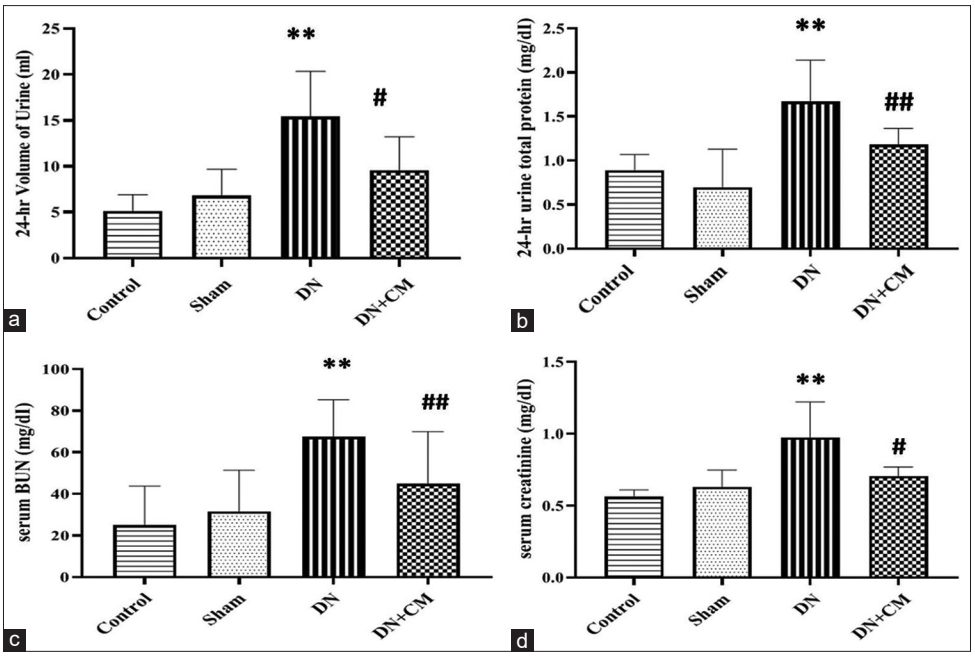

Figure 2: Effects of conditioned medium from kidney tubular cells on DN rats. Measurement of urine volume (a), urine protein (b), BUN (c), and Scr (d) levels in control and diabetic nephropathy rats before and after conditioned medium from kidney tubular cell treatment. Values expressed as mean ± standard deviation for 8 rats. $^{**}P < 0.001$, $^{\#}P < 0.05$, $^{\#\#}P < 0.001$; * and # symbols indicate comparison to control and diabetic nephropathy groups, respectively

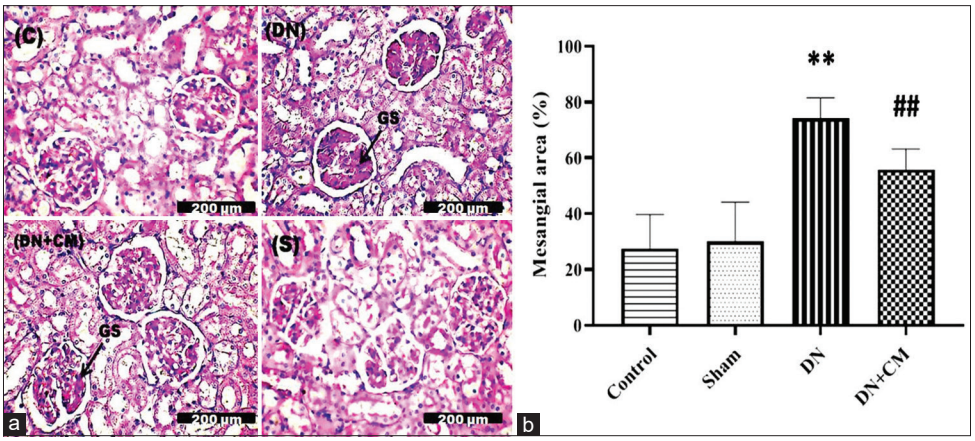

Figure 3: Light microscopy examination of tissue sections that stained by PAS in different groups. (C) Control, diabetic nephropathy ((DN) group, (DN + CM) diabetic nephropathy treated with conditioned medium from kidney tubular cells and (S) Sham group. GS: Glomerulosclerosis. Intravenous injection of conditioned medium from kidney tubular cells ameliorate pathological changes in diabetic nephropathy rats after 6 weeks. Magnification: ×400 (a). Each rat group's mean mesangial area was analyzed quantitatively. Results are expressed as the means ± standard deviation for 8 rats. $^{**}P < 0.001$, versus control; $^{\#\#}P < 0.001$, versus diabetic nephropathy (b)

previous studies, this reduction leads to increased vulnerability to oxidative stress and fibronectin accumulation in the extracellular matrix.[37] Increased expression of miR-377 has also been linked to increased expression of the matrix protein fibronectin, which collects significantly in DN, according to a previous study.[38] In this study, it was shown that miR-29a expression was significantly reduced in the diabetic group compared to the control group. Our results were similar to those of Du *et al*.[39] This study showed that administration of KTCs-CM to DN animals significantly increased miR-29a expression in these animals compared to the untreated DN group. Wang *et al*. also reported that miR-29a reduced renal fibrosis in the rat model of unilateral obstruction by releasing it into the blood serum through exosomal carriers.[40] In the present study, the expression level of AQP1 protein was studied and the results demonstrated that the expression of this protein decreased sharply 6 weeks after induction of diabetes in rats, which was in line with the previous results.[41] According to previous articles, hyperglycemia causes the production of cellular hypoxia through the production of mitochondrial

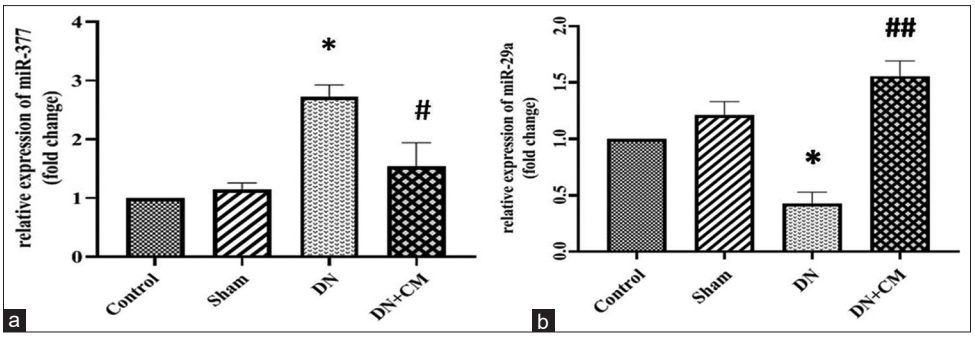

Figure 4: The impact of conditioned medium from kidney tubular cells on the expression of miR-377 (a) and miR-29a (b) following diabetic nephropathy injury. Data were presented as the means ± standard deviation for 8 rats. *$P < 0.01$ versus control; #$P < 0.05$ ##$P < 0.001$ versus diabetic nephropathy

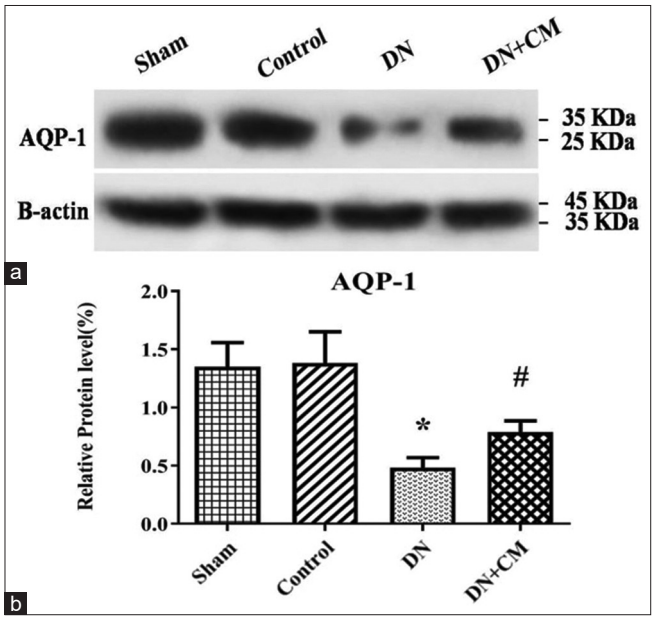

Figure 5: Expression of AQP1 protein (28 KDa) in kidney tissue of different groups using the Western blotting technique. β-actin (42 KDa) was used as an internal control (a). Values expressed as mean ± standard deviation for 8 rats. *$P < 0.01$ versus control; #$P < 0.05$ versus diabetic nephropathy. Protein expression was calculated to semi-quantitative using ImageJ software (b)

ROS (mtROS) followed by the suppression of AQP1.[42,43] Reduced AQP1 protein expression in the proximal tubule can reduce water uptake, resulting in increased urine volume and lower osmolality.[44] The present study also showed that injection of CM into DN animals significantly increased AQP1 protein expression in this group compared to the untreated DN group.

Conclusion

Now, according to these results and the results of previous studies, it can be concluded that CM can have therapeutic properties for DN due to their beneficial content, such as extracellular microvesicles, nanovesicles, proteins, microRNAs, cytokines, and effective growth factors.

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Conflicts of interest

There are no conflicts of interest.

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