Mesenchymal stem cells pretreated with melatonin ameliorate kidney functions in a rat model of diabetic nephropathy

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Abstract: The aim of this study was to investigate the effect of a regenerative therapy comprising mesenchymal stem cells (MSCs) pretreated with melatonin (MT) as a new therapy for underlying diabetic nephropathy (DN) pathogenesis in a rat model, and its possible effect on autophagy protein Beclin-1. Forty adult male albino Wistar rats were distributed among 4 groups: (i) control, (ii) DN, (iii) MSC-treated, and (iv) treated with MSCs that were pre-incubated in-vitro with MT (5 μmol·L⁻¹ for 24 h; MSCs + MT). MSCs treatment significantly improved the renal functions and ameliorated the measured underlying DN pathogenesis and elevation of Beclin-1 protein levels compared with the DN group. In-vitro pretreatment of MSCs with MT enhanced proliferation and efficiency, and thus improved the kidney functions by increasing superoxide dismutase (SOD-1) and Beclin-1, and decreasing transforming growth factor (TGF-β) markers in the kidney tissue, compared with the MSC group (P < 0.05). In conclusion: MSCs represent a promising target in DN management, and their effect can be intensified by pretreatment with MT. The elevated levels of Beclin-1 could be a mediator.

Key words: diabetic nephropathy, MSCs, melatonin, Beclin-1.

Introduction

The prevalence of diabetes mellitus (DM) has become extremely high; according to the International Diabetes Federation (IDF) 642 million people are expected to suffer from DM worldwide by the year 2040 (IDF 2015). Diabetic nephropathy (DN) is one of the microvascular complications of diabetes that may progress to a serious end-stage renal failure. DN is characterized by renal inflammation, oxidation, and progressive fibrosis leading to loss of function and efficiency, and thus improved the kidney functions by increasing superoxide dismutase (SOD-1) and Beclin-1, and decreasing transforming growth factor (TGF-β) markers in the kidney tissue, compared with the MSC group (P < 0.05). In conclusion: MSCs represent a promising target in DN management, and their effect can be intensified by pretreatment with MT. The elevated levels of Beclin-1 could be a mediator.

Mots-clés : néphropathie diabétique, CSM, mélatonine, Beclin-1.

Mesenchymal stem cell (MSC) regenerative therapy is a new approach in renal regeneration. The use of MSCs for cell therapy depends on the capacity of these cells to home, engraft, and transdifferentiate in the cell phenotype of the host’s damaged tissue to be repaired. Nephrons are known to be of mesenchymal origin, and so MSCs could differentiate to repair injured nephrons (Anglani et al. 2004).

The local inflammatory and oxidative stress state in diabetic tissues may explain the low homing capacity and survival efficiency of MSCs (Fadini et al. 2017). We hypothesized that ex-vivo pretreatment of MSCs could enhance their survival and efficiency. Thus, there was a need to search for an agent to increase the capacity of MSCs to home to the injured tissue, to survive, and to enhance their regenerative efficiency.

Melatonin (MT, N-acetyl-5-methoxytryptamine) is a neurohormone secreted mainly during the night by pineal and nonpineal cells. It is involved in the regulation of circadian rhythms (Peschke et al. 2013). We propose that restoring the efficiency of Beclin-1 could be renoprotective in DN.

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et al. 2015). Also, MT has potent anti-inflammatory anti-oxidant, and anti-apoptotic properties. Moreover, it regulates MSC differentiation (Liu et al. 2014).

Therefore, this study aimed to investigate the therapeutic effect of MSCs, alone or after incubation with melatonin, in a rat model of DN, and the possible role of modulating Beclin-1 in controlling renal pathology in that model.

Materials and methods

Animals and study protocol

Forty adult male albino Wistar rats weighing 150 to 170 g were purchased from the Animal House, Faculty of Medicine, Cairo University, to be used in this study. The animals were housed in cages exposed to normal room temperature and humidity in normal light/dark cycles with free access to water and standard rat chow in the Institutional Animal Care Unit. All of the animal procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (7th ed.) using the National Institutes of Health protocol, and were approved by the Institutional Animal Care and Use Committee of Cairo University (CU-IACUC; No. CU/III/S/33/17).

The animals were assigned to four groups (10 rats each): control group, and 30 diabetic rats. Diabetes was induced by a single intraperitoneal injection of streptozotocin (50 mg/kg) dissolved in freshly prepared citrate buffer (0.1 mol/L; pH = 4.5) (Kaur et al. 2015). At week 6 after the induction of diabetes as previously described (Kaur et al. 2015), DN was indicated by the presence of urinary albumin excretion (UAE) measured over a 24 h period (UAE > 30 mg·day−1) (Gross et al. 2005). The DN rats were randomly assigned to 3 groups: (i) the untreated diabetic group that received vehicle only (DN); (ii) the MSC treated group (MSCs); (iii) DN rats treated with a single injection of 2 × 10^6 labeled MSCs per animal in 0.5 mL serum-free medium into the tail vein (Zhou et al. 2009). In the MT-preconditioned MSCs group (MSCs + MT), the MSCs were treated with melatonin (5 μmol/L) for 24 h before being injected into the rats (Mias et al. 2008).

Preparation of bone-marrow derived MSCs from rats

Under aseptic conditions, the bone marrow of both femurs and tibiae obtained from 2 rats of 150 g, was flushed out by a 20 gauge needle containing Dulbecco’s-minimum essential medium (DMEM; Gibco, Grand Island, New York, USA) (Alhadlaq and Mao 2004).

The mononucleated cell layer of bone marrow containing MSCs was separated using Ficoll–Paque (Pharmacia Fine Chemicals, Piscataway, New Jersey, USA) and centrifuged at 300 g for 25 min. Thereafter, the supernatant was removed and the pellet was suspended in DMEM supplemented with 10% fetal bovine serum (Gibco), penicillin–streptomycin (Gibco/BRL), and incubated at 37 °C with 5% CO2. After the initial 24 h, fresh medium was added to the cells, and then the medium was changed every 2–3 days. At 70%–80% confluence, the cells were detached using 0.25% trypsin–EDTA to each dish. The dishes were re-incubated for around 5 min to allow cell detachment. Then, an equal amount of culture medium was added to inactivate trypsin. The cells were centrifuged at 200 g for 5 min, re-suspended in serum-supplemented medium, and then re-incubated. The resulting cultures were referred to as first-passage cultures.

Characterization of the cultured MSCs

When the 4th passage was reached, surface marker monoclonal antibodies for CD34 FITC and CD105 PE (Beckman Coulter) were used to identify MSCs from other bone marrow cells by flow cytometry. Cell analysis was performed using CYTOMICS FC 500 Flow Cytometer (Beckman Coulter) and analyzed using CXP Software version 2.2. Most of the adherent cultured cells expressed...
CD105 (Figs. 1A and 1B); however, the majority of the adherent cells were negative for CD34. Thus we considered that the majority of adherent cells were MSCs (Bayati et al. 2013).

In-vitro study
To assess the effect of melatonin on MSCs, the MSCs were divided into 2 equal fractions: first, the untreated MSCs, and second, the MSCs that were treated with melatonin (5 μmol/L) for 24 h (Mias et al. 2008). The treated or untreated MSCs was injected in a single dose of (2 x 10^6 cells) per rat by intraperitoneal injection in rat’s tail vein (Abdel Aziz et al. 2014). Before injecting the cells into the rats, the cells were was extensively washed and examined using a cell proliferation assay.

M TT cell proliferation assay
Cell proliferation of MSCs incubated with MT was compared with the controls (non-incubated cells). Cell proliferation was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2.5-diphenyl tetrazolium bromide) cell proliferation kit (Trevigen Inc., Gaithersburg, Maryland, USA) as per the manufacturer’s protocol. Briefly, the cells were plated in tissue culture plates in 3000 cells well^–1 in a final volume of 100 μL of medium, and were allowed to attach for the full 24 h of the experiment. The MTT reagent was added at 10 μL per well and the plate was incubated for 2 h to allow for intracellular reduction of the soluble yellow MTT to the insoluble purple formazan dye. Detergent reagent was added to each well to solubilize the formazan dye prior to measuring the absorbance of each sample in a microplate reader at 570 nm. Cell proliferation was assessed as the percentage of cell proliferation compared with the untreated cells.

MSCs were harvested during the fourth passage and were labeled with PKH26. PKH26 is a novel fluorescent reporter molecule that incorporates into the cell membrane bilayer and exhibits clear, stable, red fluorescence in precisely labeled cells. The original function and cell viability are completely retained in the labeled cells. In this study, MSCs were labeled with PKH26 from Sigma Company (St. Louis, Missouri, USA): briefly, a 10 μmol/L dye concentration was used for a cell concentration of 2 x 10^6·mL^–1, then cells were mixed with the PKH26 reagent and the mixture was incubated at 25 °C for 2–5 min, and then gently mixed by rocking the tube forward and backward during the incubation time period. The staining action was blocked by adding the same volume of serum and incubating for 1 min. Then, the cells were centrifuged at 400g for 10 min at 25 °C. The supernatant was removed, and the cells were transferred into a new tube. After washing 3 times, 10 mL of complete culture medium was added, and cells were centrifuged. Then, the cells were adjusted to an appropriate density and were observed under a fluorescent microscope. The cells were then injected intravenously into the rat’s tail vain. Kidney tissues were later examined with a fluorescence microscope to detect and trace the cells.

Sample collection
Two weeks following MSC injection, as described by Zhou et al. (2009), and after overnight fasting, 24 h urine samples were collected from rats in the metabolic cages and was used to detect creatinine and UAE (Albuwell M Kit, Murine Microalbuminuria ELISA). The body mass was measured using a digital scale (Mettler-Toledo 200) and non-invasive systolic arterial blood pressure (SABP) was measured using a blood pressure transducer (ADInstruments), and the data were analyzed using Labchart 7 software.

Retro-orbital blood samples were collected under pentobarbital sodium anesthesia [50 mg·(kg body mass)^–1, by intraperitoneal injection (i.p.)] using a capillary tube, and the samples were separated by centrifugation at 200g for 10 min. Serum urea and creatinine levels, the gold standards for renal impairment, were directly measured using the conventional colorimetric method (QuantiChrom assay kit, BioAssay Systems, Corporate Place, Hayward, California, USA). Color intensity was measured at 520 nm [for blood urea nitrogen (BUN)] and 510 nm (for creatinine). Angiotensin II levels were assessed with an enzyme immunoassay kit (RayBio). Plasma glucose level was measured by the glucose oxidase method using a commercially available kit (Diamond, Egypt); insulin levels and the soluble advanced glycation end product (AGE) carboxymethyl lysine (CML) were measured using ELISA kits (Sigma).

Calculation of creatinine clearance (CCr)
The 24 h urine samples were centrifuged to remove debris and then the volumes were measured. CCr was calculated using the following equation (Khowailed et al. 2015):

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\text{CCr} = \frac{\text{urinary creatinine (mg·dL}^{-1}\text{)} \times \text{urine volume (mL)} \times 1000 (\text{g})}{\text{serum creatinine (mg·dL}^{-1}\text{)} \times \text{body mass (g)} \times 1440 (\text{min})}
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Scarification of the animal groups was performed under pentobarbital anesthesia [50 mg·(kg body mass)^–1, i.p.] (Araujo et al. 2016). Two weeks after administering the MSCs, the right kidneys were excised and used to determine the renal expression of transforming growth factor beta (TGF-β), tumor necrosis factor-alpha (TNF-α), interleukin10 (IL-10), superoxide dismutase (SOD), and Beclin.

Determination of TNF-α, IL10, SOD, TGF-β, and Beclin-1 in renal tissue
The concentration of cytokines, SOD, TGF-β, and Beclin-1 were measured in renal tissue homogenate using the sandwich enzyme linked immunosorbent assay (ELISA) kit according to the manufacturer’s instructions (R&D Systems, Minneapolis, Minnesota, USA). The measured protein present in the samples or standard binds to specific monoclonal antibodies adsorbed to the micro-wells. A biotin-conjugated monoclonal antibody was added. Following incubation, unbound biotin-conjugated antibody was removed during the wash step. Streptavidin–horseradish peroxidase (HRP) was added, which bound to the biotin-conjugated antibody; following incubation, unbound streptavidin–HRP was removed during the wash step, and a substrate solution that reacts with HRP was added to the wells. A colored product was formed in proportion to the amount of measured protein present in the sample. The reaction was terminated by the addition of acid, and absorbance was measured at 450 nm; the concentration was calculated from the standard curve.

Histological examination
The left kidneys were used for histopathological examination. Tissue sections from the paraffin blocks were cut at 4 μm thickness and stained with haematoxylin and eosin for histopathological diagnosis. Other sections were stained with Masson’s trichrome and periodic acid – Schiff reagent (PAS) to assess the levels of sclerosis. Glomerulosclerotic injury was semiquantitatively graded by examination of 10 fields for each specimen as follows: 0 = intact glomeruli (normal); 1 = lesions affecting <25% of the glomerular area; 2 = lesions affecting 25%–50% of the glomerular area; 3 = lesions affecting 50%–75% of the glomerular area; and 4 = lesions affecting >75% of the glomerular area (Elbe et al. 2015). Homing was detected in the unstained portions of the red fluorescent stained cryosections (5 μm thick) of renal tissue.

Statistical analysis
The data presented are the mean ± SD. Statistical analysis was done using the analysis of variance (ANOVA) test, followed by Tukey’s post-hoc test for multiple comparisons between groups. Independent sample t test was used in MTT test comparison between the two MSC-treated groups. Statistical significance was
considered for values of $P < 0.05$. The Pearson correlation test was used to determine the linear relationship between CCR and Beclin-1 (quantitative variables).

Results

Cell characterization and the proliferation assay test
In the flow cytometry histograms of the MSC marker, CD105 was highly expressed (red area, Fig. 1A; colour online only), and there was negligible expression of hematopoietic marker CD34 (Fig. 1B), so we considered these cells as multipotent mesenchymal stem cells. Compared with the control group, melatonin significantly increased cell proliferation (by 72.1%, $P < 0.05$) in the group pretreated with melatonin (Fig. 1C).

Effect of treatment with MSCs and MSC + MT on glycemic state, hemodynamics, and kidney function

Induction of diabetes resulted in a significant increase ($P < 0.001$) in fasting serum glucose levels and CML. A significant ($P < 0.001$) decrease in insulin levels was shown in the DN group, which was associated with significantly deteriorated renal function, as evidenced by increased levels of BUN, serum creatinine, UAE, and glomerular injury score ($P < 0.05$), accompanied with a significant decrease ($P < 0.001$) in CCR. Serum levels of angiotensin II (Ang II) and SABP showed significant increases ($P < 0.05$) compared with the corresponding values for the control group. Glucose and CML levels were significantly decreased and insulin levels were significantly increased in the treated groups by comparison with the DN group ($P < 0.001$), and there were greater decreases in CML levels in the groups treated with MSCs + MT compared with the group treated with MSCs only.

Treatment with MSCs significantly ($P < 0.05$) improved all measured renal function parameters. Interestingly, the group treated with MSCs + MT showed even greater improvement compared with the group treated with MSCs only. Ang II levels and SABP were significantly decreased in the treated groups compared with the DN group, and there were greater decreases in Ang II levels in the group treated with MSCs + MT compared with the group treated with MSCs only (Table 1).

MSCs and MSCs + MT treatments have anti-inflammatory and antioxidative effects

Induction of diabetes caused a statistically significant ($P < 0.001$) increase in pro-inflammatory TNF-α [127.31 ± 14.03 pg·(mg protein)$^{-1}$] compared with the control group [31.53 ± 4.24 pg·(mg protein)$^{-1}$], together with a significant reduction ($P < 0.001$) in the anti-inflammatory IL-10 [58.81 ± 7.05 pg·(mg protein)$^{-1}$] and antioxidative SOD [0.52 ± 0.06 μg·(mg protein)$^{-1}$], accompanied with the corresponding values in the control group [144.80 ± 13.51, and 3.30 ± 0.35 μg·(mg protein)$^{-1}$, respectively].

Following treatment, a significant reduction ($P < 0.001$) in TNF-α was observed in the groups treated with MSCs only and with MSCs + MT [77.59 ± 5.86 and 66.06 ± 3.0 pg·(mg protein)$^{-1}$, respectively], accompanied by a significant ($P < 0.001$) increase in the levels of IL-10 and SOD in the group treated with MSCs only (109.13 ± 9.00 pg·(mg protein)$^{-1}$) and 2.07 ± 0.2 μg·(mg protein)$^{-1}$, respectively; a greater increase ($P < 0.05$) in the level of SOD was observed in the group treated with MSCs + MT (2.60 ± 0.15 μg·(mg protein)$^{-1}$), which is not statistically different from the group treated with MSCs only (Figs. 2A–2C).

Treatment with MSCs and MSCs + MT suppresses profibrotic markers

In the DN group, there was a significant increase ($P < 0.001$) in TGF-β1 [145.63 ± 15.34 pg·(mg protein)$^{-1}$] compared with the control group [42.93 ± 4.52 pg·(mg protein)$^{-1}$], and this was significantly ($P < 0.001$) reduced by MSC treatment [71.49 ± 4.42 pg·(mg protein)$^{-1}$], and there were even greater decreases in the group treated with MSCs + MT [52.43 ± 3.88 pg·(mg protein)$^{-1}$], for which the results were not statistically different from the control group ($P = 0.37$) (Fig. 2D).

The effect of treatment with MSCs and MSCs + MT on the autophagy protein Beclin-1

The levels of Beclin-1 in the treated groups were significantly ($P < 0.001$) reduced in the DN group [1.44 ± 0.36 ng·(mg protein)$^{-1}$] compared with the control group [5.67 ± 0.58 ng·(mg protein)$^{-1}$]. In the treatment groups, injection of MSCs was associated with significant ($P < 0.001$) increases in the level of Beclin-1 [3.8 ± 0.84 ng·(mg protein)$^{-1}$] by comparison with the DN group. Greater expression of Beclin-1 ($P < 0.05$) was found in the group treated with MSCs + MT [6.3 ± 0.78 ng·(mg protein)$^{-1}$], compared with the group treated with MSCs only (Fig. 3A).

Correlation between autophagy-protein expression and CCR

Creatinine clearance was strongly positively correlated to the increase in relative gene expression of Beclin-1 ($0.79$, correlation is significant by 0.01 (2-tailed)) (Fig. 3B).

Histological evaluation of the renal tissue and homing of MSCs

Figure 4 shows the homing of red fluorescence-labeled MSCs in the treated groups (hematoxilin and eosin, PAS, and Masson’s trichrome stains). There were glumerulosclerotic changes observed in the kidney tissues obtained from the untreated diabetic rats (Figs. 5D–5F), compared with the control group (Figs. 5A–5C), but these changes were more or less reversed in the groups treated with MSCs (Figs. 5G–5I). Greater improvements in renal structure were observed in the MSCs + MT treatment group (Figs. 5J–5L).
Discussion

From the results of this study, we found that in a rat model of DN, treatment with MSCs produced significant improvements in kidney function and ameliorated the underlying pathogenic conditions. Furthermore, when the injected MSCs were pre-incubated with MT, a greater significant improvement in the measured renal function parameters was found. This improvement was significantly correlated with the activation of the autophagy pathway.

Indeed, in this study, there was marked deterioration in renal function in the untreated DN group. The decreased CCr in DN suggests chronic ischemia and blood flow impairment, which may be caused by intrarenal vasoconstriction secondary to local activation of the renin-angiotensin system (Nangaku 2005). Additionally, it is proposed that hyperglycemia also causes upregulation of Ang II type 1 receptor, which can increase its sensitivity and subsequently cause hypertension (Miller et al. 2006).

The injected MSCs produced significant improvements in the blood sugar parameters, as indicated by their capacity to generate insulin-producing cells (IPCs). These IPCs express multiple genes related to the development or function of pancreatic beta cells, including the secretion of pancreatic insulin (Xie et al. 2009). As a consequence, there is improvement of the diabetic condition, and
protection from renal damage in diabetic mice has been observed (Ezquer et al. 2008).
Administration of MSCs resulted in marked reversal of the increase in Ang II levels; this could be explained, in part, by reducing reactive oxygen species (ROS) generation and ameliorating vascular endothelial dysfunction, thereby improving the hemodynamics (Baber et al. 2006).

The use of MSCs for cell therapy depends on the capacity of these cells to home, engraft, and trans-differentiate into the cell phenotype of host’s tissue to be repaired (Morigi et al. 2008). In addition, MSCs exert renoprotection via self-differentiation and local paracrine secretion of mitogenic and vasculotropic mediators, which limit renal injury and apoptosis and help the engrafted cells to survive and encourage regeneration (Tögel et al. 2007).

Our results as well as others have reported limited engraftment and differentiation under in-vivo conditions. This may be a result of excessive inflammation and oxidative stress in the host’s diseased tissues, presenting obstacles to the advancement of MSCs in DN therapy (Morigi et al. 2008; Bruno et al. 2009). We attempted to intensify the capacity of MSCs to home to and survive in injured renal tissue, and to enhance their regenerative activity. This was investigated by ex-vivo pretreatment with MT. Melatonin, through its antioxidant activity (Winiarska et al. 2016), protects cells from apoptosis and damage caused by the pathological environment of diseased tissue.
Interestingly, pre-incubation with MT (5 μmol·L⁻¹) for 24 h, resulted in a significant increase in the in-vitro cellular proliferation of the pretreated MSCs compared with the untreated MSCs, as indicated by the results of the functional proliferation assay test. MT has great potential to eliminate ROS (Zhang et al. 2010) and regulate intracellular signaling (Luchetti et al. 2010). Similarly, MT preserves the proliferative power of MSCs and reduces their senescence and dysfunction (Shuai et al. 2016).

The ex-vivo MT treatment functioned as a preconditioning agent that increases paracrine secretions favorable to MSCs activity and decreases the risk of early death of the engrafted MSCs in ischemic renal tissue (Mias et al. 2008). These beneficial effects of MT do not require concomitant injection of MT with the MSCs, as the effects were observed after extensive washing of MSCs before injecting them into the rats.

MT-pretreatment of cells enhanced the structural and functional recovery of the kidney tissue in our DN model. This was demonstrated by the greater improvements in renal function compared with the group treated with MSCs only, suggesting that MT preserved the stemness of MSCs after being engrafted into the diseased tissue.

Although there was not significant difference in the blood glucose or insulin levels in the group treated with MSCs + MT compared with the group treated with MSCs only, it was found that CML, the marker for AGE, was significantly reduced in the MT incubated MSCs than the group treated with MSCs alone. AGEs are known to cause renal damage under hyperglycemic conditions (Ojima et al. 2013). This important effect can explain the beneficial effect of MT pretreatment in limiting renal damage in diabetic rats.

In this work, MSC treatment down-regulated the kidney expression of TGF-β. There was a significant decrease after MT incubation compared with the group treated with MSCs only. Down-regulation of the fibrogenic factor TGF-β might contribute to the reduction in tubulointerstitial fibrosis and glomerulosclerosis, as described in the study done on DN by Abdel Aziz et al. (2014) and on the streptozotocin kidney of MSC-treated pigs (Eirin et al. 2012).

An important finding in our study is treatment with MSCs caused up-regulation of the autophagy protein Beclin-1. Inhibition of autophagy in diabetes was evidenced by fewer autophagosomes and decreased Beclin-1 protein (Ding and Choi 2015). Hyperglycemia promotes the interaction between Beclin-1 and Bcl-2 inducing Beclin-1 to suppress apoptosis and autophagy (He et al. 2013). The exact mechanism by which MSCs activate autophagy in kidney tissue is still unknown; however, it may be achieved through decreasing the hyperglycemia-induced oxidative stress in renal tissue.

Eirin et al. (2012) stated that both renal oxidative stress assessed by the in-situ production of superoxide anion and systemic oxidative stress were decreased in MSC-treated pigs. The antioxidant power of MSCs may be achieved through decreased expression of NAD(P)H-oxidase and peroxynitrite, and increases in SOD and glutathione peroxidase (Lanza et al. 2009).

MSC treatment down-regulated the pro-inflammatory cytokine TNF-α and up-regulated the anti-inflammatory and organ-protective IL10 in kidney tissue.

IL10 can be produced by MSCs and secreted in a paracrine fashion (Semedo et al. 2009). Moreover, MSCs help to improve the environment for the engrafted MSCs by reshaping the immune response through inducing conversion of T lymphocytes from a proinflammatory Th1 to the anti-inflammatory Th2 state, increasing the release of IL10, and decreasing the secretion of TNF-α (Asanuma et al. 2010).

In conclusion, our data indicates that MSCs are a new target in the management of diabetic nephropathy via modulation of underlying pathogenic factors, and activation of autophagy. Furthermore, preincubating MSCs with MT improves their viability and intensify their efficiency, and so better renal functions were obtained.


