ABSTRACT

Objective: To investigate the role of mesenchymal stem cells in fibrogenesis using a model of chronic renal insufficiency. Methods: Mesenchymal stem cells were obtained from tibias and femurs of Wistar-EPM rats. After three to five passages, the cells were submitted to phenotypic analyses and differentiation. Wistar rats were submitted to the 5/6 nephrectomy model, and 2.10⁵ mesenchymal stem cells were administered intravenously to each rat every two weeks until the eighth week. Results: Sex-determining region Y was observed in female rats treated with stem cells. Serum and urine analyses showed improvement of functional parameters in mesenchymal stem cells treated animals, such as creatinine, serum urea, and proteinuria. Moreover, hemocrit analysis showed improvement of anemia in mesenchymal stem cell treated animals. Masson’s Trichromium and Picrosirius Red staining demonstrated reduced levels of fibrosis in mesenchymal stem cells treated in animals. These results were corroborated by reduced vimentin, collagen I, TGFβ, FSP-1, MCP-1 and Smad3 mRNA expression. Renal IL-6 and TNFα mRNA expression levels were significantly decreased after mesenchymal stem cells treatment, while IL-4 and IL-10 expression were increased. Serum expression of IL-1α, IL-1β, IL-6, IFN-γ, TNF-α, and IL-10 was decreased in mesenchymal cell-treated animals. Conclusions: Altogether, these results suggest that mesenchymal stem cells therapy can indeed modulate the inflammatory response that follows the initial phase of a chronic renal lesion. The immunosuppressive and remodeling properties of the mesenchymal stem cells may be involved in the improved fibrotic outcome.

Keywords: Mesenchymal stem cells; Renal insufficiency, acute; Rats, Wistar
INTRODUCTION

The prevalence of chronic renal disease (CRD) has been growing worldwide. Global Kidney Day campaigns (2007 and 2008) highlight the relevance of the theme\(^\text{(1-2)}\). It is estimated that 5 to 9% of the entire adult world population has some type of renal damage, with high mortality in cardiovascular patients who also have CRD.

Among the consequences of CRD are loss of the kidney, complications resulting from a decrease in glomerular filtration rate (GFR) and an increased risk of cardiovascular diseases. Complications resulting from the reduction of GFR include hypertension, anemia, malnutrition, bone disorders, and mineral imbalances, neuropathies, and decreased quality of life\(^\text{(3)}\). Progression to CRD may lead to end-stage renal disease (ESRD), which requires transplantation or dialysis\(^\text{(4)}\).

In transplantation, time-dependent complications also lead to a clinical picture of ESRD, called Chronic Graft Nephropathy (CGN). All the stages of CRD are present, but they are more accelerated, and 10 years after transplant, ESRD is present in 58.4% of cases, with sclerosis in 37.3% of the glomeruli\(^\text{(5-6)}\).

Although the pathogenesis of progressive renal disease (PRD) is greatly complex, it is worth pointing out that regardless of the etiology of the original disease, PRD results from a pathogenic process that leads to a common final route in which glomerulosclerosis and interstitial fibrosis apparently have fundamental roles\(^\text{(7-9)}\).

Mesenchymal stem cells (MSCs) were initially isolated in 1976 by Friedenstein et al.\(^\text{(10)}\) as bone marrow cells with the capacity of forming colony units similar to fibroblasts \textit{in vitro} (CFU-F). In 1991, Caplan defined the term MSC as a cell capable of originating other cell strains different from their origin\(^\text{(11)}\). In 2005, with nomenclature standardized, the Committee for Mesenchymal Stem Cells and Tissues of the International Society for Cellular Therapy (ISCT) defined MSCs as:

- MSCs should adhere to plastic when maintained in cultures;
- MSCs should express CD105, CD73, and CD90 and not express markers CD45, CD34, CD14, or CD11b, CD79a or CD19 and HLA-DR;
- MSCs should differentiate into osteoblasts, adipocytes and chondroblasts \textit{in vitro}\(^\text{(12)}\).

Specifically in nephrology, MSCs have been largely used in experimental models of acute renal damage (ARD), of glomerulonephritis, and of unilateral ureteral obstruction.

Many of the studies carried out with stem cells (SCs) sought to analyze the role of these cells in aiding tubular regeneration after tubular damage, whether toxic or ischemic. For this, they performed ablation of the bone marrow in an animal and reconstituted it with cells marked with GFP, β-Galactosidase (β-Gal), or performed the reconstitution in animals of different genders (male $\mapsto$ female), thus investigating the presence of the Y chromosome.

The research projects that really used SCs as therapy, i.e., administering SCs from an exogenous source for ARD, demonstrated excellent results. Morigi et al. tested the hypothesis that treatment with MSC, or bone marrow hematopoietic stem cells (HSC), could improve renal function and attenuate tubular lesion in animals with acute toxic renal insufficiency (ARI). Female mice receive intravenous MSC or HSC from male animals one day after an injection of cisplatin. Analysis using hybridization with the Y chromosome showed the presence of MSC in the region of the proximal renal tubules, expressing surface markers with lectin (\textit{Lens culinaris}), indicating migration and repair of the damaged kidney. The MSCs, but not the HSCs, were capable of inducing tubular proliferation and restoring renal archiecture\(^\text{(13)}\). Duffield et al. injected MSC intravenously in mice and noted an improvement in the ARI. No MSC was found in the kidney. It was suggested that MSCs might aid in repair by modulating the inflammatory response, since MSCs disappear rapidly from circulation and may be ingested by cells of the immune system of the spleen and liver\(^\text{(14-15)}\).

Despite yet uncomprehended mechanisms, it has been suggested that they might regenerate tissues by secreting trophic factors, by fusing with damaged cells, or by differentiating into new resident renal cells. However, most of the results indicate that the curative effects of exogenous MSCs are not explained by direct repopulation/fusion of SCs to the tubules\(^\text{(14,16-17)}\). The
time of analyses carried out on animals, in most studies, was 24 to 48 hours, which is too quick for such protective effects to be justified by transdifferentiation/fusion of extrarenal cells into epithelial cells\cite{26,28-29}.

Paracrine factors secreted by MSC may explain the beneficial effects in acute renal lesions. Togel et al. found significant levels of VEGF, HGF, and IGF-I in the kidneys of animals treated with MSCs\cite{26,28,29}. Another factor that may justify the functional improvements noted after the administration of MSCs is the immunomodulating role of the SCs, since damage of ischemia and reperfusion (IR) displays a clear inflammatory picture, as mentioned before. High levels of anti-inflammatory cytokines were found in renal tissues of animals treated with MSC after renal ischemia and reperfusion\cite{21-22}.

The role of MSCs in the process of renal fibrogenesis is not known. In experimental models of Alport’s Syndrome, some results were encouraging. It is known that collagen IV is the main component of the glomerular basement membrane. Alport’s Syndrome results from mutations in collagen IV. Therefore, researchers made a knockout animal for collagen IV chain A3 (Col4A3 -/-), thus creating an animal model for Alport’s Syndrome. In Col4A3-/- animals, bone marrow transplantation provenient from normal animals resulted in an improvement of histological and functional parameters, with a significant reduction in proteinuria levels, leading to a partial restoration of expression of collagen IV alpha 3 chain, concomitant with the expression of collagen IV alpha 4 and 5 chains, improving glomerular structure\cite{20}.

In another study, researchers administered weekly MSCs using the same Alport’s Syndrome model (Col4A3 -/-) and the data indicated that there was a repair in the defective basement membrane, besides prevention of a loss of peritubular capillaries and a reduction of fibrosis, although the improvement in functional parameters, creatinine dosing, proteinuria, and survival curve, was not obtained\cite{22}.

In other solid organs, there are more experimental studies. In models of chronic cardiac disease, the administration of the conditioned MSC medium decreased fibrosis, indicating a paracrine effect of MSCs in vivo. When this phenomenon was analyzed in vitro, there was a decrease in expression of collagen I and III and a decrease in proliferation of cardiac fibroblasts when maintained in the conditioned culture medium\cite{23}.

The administration of MSCs in a model of chronic hepatic disease also resulted in a lower expression of collagen and an improvement of functional parameters with an elevation of serum albumin levels and a drop in serum ALT (alanine aminotransferase) levels\cite{24}. The authors suggest that this improvement results from the engraftment of MSCs in the damaged region leading to a change in the microenvironment of the fibrosed hepatic tissue. With their capacity to modulate various responses, the MSCs could further modulate the HSC. When activated, HSCs are the largest sources of collagen secretion, besides generating an imbalance among metalloproteinases\cite{27-28}. In an effort to analyze the mechanism responsible for this protection, Parekkadan carried out an assay of co-culture between MSCs and resident liver cells, the hepatic stellate cells. In this indirect co-culture system, once activated in contact with the MSCs, the stellate liver cells express less collagen and display less proliferation. In addition, activated hepatic cells displayed greater death by apoptosis, indicating, therefore, that MSCs can modulate hepatic cells by means of a paracrine route\cite{29}.

Models of pulmonary fibrosis were also studied and again, a reduction was observed in the deposition of collagen and in the decrease of the inflammatory scenario after treatment with MSCs\cite{30-31}. Further, another group demonstrated that after administration of MSCs in the chronic pulmonary disease model, the MSCs did not take on the phenotype of pulmonary cells, but it was also noted that the protection was mediated by a decrease in the inflammatory picture, leading to the expression of growth factors\cite{32}.

On the other hand, Kunter et al. analyzed if the MSCs were capable of preserving the renal structure in the renal model of glomerulonephritis, induced by the anti-Th1.1 antibody. Precociously, the MSCs induced a functional and histological improvement in the animal model. Nevertheless, on the long range, adipose vesicles appeared in the glomerulus. It is likely that the MSCs maldifferentiated into adipocytes, while the scenario of glomerular sclerosis was setting in\cite{33}.

Recent studies, however, have demonstrated functional and structural improvement in treatment with MSCs and negative strain cells (Lin-) therapeutically in another model of CRD, the 5/6 nephrectomy model. Punctual treatment with bone marrow cells or even MSC in the renal parenchyma resulted in a greater GFR and a decrease in proteinuria, with no histological changes\cite{34}. Whereas treatment with Lin- cells resulted in an improvement in indices of proteinuria, glomerulosclerosis, anemia, infiltration of immune cells in renal tissue, and less expression of MCP-1\cite{35}. In a similar paper, albeit using a different 5/6 nephrectomy model (in this experiment, the 5/6 model was obtained by total nephrectomy of the right kidney, and the left kidney was previously clamped for 40 minutes, followed by reperfusion and ablation of half the kidney), Choi et al. also observed an improvement in proteinuria and a lower index of glomerulosclerosis in animals treated with MSCs after four months\cite{36}.
Therefore, in CRD models, the role of MSCs is not clear\(^{(37)}\). Studies published in literature are few and controversial. Seeking to analyze the role of MSCs in CRD, we administered MSCs in the 5/6 nephrectomy model and evaluated the inflammatory and fibrotic mechanisms, clearly characteristic of PRD, which would be related to the modulation of the fibrogenic process through the MSCs.

**OBJECTIVE**

To analyze the immunomodulation and antifibroblastic role of MSCs in a model of chronic renal disease, renal ablation.

**METHODS**

**Isolation and characterization of the MSCs**

Male Wistar rats, weighing approximately 150 g each, were euthanized and had the bone marrows from the femur and tibia removed. The bone marrow was washed with sterile PBS and accommodated in a sterile 15 ml tube. Using laminar flow, the material collected was washed three times with sterile PBS (900 g, two minutes). The material was resuspended in 4 ml of DMEM-low glucose medium (Gibco/Invitrogen, Carlsbad, CA, USA). To separate the mononuclear fraction of the bone marrow, the resuspended material was submitted to the Histopaque protocol (Sigma-Aldrich, St. Louis, MO, USA). Next, 4 ml of the material collected and centrifuged at 400 g for 30 minutes was gently added to 4 ml of Histopaque. After centrifugation, an interface is formed between the Histopaque and the DMEM medium containing the mononuclear cells. These are selected and washed with PBS five times (900 g, five minutes). The pellet formed was then resuspended in DMEM-low glucose, supplemented with 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil). Then, 2x10\(^5\) cells per mm\(^2\) of surface were plated. The cells were maintained in a culture with 5% CO\(_2\), at 37 ºC, for 10 to 15 days.

**Immunophenotyping of MSCs**

The MSCs were removed from the culture plates using 10% trypsin and counted in a Neubauer chamber. Approximately 5x10\(^6\) cells were incubated for 30 minutes, at 4 ºC with the antibody to mark surface CD45 cells (BD Biosciences, San Jose, CA, USA), CD44 (BD), CD73 (BD), CD29 (BD), CD 31 (BD), CD34 (Santa Cruz, Santa Cruz, CA, USA), and CD 90 (BD), at a dilution of 1:10. After this, the cells were washed twice with PBS and fixed (1% paraformaldehyde, 0.1% sodium azide and 0.5% SFB). The cells were then analyzed in a FACSCalibur™ flow cytometer (BD).

**Differentiation of MSCs in rats**

As to differentiation assays, two plasticity tests were used to functionally characterize the SCs. Adipogenic differentiation: first or second passage MSCs were cultivated in 6-well plates with a DMEM culture medium until they reached confluence. At this point, the DMEM culture medium was exchanged for the adipogenesis induction culture medium consisting of DMEM medium (high glucose, 4.5 g/l) supplemented with 1 µM dexamethasone (Sigma), 10 µM indomethacin (Sigma), 10 µg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma) and 10% SBF, for three days. Next, the adipogenesis induction medium was exchanged for a maintenance medium - DMEM (high glucose, 4.5 g/l) supplemented with 10 µg/ml insulin and 10% SBF. This stimulation was repeated twice, for a total of 18 days of treatment. The control cells were cultivated in DMEM medium supplemented only with 10% SBF. Levels of differentiation were examined by microscopy and histological tests. Analysis of the expression of genes specific for adipogenesis, such as PPAR\(_{\gamma}\), was performed on the 18\(^{th}\) day of differentiation. Osteogenic differentiation: osteogenic differentiation was induced in the first or second passage of MSC cultures. The MSCs were incubated in a culture medium containing 0.1µM dexamethasone, 10 mM sodium \(\beta\)-glycerophosphate, and 0.2 mM ascorbic acid (Sigma). The medium was changed every 3 days and the cells maintained in culture for 24 days. The level of osteogenic differentiation was analyzed by microscopy using Von Kossa staining.

**Remanescent kidney model (5/6 nephrectomy)**

Operative technique: in animals anesthetized with ketamine/xylazine, laparotomy was performed with exposure of the left kidney. Before surgery, retro-orbital blood was collected. Two of the three branches of the left renal artery were ligated, for ischemia of approximately 2/3 of the kidney. After two weeks, the same animal was submitted to right nephrectomy. The group of animals treated with MSCs received 2x10\(^5\) MSCs intravenously (tail vein) at the 2\(^{nd}\), 4\(^{th}\), and 6\(^{th}\) weeks. The animals were euthanized at the 8\(^{th}\) week. The use of the animals was approved by the Ethics Committee of UNIFESP (2006/0210).

Assessment of renal function: creatinine in the serum and urine was analyzed with Jaffe’s method, whereas serum urea was dosed with the Urease kit (Labtest). In order to quantify 24-hour protein in the
urine, the samples were submitted to the Sensiprot kit (Labtest). Serum albumin was dosed with the intention of indirectly observing the excretion of albumin as well as assessing the nutritional state of the animals and their hepatic function. For this, the albumin colorimetric kit (Labtest) was used. Serum glucose levels were dosed using the Glucose PAP Liquiform test (Labtest). Total cholesterol was measured in sera of the animals at the eighth week with the Cholesterol Liquiform test (Labtest).

Inulin clearance: to determine GFR, inulin clearance assays were conducted. On the day the animals were sacrificed (eighth week), they were anesthetized intraperitoneally with pentobarbital (50 mg/kg of body weight). The trachea was cannulated with a PE-240 catheter, and the animals were maintained on spontaneous respiration. In order to preserve arterial pressure and allow the collection of blood, a PE-60 catheter was inserted into the right carotid artery. For infusion of inulin and fluids, another PE-60 catheter was inserted in the left jugular vein. In order to collect urine samples, a suprapubic incision was made and the bladder was cannulated with a PE-240 catheter. After the surgical procedure was complete, a dose of inulin (100 mg/kg of body weight diluted in saline solution) was administered through the jugular vein. Subsequently, a constant infusion of inulin was initiated (10 mg/kg of body weight in saline solution) which remained until the end of the experiment, at a rate of 0.04 ml/min. Three urine samples were collected with 30-minute intervals. The blood samples were obtained at the beginning and end of the experiment. Blood pressure was measure at the beginning of the experiment, as well as the hematocrit. Serum and urine inulin was determined by the antrona method. The GFR was expressed in ml/min/100 g.

Analysis of histomorphometric fibrosis
The kidneys were removed in aseptic, but not sterile, conditions, with the animals under the effect of the anesthetic (Ketamine-Xylazine). After sectioning and removing the renal capsule, the kidney was cut and fixed in 10% buffered formaldehyde until blocking in paraffin. Fragments of 3 μm were stained with Masson’s trichromium in which interstitial fibrosis was characterized by the expansion of the cellular matrix with distortion, collapse, and thickening of the basement membrane. Additionally, 3 μm slices were stained with Picrosirius in order to quantify collagen I and III present in the kidneys. The slides were also stained with Schiff’s periodic acid stain (PAS) for analysis of glomerulosclerosis. The slides were analyzed by pathologists in a blind assay.

Analyses of genic transcripts
The renal tissue was macerated in Trizol and processed according to the manufacturer’s procedure. The cDNA was obtained by means of the M-MLV Reverse Transcriptase enzyme (Promega). To amplify the genic transcripts, the following primers were used: TaqMan (Applied Biosystem) for vimentin (Rn00579738_m1), TIMP-1 (Rn01430875_g1), Mmp-9 (Rn01423075_g1), fibronectin (Rn01401510_m1), HO-1 (Rn00561387_m1), IL-1β (Rn00580432_m1), IL-4 (Rn01456866_m1), IL-6 (Rn00561420_m1), IFNγ (Rn00594078_m1); IL-10 (Rn00563409_m1), TNFa (Rn99999017_m1), HGF (Rn00690368_m1), HPRT (Rn01527838_g1).

Analysis of animal sera cytokines
The panel used was the Rat 9 Plex (Bio-Rad, Hercules, CA, USA) to analyze cytokines IL-1β, IL-1α, IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN-γ, TNF-α. The assay was developed as per the manufacturer’s protocol.

Statistical analysis
Data were presented on graphics, and showed means and standard deviation (SD). Stundent’s t-test, Mann-Whitney’s test and ANOVA were used to compare data. Differences were considered statistically significant with a p-value less than 0.05.

RESULTS
MSC isolation and culture
Rats of approximately 120 to 150 g (four weeks) were utilized for isolation of MSCs. Figure 1 shows that in cultures, MSCs form colonies and CFU-Fs, which also characterize the acquisition of these cells. The expression of CD44, CD45, CD31, CD34, CD29, CD73, and CD90 was analyzed by flow cytometry. As described, the cells were incubated with a medium that induced the differentiation into adipocytes, and were later stained by the Oil Red method that allows visualization...
of adipose vesicles present in the cells. The cells were also differentiated into osteocytes. Using Von Kossa’s stain, we were able to analyze some cells with calcium deposits (Figure 3).

![Image of adipose vesicles and osteocytes]

**Figure 1.** Mesenchymal stem cells isolated from the bone marrow of male rat tibias and femurs. In (A), MSCs during the first passage. In (B), MSCs in the second passage. In (C), image of MSCs cultivated for 4 weeks and stained with Harris’s hematoxylin, the formation of colonies.

**Figure 2.** Analysis of the expression of CD44, CD45, CD34, CD29, CD31, CD73, AND CD90. In (A), a cell population (P1) selected for analysis used in all the preparations. From (B) to (H), the analysis of cells in histograms, assessing the intensity of fluorescence, looking for the presence of the fluorophore according to the antibody used. The light gray line shows the profile of non-marked MSCs, and in black, the same MSCs marked with the conjugated antibody. In (B), CD90; in (C), CD29; in (D), CD44; in (E), CD34; in (F), CD45; in (G), CD73, and in (H), CD31.

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**Functional analysis**

We noted a significant decrease in the serum levels of creatinine and urea in the animals treated with MSCs, as well as a reduction in the ratio between urinary protein and creatinine. The values represent mean ± standard deviation with n = 7 and sham = 5 (p < 0.05). For the inulin clearance a group of four animals was used. However, inulin clearance showed no significant difference despite displaying a small increase in the animals treated with MSCs (0.25 ± 0.04 0.34 ± 0.009 ml/min/100 g, respectively, p = 0.25). The results are represented in Figure 4 (A, B, C and D).

![Figure 3. RAT MSCs differentiated into adipocytes (A) stained by Oil Red and its control (B). At C, MSC differentiated osteocytes, stained by Von Kossa and its control in D.]

In order to verify if the treatment led to systemic modifications, we analyzed biochemical parameters such as serum albumin, total cholesterol and blood glucose. Also measured were pressure data and hematocrit. Pressure data and those of serum albumin and blood glucose showed no statistical differences, although the hematocrit of treated animals proved significantly higher, as may be noted on Table 1. Interestingly, total cholesterol was reduced in animals treated with MSC at the eighth week (Table 1).

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**Histomorphometric analyses and expression of fibrosis-related molecules**

Since treatment resulted in a functional improvement, we analyzed if this improvement was reflected in modifications of the fibrosis pattern. For this, the degree of fibrosis was quantified by different histological techniques.
Immunosuppressive and remodelling properties of mesenchymal stem cells in a model of chronic kidney disease

The morphometric analyses were performed by a pathologist in a blinded assay. The values are arithmetic means ± standard deviation, with n = 7 for each group and n = 5 for the shams, p < 0.05. Masson and Picrosirius stains allowed the quantification of collagen fibers and PAS evidenced the glomerulosclerosis. Treatment with MSCs in the 5/6 nephrectomy model led to a significant reduction in tissue fibrosis. These results are demonstrated on Figure 5 (A-C), besides images representative of each condition (Figure 5, D-I).

The animals’ kidneys cDNA was obtained and submitted to amplification of certain genes quantitatively by Real Time PCR. The values are arithmetic means ± standard deviation, normalized by the expression of HPRT and calculated by ∆∆Ct relative to a control animal with n = 5 for each group, p < 0.05. The pattern observed in the histological analysis was repeated in the quantification of molecules related to the process of fibrosis. The renal tissues of animals treated with MSC showed lower levels of vimentin (Figure 6A), collagen 1 (Figure 6C), TGF-β (Figure 6E), FSP-1 (Figure 6F), Smad 3 (Figure 6H), and MCP-1 (Figure 6I). The other molecules analyzed, collagen 3 (Figure 6D), α-SMA (Figure 6G) and fibronectin (Figure 6J) showed a tendency towards lower expression when compared to non-treated animals, but with no statistical significance. Tissue remodeling also showed modifications, since the TIMP-1/MMP-9 ratio was well reduced in Nx animals treated with MSCs (Figure 6B).

Presence of MSCs in renal tissue
As the model was developed in female animals that received MSCs from male animals, we sought the expression of the SRY gene in renal tissue. As can be observed in Figure 7, most of the animals of the group displayed SRY expression, although they had all received the same treatment with the same MSCs at each administration, suggesting a disparity in expression.
Since the MSCs are found in renal tissue, we analyzed some molecules that could possibly be modulating this response. We studied some antifibrotic molecules, such as HGF, Smad7, BMP-7, and HO-1. Of these, only HGF and HO-1 displayed significantly greater expression in the renal tissue after treatment with MSC (Figure 8). The presented values are arithmetic means of $\Delta\Delta$CT ± standard deviation, normalized by the expression of HPRT and calculated by $\Delta\Delta$Ct in relation to a control animal, with n = 5 for each group, p < 0.05.

Analyses of the expression of cytokines in renal tissue and sera of animals

Considering the immunomodulating role of MSCs, we quantified some cytokines in the renal tissue of animals treated or not treated with MSCs. The values presented are arithmetic means of $\Delta\Delta$CT ± standard deviation, normalized by the expression of HPRT and calculated by $\Delta\Delta$Ct in relation to a control animal, with n = 5 for each group, p < 0.05. We observed that IL-6 and TNF-α are expressed less in the renal tissues of animals treated with MSCs (Figures 9, B, and F; respectively). Inversely, cytokines Th2 (anti-inflammatory) showed a
greater expression among those analyzed, IL-4 (Figure 9C) and IL-10 (Figure 9D).

After eight weeks the animals were sacrificed and their serum was collected and analyzed by Multiplex. Therefore, in analyzing the profile of cytokines in the sera of animals, we noted that all the cytokines analyzed showed less expression in animals treated with MSCs (Figure 10). The

In this aspect, the administration of MSCs has been increasing as an attractive perspective. The advantage of using MSCs is due to the ease in obtaining them, their rapid expansion in culture, and especially from the fact that MSCs are not recognized by the acquired immune system, allowing allogenic transplantation without the risk of rejection, due to the low expression of Class I and II MHC (43-45). However, the most attractive feature of therapy with MSCs is their anti-inflammatory, regenerative, and immunosuppressive capacities (43,45-46).

In this project we observed an improvement of the levels of creatinine and urea, as well as of proteinuria with the treatment using MSCs in the 5/6 Nx model, indicating less kidney tissue damage in the animals. This was reflected in the levels of the hematocrit that were much higher in animals treated with MSCs, which could suggest that in the animals treated with MSCs there is an improvement in tissue structure that guarantees the expression of erythropoietin, reversing the scenario of anemia that is characteristic of the chronic model. This concurs with our morphological results in which we observed a small area of fibrosis and less glomerulosclerosis.

Pressure levels still remained high. The 5/6 Nx model led to the ablation of ~80% of the animals' nephrons. The adaptation of the remaining nephrons to the new condition is inevitable, and a consequence of this is the maintenance of high pressure indices in these animals, regardless of the treatment. However, with the administration of MSCs, the renal structure is not as damaged as in 5/6 Nx animals not treated with MSCs, which was observed by less interstitial fibrosis and primarily by the lower degree of proteinuria. New experiments combining anti-hypertensives with MSCs may lead to an improvement of the results obtained.

By means of the analysis of MCP-1, it can be inferred that there is less mononuclear inflammatory influx in animals treated with MSC since MCP-1 is a molecule that attracts monocytes/macrophages. Tissue remodeling also is altered, since the TIMP-1/MMP-9 ratio is reduced in animals treated with MSCs, indicating that the renal tissue expresses less TIMP-1 and more MMP-9, thus maintaining the renal structure. In analyzing the presence of MSC in the 5/6 Nx kidney, we noted that the MSCs were in the kidney. Likely the more frequent administration enabled the MSCs to be found in the renal tissue, as we could observe the presence of SRY in the kidneys of females treated with male MSCs. HGF and HO-1 are molecules expressed by MSCs, and their effects on fibrosed renal tissue are well-known.

HGF inhibits the fibrotic progression, as has already been demonstrated by various studies in literature (47, 50). In our project, we observed high levels of HGF expression in the animals treated with MSCs, probably

Figure 10. Serum quantification of cytokines. There was a significant decrease of all levels of cytokines of the animals treated with MSC. In (A) IL-1 a; (B) IL-1 b; (C) IL-6; (D) IL-10; (E) IFNg, and (F) TNFa.

values represent arithmetic means ± standard deviation, with n = 4 for each group, p < 0.05.

DISCUSSION

Today, CRD may be considered an epidemic, with high mortality and morbidity rates, despite all the advances already obtained in these areas (1,39-41). Thus, alternative therapeutic forms are necessary.

As of 1999, studies with SCs became well-known due to their therapeutic potential. Much was made of the use of SCs in various diseases. There was a focus on embryonic SCs since these have the capacity for pluripotency. However, with studies on plasticity and the absence of immune response, the MSCs became attractive for therapeutic uses.

Studies of SCs and the kidneys began in 2001, with the research of Grimm et al. who analyzed renal transplantation biopsies of six male receptors of organs coming from female donors, with grafts that had a diagnosis of CGN (42). Interestingly, they observed that the kidneys of female donors had cells positive for the Y chromosome when analyzed by in situ hybridization. Further, these cells also were positive for α-SMA (smooth muscle actin), a marker of myofibroblasts and of mesenchymal cells, including MSCs.
expressed by the MSCs present, which could lead to inhibition of the fibrotic process in the kidneys of 5/6 Nx animals. Additionally in our model, HO-1 shows greater expression in animals treated with MSCs. Since the MSCs are found in renal tissue, and we know that HO-1 is well expressed by MSCs, we may infer that the increase in HO-1 is a consequence of the MSCs present in the kidney, as well as HGF.

The immunomodulating role of MSCs is well recognized in in vitro assays. MSCs increase the proportion of regulator T-cells CD4+CD25+, increase the secretion of IL-4, induce the production of IL-10, delay maturation of antigen-presenting cells (APCs), and decrease the expression of HLA-DR in APCs. This action may influence the development of the epithelial-mesenchymal transition (EMT). Chuang et al. analyzed the role of cytokines in EMT and observed that, among the cytokines tested, TNFα showed a high dose-dependent correlation with EMT. Since the pro-inflammatory cytokines are reduced in animals treated with MSCs, we may infer that there is less EMT, and consequently, less fibrotic progression. In our case, this was observed by means of analyses of vimentin, TGF-β, and Smad3, which at the eighth week were reduced in animals treated with MSCs.

The Th1/Th2 response is polarized in renal tissue submitted to chronic damage in animals submitted to treatment with MSCs. When analyzed systemically, we noted significant reductions in the levels of serum cytokines (IL-1α; IL-1β; IL-6; IL-10; IFNγ, and TNFα) in these animals, demonstrating that they are systemically immunodepressed.

Periodical treatment with MSCs in the 5/6 model led to a decrease in renal progression and partial improvement of renal function by a decrease in fibrotic areas, probably due to the modulation of the inflammatory response (decrease of pro-inflammatory Th1 cytokines), leading to the reduction of EMT and maintaining the tissue structure. Interestingly, treatment with MSCs induced a systemic immunosuppression, which needs to be studied further, but that opens perspective for transplantation immunosuppressive therapy.

**CONCLUSIONS**

Treatment with MSCs in CRD led to an improvement in renal function, as was observed in the reduced levels of serum creatinine and urea and proteinuria; it reduced the fibrotic area and reduced the glomerulosclerosis, as observed in staining as per Masson and Picrosirius; it was also confirmed by the decrease in expression of RNAm of fibrotic molecules, primarily of vimentin, collagen 1, TGF-β, FSP-1, Smad 3, MCP-1, and the TIMP-1/ MMP-9 ratio. The MSCs were found in renal tissue, corroborating the increased expression of HO-1 and HGF, which correlates with the antifibrotic properties and systemically decreases the expression of serum cytokines, likely characterizing immunosuppression resulting from treatment with MSCs.

**ACKNOWLEDGEMENTS**

This study was undergirded by the support of Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, 04/08226-9, 06/0620-5, and 07/07139-3), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, 573815/2008-9), Instituto Nacional de Ciência e Tecnologia (INCT) de Fluidos Complexos and the Brazilian Ministry of Health (MS/DECIT). The assays were performed in collaboration with Maria Heloísa Shimizu, MD, from Prof. Dr. Antonio Carlos Seguro’s laboratory.

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