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Mitigation of Renal Injury in Wistar Rats Using Adipose Tissue-derived Mesenchymal Stromal Cells and Simvastatin

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What's known on the subject? and What does the study add?

Ischemia-reperfusion injury (IRI) remains one of the primary risk factors contributing to adverse outcomes in kidney transplantation. Combining adipose-derived mesenchymal stem cells and simvastatin enhances renal function after IRI, indicating synergy. Neutrophil gelatinase-associated lipocalin was identified as a potential early biomarker for renal IRI.

Abstract

Objective: Renal ischemia-reperfusion injury (IRI) is a major cause of acute kidney injury, negatively impacting short- and long-term kidney transplantation results. No effective treatment is available to protect or treat IRI currently. We aimed to investigate the role of pre-injury oral simvastatin and adipose tissue-derived mesenchymal stem cells (MSC) infusion, alone or in combination, to prevent and treat renal IRI, and to evaluate neutrophil gelatinase-associated lipocalin (NGAL) as an early biomarker for IRI in a rat model.

Materials and Methods: The study was conducted on adult male Wistar rats (n=75, 8-12 weeks old). Rats were divided into the following groups: healthy group (H) (no surgery, no treatment); control (C) (lesion animals + no treatment); oral simvastatin + lesion animals (S); MSC infusion + lesion animals (SC); MSC infusion + oral simvastatin + lesion animals [stem cells plus simvastatin (SC+S)]. Blood samples were collected at days 0, 15, and 30 for measurement of serum creatinine (Cr) and on day 1 for measurement of NGAL protein. The animals were followed up for 30 days, at which time a histopathological analysis was performed.

Results: The model used was able to establish IRI, as NGAL levels were significantly higher in the interventional groups. Cr increased at 15 days and returned to baseline, showing a pattern that was significant in the SC+S group. The combination of MSC and simvastatin resulted in lower renal IRI morphologic scores.

Conclusion: The combination of pre-injury oral simvastatin and MSC infusion synergistically prevents experimental renal IRI.

Keywords: Basic science, reconstructive urology, transplantation and vascular surgery

Introduction

Chronic kidney disease is a highly prevalent disease. Data from the global database on donation and transplantation reveal a high rate of patients using replacement therapies such as renal transplantation and dialysis (1-3). A comparison of these two substitutive therapies reveals the superiority of renal transplantation in terms of quality of life and other aspects (4). However, renal transplantation promotes ischemic tissue injury, experienced by the organ during transplantation, the so-called

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renal ischemia-reperfusion injury (IRI). An inopportune injury can jeopardize renal function during warm ischemia during partial nephrectomy. Considering both circumstances renal transplantation and partial nephrectomy to mitigate IRI are essential (5-9).

For decades now, several clinical protocols and drugs have been used with the aim of reducing renal tissue degradation. Considering the post-transplant scenario, losses of implanted grafts are occurring (2). Therefore, promising therapeutic alternatives, such as stem cell (SC) therapies and anti-oxidative stress drugs, are now being studied (6,10,11).

Mesenchymal stromal cells (MSC) are adult and multipotent cells that can differentiate into mesodermal cell lines (12). These cells have important properties such as the production and release of anti-inflammatory and immunomodulatory molecules, growth factors, and angiogenic factors (10). Their low immunogenicity allows these cells to be widely used in research and allogeneic transplants without the need for compatibility tests (13,14). The use of MSC or their extracellular vesicles as a treatment for IRI resulted in better renal function and faster recovery of renal epithelial cells, decreased cellular apoptosis, and increased anti-inflammatory cytokines, antioxidants, and renal-specific growth factors in acute renal tubular necrosis (11,12,14-17).

Simvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A inhibitor, was previously demonstrated to modulate inflammation and oxidative stress in experimental models. In addition to the known beneficial effects of simvastatin on cholesterol reduction, recent studies have shown a potential renoprotective effect during warm ischemia (18-20).

However, to date, studies investigating the combined use of simvastatin and adipose tissue-derived mesenchymal stromal cells (ADSCs) to prevent kidney damage have not been performed. These therapies used synergistically led to beneficial results in bone fracture recovery and ADSC growth acceleration (21,22).

Therefore, our study aims to investigate the potential use of simvastatin and ADSC in improving renal function when used either alone or in combination, as a form of warm IRI treatment in an experimental model. Additionally, we aim to evaluate neutrophil gelatinase-associated lipocalin (NGAL) as an early biomarker for IRI.

Materials and Methods

All procedures performed in this study involving human subjects were in accordance with the ethical standards of the Research Ethics Committee (approval number 31647514.7.0000.0020, date: 09.06.2015 - Pontifical Catholic University of Puerto Rico Ethics Committee) and with the 1964 Helsinki Declaration or comparable ethical standards. All procedures performed in this study involving animals were in accordance with the ethical standards of the Ethics Committee for Animal use PUC-PR (approval number 0882, date: 05.06.2014 - Pontifical Catholic University of Puerto Rico Ethics Committee).

ADSC samples were isolated from the liposuction of two healthy human donors who agreed to participate in the study and signed the informed consent form.

Animals

Adult male Wistar rats (*Rattus norvegicus*) were used (n=75) with a mean age of 8-12 weeks and averaging 300 g of body mass. The rats were housed in polypropylene cages measuring 41×34×16 cm, with four rats per cage. Temperature, humidity, and light were controlled (18-21 °C, 55-65% relative humidity, 12-hour light-dark cycle). They had ad libitum access to standard rodent feed (NUVITAL®, PR - Brazil) and water. The bedding material, consisting of pine wood shavings (Inbrasfama, PR - Brazil), was replaced daily. Prior to the commencement of the experimental protocol, the animals underwent a ten-day acclimatization period.

All efforts were made to minimize suffering while also minimizing the number of animals used. Sixty animals underwent the ischemia/reperfusion surgical procedure (as described below). Rats were divided into six groups. The control group (group C, n=15) underwent the surgical procedure but did not receive any therapeutic intervention, as they were inoculated with a sterile infusion of DMEM-F12 (Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12) culture medium (Gibco™ Invitrogen Corporation, NY, USA) below the renal capsule. Additionally, a group was used as a control, aiming to investigate the potential use of the accuracy of NGAL as an IRI biomarker. A healthy group (group H, n=15) was kept under the same conditions but was not submitted to the surgical procedure or receive any treatments. In the remaining three groups, the ischemia/reperfusion surgical procedures were performed, along with simvastatin (Pharmacy of Manipulation, Viaflora, Curitiba) and/or ADSC infusions, oral simvastatin (S, n=15), ADSC infusion (SC, n=15), ADSC infusion + oral simvastatin (SC+S, n=15) (Figure 1).

Experimental Warm Renal Dysfunction Model (Surgical Procedure)

This experiment was designed to investigate whether Simvastatin and ADSC, alone or in association, would prevent renal degradation. After analgesia with morphine (DIMORF®, Cristalia, SP - Brazil) 2.5 mg/kg and anesthesia with ketamine (Ketamin®, Cristalia, SP-BR) 75 mg/kg combined with xylazine (ANASEDAN®, Ceva, SP-BR) 10 mg/kg, the renal I/R procedure was performed according to Cai et al. (23), with modifications. Briefly, a xiphopubic incision was made,

followed by moving away viscera and locating and dissecting the right kidney. The right renal vascular pedicle and ureter were ligated, and a right nephrectomy was performed. The left kidney was then located and dissected. The left renal hilum was clamped using a Bulldog clamp (Kent Scientific Corporation©, CT, USA) for 60 minutes. After observing kidney reperfusion for five minutes, the peritoneal cavity was reviewed, and the abdominal wall was closed using a continuous suture.

Cell Isolation and Culture of Adipose-derived Mesenchymal Stem Cells

A total of 200 mL of adipose tissue were obtained from donors who underwent liposuction. ADSCs were isolated using the enzymatic digestion method according to Rebelatto et al. (24). Briefly, the adipose tissue was washed with phosphate-buffered saline (PBS) (Gibco™ Invitrogen Corporation, NY, USA), and the tissue was digested with collagenase type I (Gibco™ Invitrogen Corporation, NY, USA) at 37 °C for 30 minutes. The material was subsequently filtered through a 100-µm filter (BD FALCON™, BD Biosciences Discovery Labware, Belford, USA). Next, a homemade red blood cell lysis buffer was used, followed by another wash with PBS. The cells were cultured in 75 cm^2 flasks with DMEM-F12 medium supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 µg/mL) (Gibco™ Invitrogen Corporation, NY, USA). The culture medium was replaced twice a week. When the cultures reached approximately 80-90% confluency, cells were dissociated using 0.25% trypsin/EDTA (Invitrogen, Auckland, NZ) and replated (passage 1). When the optimal number of cells for transplantation was reached, the cell viability test was performed using the vital dye 7-amino-actinomycin D (7-AAD) and Annexin-V (BD Pharmingen®, Becton Dickinson and Company, NJ, USA).

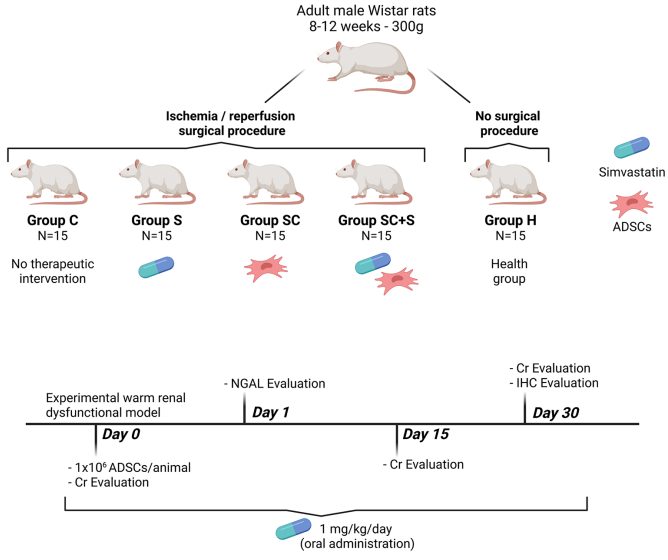

Figure 1. Study design and schematic representation of the days before and after the treatment until the end of the experiments

NGAL: Neutrophil gelatinase-associated lipocalin, ADSCs: Adipose tissue-derived mesenchymal stromal cells, Cr: Creatinine, IHC: Immunohistochemistry, SC+S: Stem cells plus simvastatin

Immunophenotypic analysis was performed by staining 5×10^5 ADSC cells. The cells were incubated with conjugated monoclonal antibodies against the following antigens: CD90, CD29, CD73, CD166, CD105, and CD34 (all PE-conjugated), CD29, CD45 [peridinin chlorophyll protein (PerCP)-conjugated]; CD14 and CD19 (both fluorescein isothiocyanate isomer-conjugated); and human leukocyte antigen-DR isotype (HLA-DR) (PerCP-conjugated). All antibodies are from Becton Dickinson, San Diego, CA, USA. The incubations were performed at room temperature for 30 min. Isotype-identical antibodies served as controls. After incubation, the cells were washed with PBS and fixed with PBS containing 1% paraformaldehyde (Exodo Cientifica/F09640SO, Sumare, SP, Brazil). The FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) was used for data acquisition, and FlowJo software (FlowJo, Ashland, OR, USA) was used for flow cytometry analysis.

Treatments

The S, SC, and SC+S groups received simvastatin, ADSC infusion, and both, respectively. Oral simvastatin (1 mg/kg/day) was administered for 30 days through the gavage technique. ADSC ($1x10^6$ cells/animal) were infused directly into the medial portion of the renal capsule five minutes after organ reperfusion.

Biomarkers

Blood samples were obtained from jugular vein punctures at days 0 (preoperative values) and after 15 and 30 for checking serum creatinine (Cr) and on day 1, to check NGAL serum concentration in order to demonstrate acute IRI. Serum NGAL protein was measured by enzyme-linked immunosorbent assay (ELISA) using Abcam's Rat Lipocalin-2 ELISA Kit (ab119602) (Abcam©, Cambridge, UK), according to the manufacturer's instructions. This experiment was designed to investigate NGAL serum concentration as a biomarker for an experimental IRI model (25,26). The absorbance was read with a 450 nm filter.

According to the manufacturer's instructions, Cr was quantified to evaluate kidney function using the Laborclin serum Cr detection kit (cat. #742071; Laborclin Laboratory Products LTDA, Pinhais, PR, BR). The output was read in the semi-automated biochemical analyzer, Quick Lab Drake (Drake©, São José do Rio Preto, SP, BR).

Renal Histopathology

At the end of 30 days, histochemical evaluations were performed using hematoxylin/eosin (HE). Histological fixation was properly performed, sections were made and stained with HE and analyzed under light microscopy (Nikon/E100/Tokyo/Japan). Histopathological analysis of renal tissue was performed

according to the criteria described by Jablonski et al. (27) and Kocak et al. (28). Because the glomeruli of the animals showed no significant histopathological alterations, the renal damage was characterized as tubular necrosis, following the score described (Table 1).

Statistical Analysis

The quantitative variables are described as mean ± standard deviation, median ± interquartile, and/or minimum and maximum when appropriate. NGAL protein and Cr were compared using the Kruskal-Wallis test. A non-parametric test (Friedman test) was used to compare Cr concentration within each group at time points 0, 15, and 30 days. The Kruskal-Wallis was also applied to compare renal histopathology, followed by Dunn's multiple comparison test. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Statistical significance was accepted at $p<0.05$.

Results

Serum NGAL Protein

We tested blood samples from the study groups, then compared these to a healthy group (no surgery, no treatment). NGAL levels were significantly higher in all interventional groups compared to healthy rats ($p<0.05$). NGAL was also different in the comparisons Healthy × other groups ($p<0.05$), Control vs. SC ($p=0.003$), and SC vs. S ($p=0.028$) (Kruskal-Wallis non-parametric test). Figure 2 demonstrates NGAL concentrations 24 hours after surgery. The study successfully demonstrated that NGAL serum concentration was directly associated with acute IRI.

Histopathological Analysis

The combination SC+S group had a median histopathological score of 2, indicating tissue injury confined to the renal cortex. Control, S, and SC groups were classified with a median score of 3. Score 3 indicates that the necrotic lesions of the tubular cells extend to the renal medulla and are thus considered more severe lesions. Group S ($p>0.05$) and SC ($p>0.05$), both with a score of 3, failed to exhibit reduced renal tubular changes when compared to the control. However, the combination SC+S group showed a significant decrease in renal tubular damage with a score of 2 ($p<0.0001$) (Figure 3).

Table 1. Classification of renal injury

Score	Criteria
0	Normal histology
1	Necrosis of individual cells
2	Necrosis of adjacent cells of the proximal convoluted tubules, confined to the renal cortex
3	Necrosis of the proximal tubules extending across the cortex
4	Necrosis affecting all segments of proximal convoluted tubules

Source: Adapted from Kocak et al. (28)

In addition to tubular necrosis, persistent qualitative aspects were observed, which involved the abnormal congestion of tubular capillaries and the presence of an inflammatory process (Figure 4).

Adipose Tissue-Derived Mesenchymal Stromal Cell Expansion and Characterization

The 7-AAD assay (viability of 92.33%) and Annexin V assay (apoptosis of 5.91%) results demonstrated that the cells were viable. Visual observation under bright field microscopy showed that cells had fibroblastic morphology and the capacity to adhere to plastic (Figure 5A). Immunophenotypic characterization of surface antigens from adipose tissue-derived MSCs exhibited positive signals for CD29, CD73, CD90, CD105, and CD166 in >95% of the cells, and negative or reduced (<2%) expression of CD14, CD19, CD34, CD45, and HLA-DR (Figure 5B), in accordance with the minimum criteria established by the International Society for Cellular and Gene Therapy (29).

Serum Creatinine

The analysis showed no significant difference in Cr concentration between the groups at baseline (day 0) ($p=0.751$). Control and S groups showed elevated Cr on day 15. Such alteration persisted until day 30 ($p<0.05$). However, considering both groups infused with SC (group SC only and SC+S), a decrease in Cr was observed on day 30 regardless of a negative Cr increase on day 15. The observed improvement was statistically significant in the combination (SC+S) group ($p<0.05$), where Cr levels returned to baseline. Nevertheless, this improvement in Cr levels was not significant in the SC group. These data are shown in Figure 6.

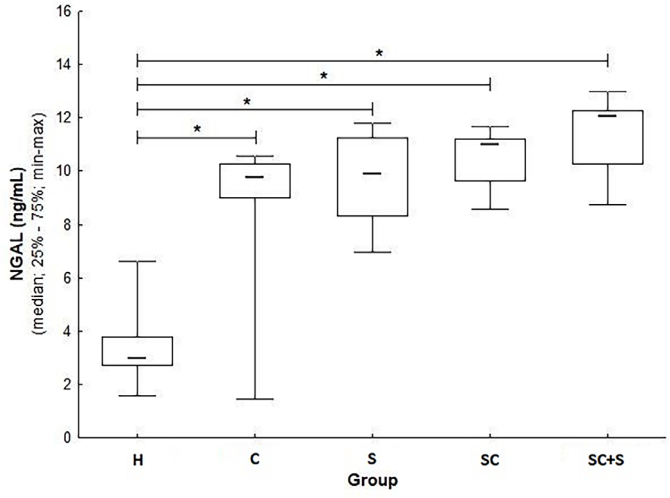

Figure 2. Serum NGAL protein concentration in the groups: healthy (H), control (C), S, SC and SC+S 24 hours after the ischemic process

NGAL: Neutrophil gelatinase-associated lipocalin, Min-max: Minimum-maximum, SC+S: Stem cells plus simvastatin, S: Simvastatin, SC: Stem cells

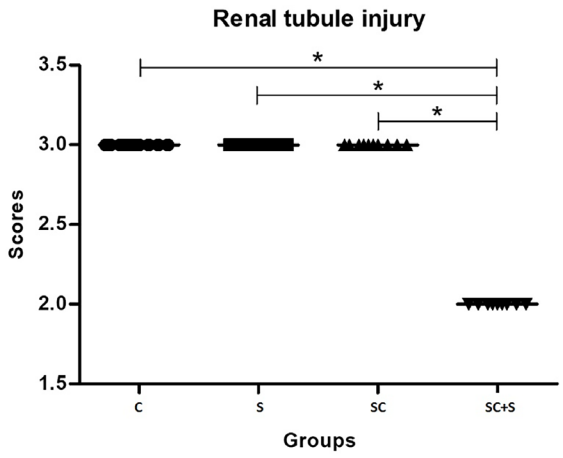

Figure 3. Renal histopathology in the groups: control (untreated animals), S, SC and SC+S submitted to ischemia-reperfusion ($p<0.0001$). The combination SC+S group showed a significant decrease in renal tubular damage compared to the other groups studied

SC+S: Stem cells plus simvastatin, S: Simvastatin, SC: Stem cells

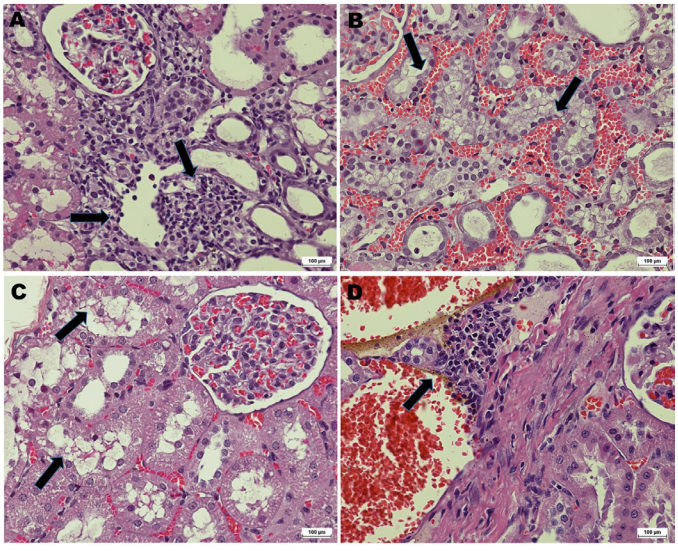

Figure 4. Qualitative histopathological aspects characteristic of tissue lesions at 40x magnification are specifically demonstrated in images A, B, and D. The histological technique used was hematoxylin/eosin. The C image specifically demonstrates the points analyzed in the characterization of the score of Jablonski et al. (27). Arrows indicate (A) mononuclear infiltrate, (B) congestion of red blood cells in the capillaries, (C) areas of necrosis in proximal convoluted tubules, such as vacuolization of epithelial cells, nuclear degradation derived from cell death and intratubular material, and (D) migration of inflammatory cells together with congestion of blood capillaries

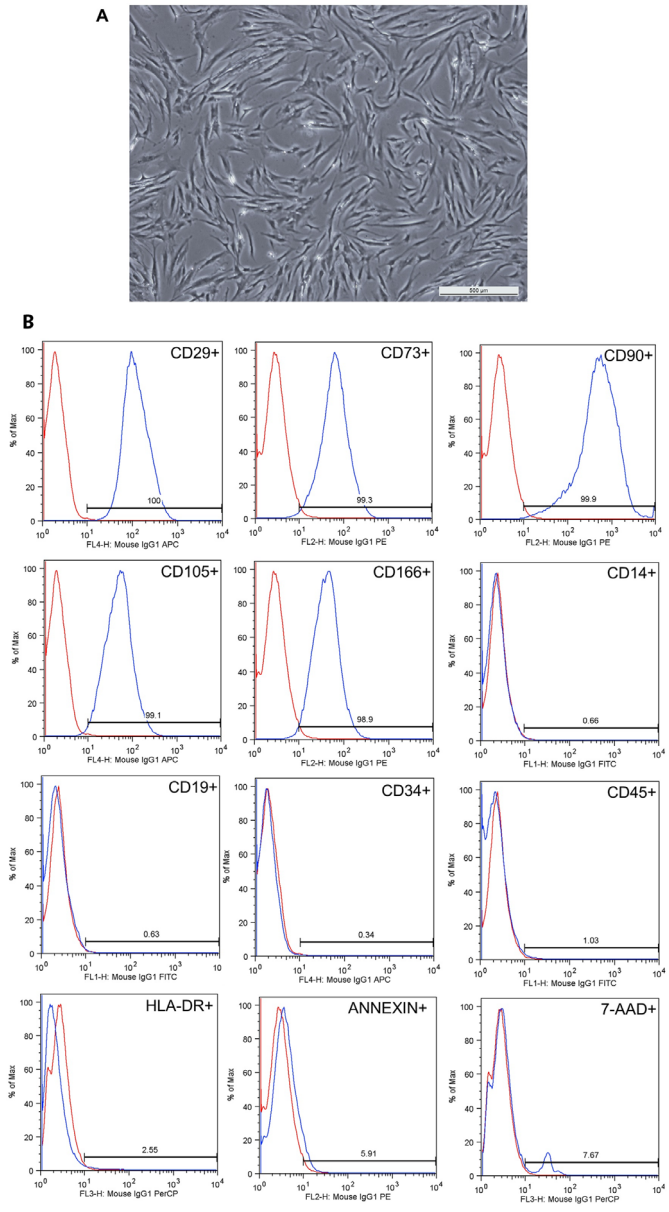

Figure 5. Adipose tissue-derived mesenchymal stem cells (MSCs) in culture and immunophenotypic characterization at passage 3. A. Representative fields showing the fibroblast-like morphology of the MSCs (magnification 50x, scale bars 500 µm). B. Representative flow cytometry analysis of cell surface markers of MSCs. The isotype control is shown as a red line histogram

Discussion

Renal injury related to ischemia and reperfusion is a consequence of warm ischemia after arterial clamping during partial nephrectomy or kidney transplantation. IRI is associated with short- and long-term postoperative complications. Treatment options to prevent or treat IRI are limited (6). In the current study, we show that a combination of ADSC and simvastatin resulted in less histological injury as well as a significant functional improvement in Cr levels in an IRI experimental model.

It has been hypothesized that NGAL could be a biomarker for IRI. Although not all studies have been consistent, there is a

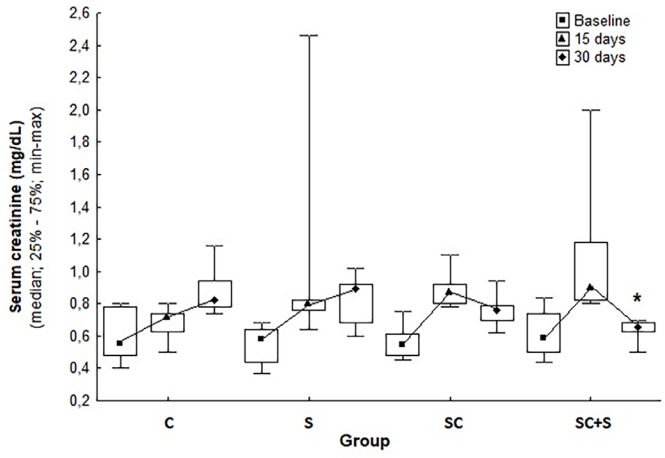

Figure 6. Serum creatinine concentration in the groups: control (untreated animals), S, SC and SC+S

SC+S: Stem cells plus simvastatin, S: Simvastatin, SC: Stem cells

general trend towards the observed outcome (25,28,30). Our results demonstrated a significant NGAL increase in all groups subjected to acute IRI compared to healthy animals. This increase in serum NGAL associated with renal damage was consistent with the findings of Corbacıoglu et al. (31) (year). Those authors successfully differentiated acute from chronic renal injury using serum NGAL. The NGAL responses, as an early biomarker of acute renal injury, we reported here are promising, but further studies involving this protein are needed. NGAL protein was not followed. Therefore, we do not yet know if the infusion of SC plus simvastatin directly into the renal capsule could generate a faster IRI recovery.

Serum Cr level was used as a standard marker of renal function. In our C and S groups, we observed a continuous increase in Cr on day 1, day 15, up to 30 days after the IRI. These data demonstrated that simvastatin alone has no therapeutic effect on the S group. Considering both groups, SC and SC+S, after an elevation in Cr on the 15th day compared with the baseline level, a subsequent reduction was observed by the 30th day. An initial reduction in glomerular filtration rate followed by an improvement was suggested. This improvement was statistically significant in the SC+S group. In this group, Cr at day 30 was similar to baseline (median at baseline of 0.59 mg/dL, median at 30 days of 0.65 mg/dL). Regardless of conflicting results, it remains to be seen if simvastatin has a potential benefit on renal function. Our findings demonstrate that simvastatin could have a synergistic role in conjunction with MSC, as we do not observe a significant reduction in Cr with ADSC infusion alone.

Particularly, there are no studies showing the beneficial effect of the combination of simvastatin and MSC in the treatment of renal injury. However, the beneficial effects of this combination have been described in the recovery of bone fractures and the acceleration of *in vitro* cell growth of SC extracted from dental pulp when exposed to simvastatin (22,32,33). Recently, Jang et al. (34) observed the same synergistic effect of bone marrow-derived MSC and simvastatin in treating induced hepatic fibrosis in rats. Through secretion of cytokines and growth factors, MSCs show immunomodulatory activity, inhibiting the activation and proliferation of immune cells, such as T-cells, B-cells, and natural killer cells, while promoting the induction of regulatory T-cells, resulting in anti-inflammatory properties. Moreover, MSCs possess the intrinsic capacity to migrate to injured tissues and release paracrine factors that promote tissue regeneration and reduce apoptosis (34). Simvastatin mitigates oxidative stress by enhancing antioxidant enzyme activity and reducing the production of reactive oxygen species. Additionally, it inhibits the expression of pro-inflammatory cytokines and adhesion molecules, improves endothelial function, and increases the bioavailability of nitric oxide (33).

Regarding the histological analysis, de Matos et al. (35) showed that cellular changes associated with inflammation, fibrosis, and necrosis occurred in the tubular epithelium after the ischemic procedure. In our study, we reported the distribution of tubular lesions for quantitative analysis because the glomeruli were intact. In consequence, tubular aspects characteristic of cell necrosis was observed, such as cell atrophy and cell and nuclear vacuolation, loss of the brush border of the epithelium of the proximal convoluted tubules, and migration of the cell nucleus and necrotic material to the center of the tubules.

Additionally, we found that the combination SC+S group had a higher mean histopathological score. According to our histological observations, repetitive events were frequent and indicative of tissue damage, such as inflammatory infiltrate and congestion of blood vessels with abnormal red blood cells, consistent with what has been previously described (36). The SC+S group was classified as having lesions confined to the cortex which are considered milder lesions than the other groups. The SC+S group underwent the same ischemic process as the other study groups and obtained a lower score. We postulate that the combined treatment effectively reduced tissue injury through synergistic anti-inflammatory and antioxidant mechanisms, which is consistent with our Cr data.

Study Limitations

In interpreting our results, we must acknowledge strengths and limitations. A strength is undoubtedly the use of standardized outcomes such as Cr and a histological Likert score. Secondly, it was clear that NGAL serum levels are associated with IRI in this experimental model and could represent a potential early biomarker of IRI as previously proposed (24). Unfortunately, several questions arose during our study. Firstly, we did not follow NGAL or accurately investigate its role as a prognostic factor for the recovery of renal function. Secondly, we have

no insight on how the protective effects of simvastatin could synergistically act with SC to promote a faster recovery of the IRI. In light of these considerations, we suggest that future studies clarify those issues.

Conclusion

Our findings indicate that the NGAL protein is associated with renal injury induced by the renal ischemia/reperfusion experimental model, suggesting it could be further explored as an early biomarker of IRI. Furthermore, the combined pleiotropic effects of simvastatin and the modulating inflammatory effects of MSC may result in a cross-potentiation, leading to improved renal function after IRI.

Ethics

Ethics Committee Approval: All procedures performed in this study involving human subjects were in accordance with the ethical standards of the Research Ethics Committee (approval number 31647514.7.0000.0020, date: 09.06.2015 - Pontifical Catholic University of Puerto Rico Ethics Committee) and with the 1964 Helsinki Declaration or comparable ethical standards. All procedures performed in this study involving animals were in accordance with the ethical standards of the Ethics Committee for Animal Use PUC-PR (approval number 0882, date: 05.06.2014 - Pontifical Catholic University of Puerto Rico Ethics Committee).

Informed Consent: ADSC samples were isolated from the liposuction of two healthy human donors who agreed to participate in the study and signed the informed consent form.

Footnotes

Authorship Contributions

Surgical and Medical Practices: F.M., D.B., L.F., Concept: F.M., D.B., A.S., S.A.B.d.M., Design: F.M., D.B., S.A.B.d.M., P.R.S.B., Data Collection or Processing: F.L.H., A.S., Analysis or Interpretation: F.M., D.B., A.S., C.L.K.R., R.P-F., Literature Search: F.L.H., D.B., S.A.B.d.M., L.F., Writing: F.L.H., L.F., R.P-F.

Conflict of Interest: No conflict of interest was declared by the authors.

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