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Biochemical and histological effects of embryonic stem cells in a mouse model of renal failure

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Abstract

Kidney diseases are a global public health problem. Despite significant advances in the understanding of renal failure (RF) and replacement therapies, this condition carries a series of complications and the life's quality of patients decreases. Differentiation capability of stem cells and their beneficial effects when they are implanted in animal models have been reported. Therefore, this work aimed to induce a long-term RF in mice, evaluating the biochemical and histological effects after implanting mouse embryonic stem cells (mESC). Mice were subjected to renal failure induction (RFI) employing cisplatin, subsequently received intraperitoneal (i.p.) injections of salt solution (control group, *n*=19) or 50 000 mESC (experimental group, *n*=19) at 24 hours, 7 days, and 13 days post-RFI. Ten animals in each group were used to analyze functional damage through serum biochemical analysis, and the mortality. For histopathological examination, three animals of each group were sacrificed at 5, 10, and 20 days post-RFI, analyzing the tubular system and glomeruli. Both groups showed blood urea nitrogen (BUN) and creatinine elevation three days post-RFI. Accumulated mortality was lower in the experimental group, presenting statistical significance. Respect to histopathological effects, the control group showed tubular dilatation, segmental focal glomerulosclerosis data, and collapsed glomeruli, while in the experimental group, glomerulosclerosis or collapsed glomeruli were not observed, evidencing regenerative data as characterized by large nuclei with prominent and binucleate nucleoli. In conclusion, mESC implant in mice with RFI significantly decreased the mortality, avoiding a greater histological deterioration related to the disease.

Keywords: renal failure, embryonic stem cells, serum biochemical analysis, kidney histology, regenerative medicine.

Introduction

Kidney diseases are a global public health problem, mortality rate between 50% and 80%, highlighting that there is an increase in this epidemiological tendency correlated to longevity and renal failure (RF) complications, such as cardiovascular disease, arterial hypertension, and diabetes [1].

When chronic renal damage is sufficiently extensive, irrespective of its origin and cause, renal function decreases until reaching end-stage renal disease, and unfortunately, there are not treatments capable of reversing the process. Although it has been shown that in damaged adult tissue, there is a cell renewal process, in specific organs this process is facilitated by the presence of stem cells (SC) or progenitors' cells, which allow periodic cell renewal and regeneration. However, the kidney regeneration is slow, and for years, the kidney was considered as an incapable organ of recovery [2].

Nowadays, it is known that renal injury is followed by regenerative hypertrophy rather than a development of new nephrons since no new nephrons are formed after 36 weeks of gestation [2, 3]. This kidney regeneration response precedes the restoration of renal function; nevertheless, renal regeneration is frequently inadequate, approximately in the 40% of patients the recovery of the function fails entirely, and 10% of these patients require a renal replacement therapy after five years of injury [4].

Despite significant advances in the understanding of RF and replacement therapies, the disease carries a series of complications and the quality of life of patients' decreases. It is important to mention that there are few alternatives for its treatment. So that, new options are being investigated focusing on regenerative medicine, seeking that these strategies improve the quality of life of the patient, decrease complications, and reduce the expenses of health institutions [1].

Regenerative medicine attempts to repair, replace or regenerate damaged tissues from diseases or injuries, being a great utopia in the field of biomedical research. However, during the last decades, this vision has become feasible and recently has been introduced into medical practice.

SC by definition are characterized by self-renewal and potentiality, particularities that allow them to give rise more SC and differentiate into different cell lineages under appropriate conditions [1]. Due to these characteristics, SC have been considered as the origin of life and consequently as a possible source of cell replacement, becoming a biological innovation since different cells, tissues and organs can be obtained from them [5].

There are two main types of SC, embryonic and adult. According to their potentiality, embryonic stem cells (ESC) can be considered totipotent and pluripotent, which can be differentiated into the three germinal lines (endoderm, mesoderm, and ectoderm). On the other hand, non-embryonic or adult SC are multipotent and unipotent, and their differentiation potential is minimal [6].

In previous works, we have reported the differentiation capability of ESC [7–9] and their beneficial effects when they are implanted in animal models [10], decreasing the inflammation process and restoring the histological

integrity. Therefore, the objective of this work was to induce a long-term (20 days) RF in mice, evaluating the biochemical and histological effects after implanting mouse ESC (mESC), once these effects have not been reported in long-term.

Materials and Methods

Animal and study groups

All procedures involving animals were approved by the Ethics Committee for Animal Research of *Escuela Militar de Medicina*. This experimental work followed the guidelines of the *Norma Oficial Mexicana* Guide for the use and care of laboratory animals (NOM-062-ZOO-1999) and the disposal of biological residues (NOM-087-ECOL-1995).

Thirty-eight adult mice were randomly divided into two groups (n=19). All members of the groups were subjected to renal failure induction (RFI), and subsequently received intraperitoneal (i.p.) injections with different treatments, according to the group that they belonged, at 24 hours, 7 days, and 13 days post-RFI. The control group received i.p. injections of 500 µL of isotonic salt solution (ISS) and the experimental group received i.p. implants of 50 000 mESC resuspended in 500 µL of ISS. Ten animals in each group were used to analyze functional damage, which was assessed through serum biochemical analysis including blood urea nitrogen (BUN), creatinine, and glucose levels, and also were used to analyze mortality. For histopathological analysis, kidney sections were obtained and analyzed under a light microscope. Figure 1 schematizes the timeline of all experiments developed in this research.

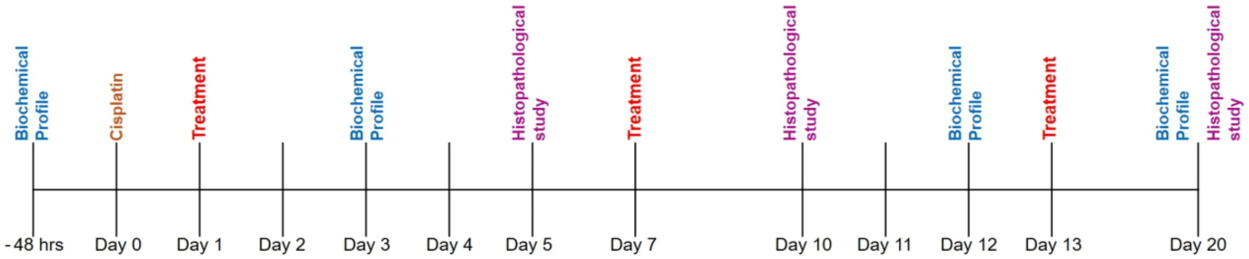

Figure 1 – ***Timeline of the study groups. The established times shown in this timeline were followed in both study groups.***

RFI in the mouse model

The animals were males of the *National Institutes of Health* (NIH) strain and two months old. They were kept in metabolic cages (Allentown Inc.; EcoFlo Rack) in humidity (50–60%) and constant temperature conditions (21±1°C) with a 12-hour light/dark cycle and had free access to food and water at all times.

As mentioned previously, 38 mice were randomly divided into two study groups (control group and experimental group), each group consisted in 19 animals, all members of both groups were subjected to RFI employing 18 mg/kg of i.p. *cis*-Diamminedichloroplatinum II (CDDP) (Sigma-Aldrich; P3494).

ESC culture

mESC (ATCC; SCRC-1011) were seeded at a density of 50 000 cells/cm^2 on a monolayer of mouse embryonic fibroblasts. mESC basal medium (ATCC; SCRR-2010) was supplemented with 15% fetal bovine serum, 0.1 mM 2-Mercaptoethanol (Invitrogen; 21985023), and 1000 U/mL mouse leukemia inhibitory factor (Chemicon; ESG1107). The cells were incubated at 37°C in a humidified 5% CO_2 and 95% air incubator. When cultured cells reached 70% confluency, doses of 50 000 mESC were obtained and resuspended in 500 µL of ISS.

Serum biochemical analysis and mortality

Ten animals of each group were used to analyze the biochemical profiles 24 hours before RFI, 3, 12, and 20 days post-RFI. For which purpose the facial vein was punctured with a 21 G/32 mm needle in the vascular bundle of the facial vein prior asepsis and antisepsis techniques, obtaining 10 drops of blood from which BUN, creatinine, and glucose levels were measured using an automated clinical chemistry analyzer (QCA; Mini line).

To assess the accumulated mortality, the number of deaths in each group was quantified every day.

Histopathological analysis

Finally, for histopathological analysis, nine mice from each group were employed, sacrificing three mice at days 5, 10, and 20 post-RFI. Tissue sections from the kidneys were obtained, embedded in Tissue Tek (Sakura; 4583) and frozen; subsequently, cryosections of 5 µm were obtained using a freezing microtome (Ecoshel; ECO-1900). After that, tissue sections were fixed in 4% paraformaldehyde for 30 minutes, at room temperature, and rinsed with phosphate buffer solution. Afterward, Hematoxylin–Eosin (HE) staining was performed according to standard methods. Stained sections were analyzed using a light microscope (Nikon; Eclipse Ti-U) and the software Image-Pro Premier 9.1 (MediaCybernetics).

Statistical analysis

Statistical significance of raw data between the groups in each experiment was evaluated using unpaired Student's t-test followed by the Bonferroni *post-hoc* test. Results were expressed as means ± standard deviation (SD) of the mean.

Results

I.p. injections were carried out at a set time and were uneventful; abscesses, peritonitis, or any other secondary complications, such as macroscopic damage, were not observed.

Serum biochemical analysis between groups

BUN, creatinine, and glucose levels are summarized in Figure 2A. All parameters were compared between

the control group and the experimental group. The results showed an elevation of BUN and creatinine three days post-RFI in both groups (Figure 2, B and C), without significant changes in glucose levels (Figure 2D). At days 12 and 20 post-RFI, BUN and creatinine values decreased up to normal values (Figure 2, B and C) in both groups. No statistically significant difference between the groups was shown.

Mortality evaluation

Although there was no statistical significance on biochemical profiles, it is important to mention that at day four there were cases of mortality, which increased over time in both study groups. However, as observed in Figure 3, accumulated mortality was higher in the group treated with ISS (70%) compared to the group treated with mESC (40%), presenting statistical significance.

A.

Group	BUN mg/dL ± SD	Creatinine mg/dL ± SD	Glucose mg/dL ± SD
		Pre-RFI	
ISS	27.20 ± 4.70	0.77 ± 0.14	156.70 ± 35.76
mESC	30.80 ± 4.13	0.69 ± 0.11	128.89 ± 23.76
		3 days Post-RFI	
ISS	50.00 ± 12.19	1.76 ± 0.73	160.13 ± 9.83
mESC	40.80 ± 2.16	1.62 ± 0.74	167.20 ± 34.18
		12 days Post-RFI	
ISS	28.00 ± 2.00	1.433 ± 0.47	140.333 ± 17.21
mESC	31.83 ± 8.58	1.17 ± 0.38	109.50 ± 16.73
		20 days Post-RFI	
ISS	23.33 ± 3.06	1.00 ± 0.20	148.00 ± 4.00
mESC	28.40 ± 7.67	1.04 ± 0.17	128.00 ± 17.44

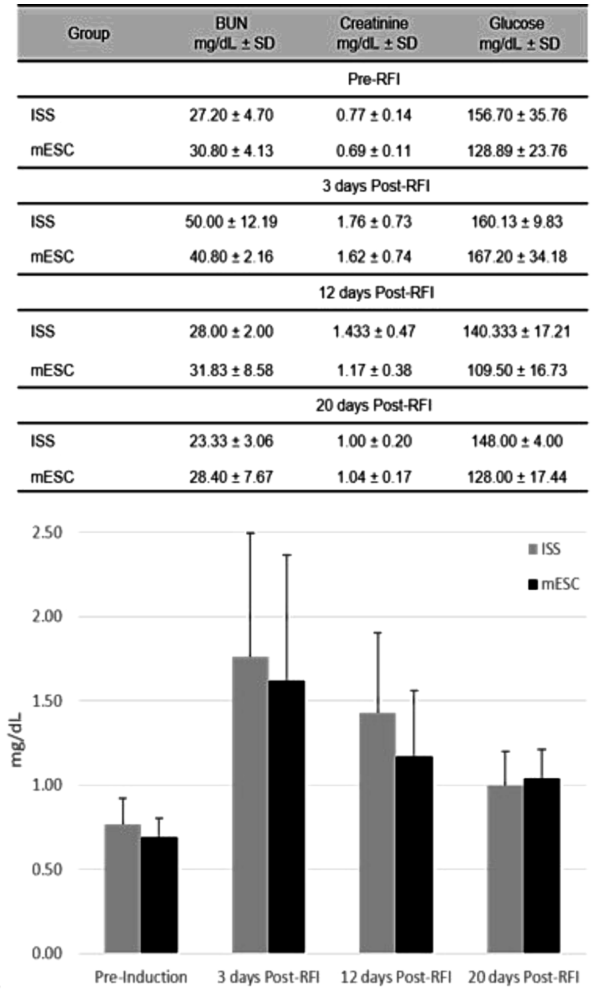

Figure 2 – ***Serum biochemical analysis in the group treated with isotonic salt solution (ISS) and the group treated with mouse embryonic stem cells (mESC) at different days post-renal failure induction (RFI): (A) Summary of the serum biochemical analysis; (B) Blood urea nitrogen (BUN); (C) Creatinine; (D) Glucose. The bars represent the means ± standard deviation (SD) (p>0.05).***

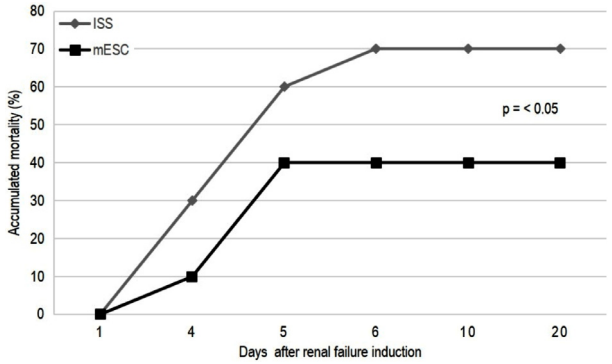

Figure 3 – ***Accumulated mortality. The group treated with isotonic salt solution (ISS) presented higher accumulated mortality (70%) at day 20, compared to the group treated with mouse embryonic stem cells (mESC) (40%) (p<0.05).***

Histopathological analysis

For histopathological analysis, three animals of each group were sacrificed at 5, 10, and 20 days post-RFI, obtaining histopathological kidney samples, analyzing the tubular system and glomeruli. Mostly, interstitial nephritis and a decrease in the number of glomeruli directly proportional to the time of injury were shown.

Five days post-RFI, in the tubular system of the control group (ISS group) interstitial nephritis was observed (Figure 4), characterized by inflammatory infiltrate {1}, and also an overall tubular lesion was evidenced, showing tubular dilatation with epithelial cell desquamation. Regarding glomerular microarchitecture (Figure 5), segmental focal glomerulosclerosis {2} with a tip lesion in the urinary pole {3} was also identified, and pyknotic nuclei were seen in most podocytes and endothelial cells. In animals treated

with mESC, although interstitial nephritis was also observed, the inflammatory infiltrate was smaller than in the control group, and the tubules were less dilated (Figure 4). On the other hand, the glomerular microarchitecture was altered, but without evidence of glomerulosclerosis or any other type of lesion, although some of the nuclei seemed to be pyknotic (Figure 5).

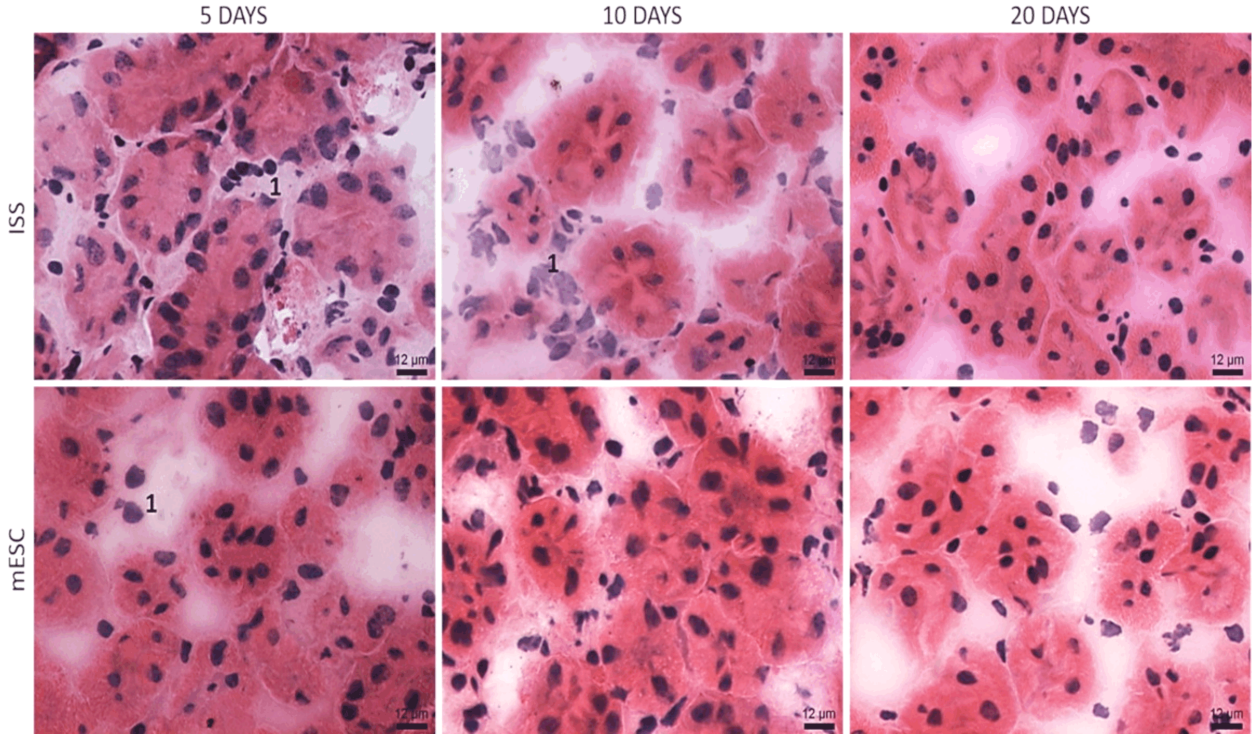

Figure 4 – ***Representative microphotographs of the tubular system. A greater dilatation of the tubules in the control group treated with isotonic saline solution (ISS) was evidenced versus the experimental group treated with mouse embryonic stem cells (mESC). Likewise, a greater inflammatory infiltrate was observed {1} on day 5, in the control group. Hematoxylin–Eosin (HE) staining, scale bars represent 12 µm.***

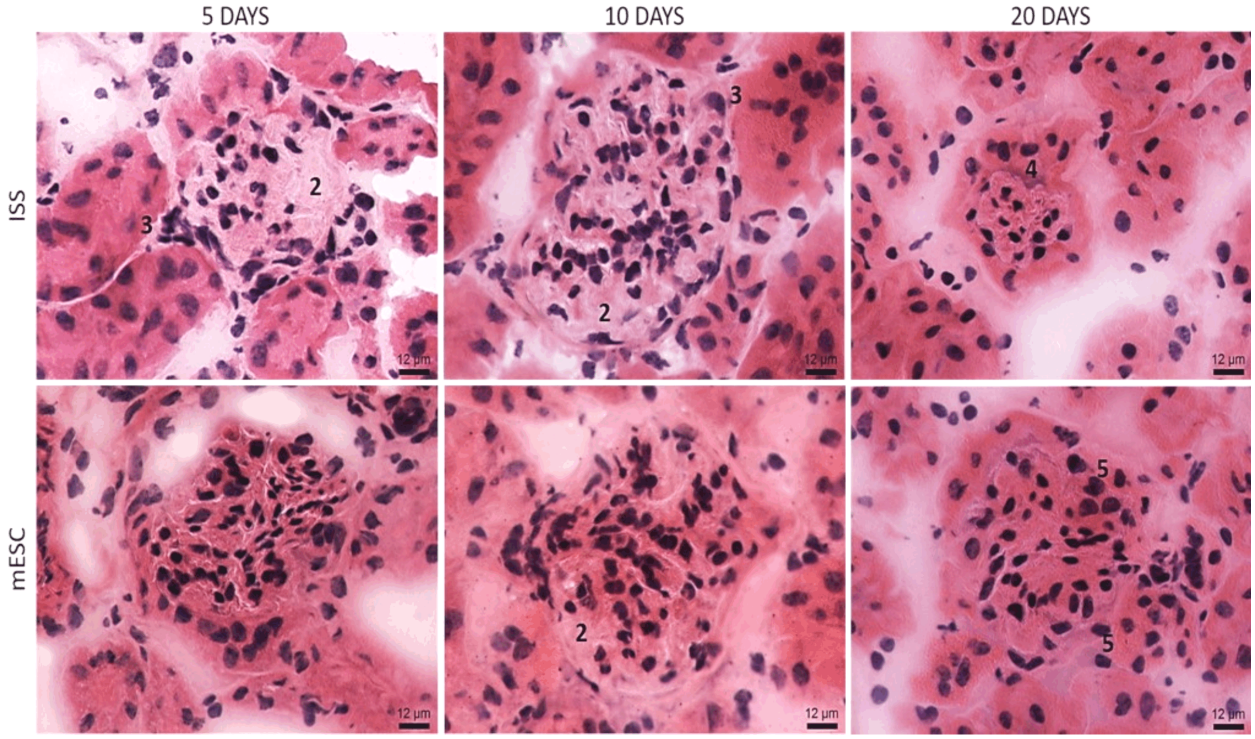

Figure 5 – ***Representative microphotographs of the glomeruli. At day 5, segmental focal glomerulosclerosis {2} was identified in the group treated with isotonic saline solution (ISS), distinguishing a lesion in the tip of the urinary pole {3}. After 20 days of renal failure, some collapsed glomeruli {4} were identified in an almost global manner. However, in the mouse embryonic stem cells (mESC) treated group, although some alterations were observed, the microarchitecture was restored at day 20, and even some regeneration data were found characterized by large nuclei with prominent and binucleate nucleoli {5}. Hematoxylin–Eosin (HE) staining, scale bars represent 12 µm.***

At day 10 post-RFI, the control group showed a smaller dilatation of the tubules; however, the inflammatory infiltrate was still evident (Figure 4). Respect to glomerular microarchitecture (Figure 5), segmental focal glomerulosclerosis {2} was yet detected with a tip injury in the urinary pole {3}, accompanied by a loss of the microarchitecture. In contrast, the mESC-treated group even though it presented inflammatory infiltrate, the microarchitecture of the tubular system and glomeruli were preserved (Figures 4 and 5).

Finally, at day 20 post-RFI, although tubular dilatation decreased in the control group (Figure 4), data of segmental focal glomerulosclerosis were still found (Figure 5), and it was even possible to identify collapsed glomeruli {4}. Nevertheless, in the mESC-treated group, glomerulosclerosis or collapse of the glomeruli were not observed, and some regenerative data were shown, characterized by large nuclei with prominent and binucleate nucleoli {5}.

Discussions

Currently, the therapies used for RF are the replacement of renal function with dialysis or transplantation. Unfortunately, dialysis is associated with high morbidity and mortality and, on the other hand, despite advances in kidney transplantation, the scarcity of donor organs limits this treatment, besides the risk to immune rejection and the need for immunosuppressants for life [5, 11–13].

In this regard, there is a great concern in the development of new therapies for renal diseases with the capacity to replace the full range of kidney functions, seeking to reduce morbidity, mortality, and the overall economic impact associated with this condition [14]. Although it has been considered that the kidney is an organ incapable of regeneration, new treatments are currently being investigated to improve renal function, emerging new therapeutic proposals in regenerative medicine [1, 12]. In this sense, the main objective of this work was to support the basic research in the treatment of RF, in the field of regenerative medicine. For which purpose, we evaluated the effects of mESC implants in a murine model with RF, assessing the BUN, creatinine, and glucose levels, as well as a histopathological analysis.

Regarding biochemical analysis, the baseline values for BUN and creatinine prior-RFI in the control and experimental groups agree with the results reported by Takai *et al.* (2015), who informed BUN and creatinine values of 27.9±4.3 mg/dL and 0.63±0.097 mg/dL, respectively [15]. In the same way, the results are similar to those reported by Aleksunes *et al.* (2008), who reported BUN values of 26 mg/dL [16].

Similarly, once the renal damage was induced using CDDP, we found a significant increase in BUN and creatinine levels three days after RFI. Our results were similar to those reported by Aleksunes *et al.*, who also used a dose of 18 mg/kg, stating BUN values of 63 mg/dL four days post-induction of damage [16]. On the other hand, Ciarimboli *et al.* (2010) used a CDDP doses of 15 mg/kg, reporting a BUN increase of 59±17 mg/dL four days after RFI [17]; likewise, Liu *et al.* (2016) used a dose of 20 mg/kg, reporting a significant BUN increase of 87.4±3.3 mg/dL, 72 hours post-induction [18]. As shown in all of the studies mentioned above, there was an increase in BUN and creatinine, although in different degrees, probably due to the different strains, doses used, and the days on which the blood chemistries were obtained.

It is important to mention that none of the previously mentioned works [15–18] carried their experiments in the animal model beyond four days after RFI. However, in this work, we carried the animal model until day 20, observing at day 12 a decrease in BUN and creatinine values in the animals that were still alive, reaching normal values; this is probably due to the fact that the acute RF is reversible in more than half of the cases [19], and in some cases, a complete regeneration has been reported [20].

In this sense, the animals that survived after day 6 in the ISS-treated group, presented a renal function restoration, reason by which a statistical significance of the BUN and creatinine values between the study groups was not observed. However, a statistical significance was found between the groups in accumulated mortality, showing a higher accumulated mortality at day 20 in the ISS-treated group, what makes us think that the mESC implant as an early treatment improve the renal function and regeneration response, preventing a more considerable damage in the kidney, which improves the animal survival.

On the other hand, about the histopathological analysis, as previously mentioned, interstitial nephritis characterized by inflammatory infiltrate was observed in the ISS-treated group five days after RFI. Besides, a global tubular lesion with scaling of epithelial cells in some tubules was shown, as well as segmental focal glomerulosclerosis with the presence of endothelial cells with pyknotic nuclei. These results agree with those reported by Aleksunes *et al.*, who reported degeneration, necrosis, and detachment of proximal tubule epithelium, as well as pyknotic and necrotic tubular cells [16]. In the same way, agree to Takai *et al.*, Ciarimboli *et al.*, and Liu *et al.*, who reported on their works damage of renal tissue characterized by tubular dilatation and necrosis [15, 17, 18].

Nevertheless, Imberti *et al.* (2014) injected human induced pluripotent SC (iPSC) in mice with renal damage induced with CDDP, and assessed renal function four days after RFI, reporting that iPSC had no protective effect on renal function or renal histology [21]. In contradiction, we found that in the mESC-treated group, although interstitial nephritis was also observed, the inflammatory infiltrate was smaller than in the ISS-treated group, and similarly, the tubules seem to be less dilated. Respect to glomerular microarchitecture, it was altered but without evidence of glomerulosclerosis or any other type of injury.

Finally, at day 20 in the ISS-treated group, we observed that although tubular dilatation decreased, we still found data of segmental focal glomerulosclerosis, identifying some collapsed glomeruli. On the other hand, in the group treated with mESC, glomerulosclerosis or collapse of the glomeruli were not observed; nevertheless, some open-face nuclei were distinguished, as well as regenerative changes characterized by large nuclei with prominent and binucleate nucleoli, probably due to the regenerative properties of SC. In this regard, Heng *et al.* (2005) have stated that human ESC function as a biological "catalyst" to stimulate tissue repair and regeneration [22], which seems to be very promising in the field of regenerative medicine.

The main strength of cell therapy in RF is the incorporation of functional cells into a damaged kidney, aiming to repair and regenerate the damaged tissue. The main attractions of the use of ESC as a therapeutic strategy are its potentiality, which refers to the ability to

differentiate into all germ layers and become a specific cell type, as well as its low immunogenicity when these cells are cultured *in vitro* and reintroduced as a therapeutic agent. Although the phenotype of differentiated cells may not be identical to their standard counterpart, the fact that they work *in vivo* raises hopes of generating "on-demand" cell therapy [10, 23].

It is important to consider that, if the cells that will be implanted are obtained from a differentiation process, an extensive study of the genotype and phenotype must be done, as well as histocompatibility tests between the implanted cells and the host. However, despite the significant progress in the field of regenerative medicine, we are still a long way from using any of its techniques in humans, because it is necessary to evaluate the side effects, biodistribution, dosage, type of administration, vehicle of the cellular therapy, as well as the response to treatment, and long-term follow-up.

Conclusions

Mice with long-term RF that survived after day 6 without treatment showed a renal function restoration. Additionally, the mESC implant in long-term RF mice model significantly decreased the mortality, avoided a greater histological deterioration related to the disease, and stimulated some regenerative changes. With these findings, we can emphasize that the main objective of regenerative medicine using cell therapy is to increase organic functionality, restore the microstructure, and reduce mortality. Moreover, with these studies, the most important fact is the change in the paradigm of organic deterioration, disease, and regeneration.

Conflict of interests

The authors declare that they have no conflict of interests.

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