Assessment of the potential therapeutic effects of Mesenchymal Stem Cells in an experimental model of Cardiorenal Syndrome

Lamis Abdelghani Salamah [a], Ghada Mahmoud Abd Elaziz [a], Hanan Hassan Fouad [b] and Marwa Fathi Abd Alla [a]

[a] *Medical Biochemistry and Molecular Biology department, Faculty of Medicine, Beni-Suef University, Egypt*

[b] *Medical Biochemistry and, Molecular Biology department, Faculty of Medicine, Cairo University, Egypt*

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Abstract:

The goal of this study is to evaluate the efficacy of bone marrow derived mesenchymal stem cells (BMMSCs) in the prevention of fibrosis following induction of CRS type 3 in rats, and in addition, to assess their effect on FGF23 and Klotho genes, renal functions, and histopathological features. A total of eighteen male albino rats were enrolled in this study divided equally into 3 groups; group I: healthy control, group II: CRS without treatment, group III: CRS treated with BM-MSCs. Rats were used to generate Cardiac Hypertrophy (in vivo), by ligating their right renal artery, and group III was injected with stem cells after the insult. Serum levels of urea and creatinine were assessed, FGF23 and Klotho genes expression in cardiac and renal tissue by quantitative real time PCR were evaluated. Histopathological examination of tissues was performed. Results revealed that serum levels of urea and creatinine were elevated with significant increase in FGF23 and decrease in KLOTHO levels in diseased group compared to control and treated group. In treated group with BMMSCs, FGF23 was markedly inhibited, and KLOTHO level was upregulated with little significant difference between treated group and normal control. In addition, there was decrease in urea and creatinine levels. Histopathological examination of treated group revealed improvement of induced fibrosis in renal and cardiac tissues compared to diseased group. The data suggests that MSCs might have therapeutic effect on cardiorenal syndrome type 3.

1. Introduction:

Mesenchymal stem cells (MSCs), also known as mesenchymal stromal cells, are a heterogeneous population of cells with a variety of potential therapeutic uses for various organs and tissues. MSCs can be obtained from many tissue sources. In the past, guinea pig spleen and bone marrow (BM-MSC) were used to isolate MSCs [1]. Renal impairment that occurs between a few hours to seven days characterizes a disease known as acute kidney injury (AKI), which has a quick onset. This illness results in renal and extrarenal issues like heart and brain due to electrolyte imbalance and waste product buildup [2].

A promising approach to speeding up kidney recovery, repairing and rebuilding tissue damage after an acute injury caused by ischemia-reperfusion, kidney transplant, and drug-mediated toxicity is MSC-based therapy. Despite the fact that MSCs are initially trapped inside the pulmonary vasculature, the intravenous route is more usually selected for treating kidney disorders due to the relative simplicity of the technique. Kidney injury is more effectively repaired using intra-arterial pathways, such as intra-aorta, intra-renal artery, and intracarotid routes. Although improving kidney function can be achieved via the intraparenchymal pathway, such as under the renal capsule, this route is less useful for clinical applications [3].

Furthermore, MSC therapy has been intensively investigated as a treatment for myocardial infarction, peripheral ischemic vascular diseases, dilated cardiomyopathy, and pulmonary hypertension [4].

Fibroblast growth factor (FGF23) levels increase in individuals with acute or chronic renal disease in response to a number of variables, such as phosphate retention, Klotho insufficiency, and decreased filtration and/or degradation by the diseased kidney [5].

A protective role for KLOTHO against endothelial dysfunction has been noted, but fibroblast growth factor FGF23 levels have been linked to decreased vasoreactivity, increased arterial stiffness, and cardiovascular morbidity and mortality. The proportional contributions of FGF23 expression with concomitant Klotho deficit in type 3 cardiorenal syndrome have not yet been explored, and expression of the FGF23/KLOTHO signaling system in the human cardio-vascular system has not yet been demonstrated [6].

We hypothesized that MSCs could treat cardiorenal syndrome by regulating the expression of the FGF23 and Klotho genes and enhancing the pathology markers of tissue injury. We conducted a research of a healthy control group and experimental models of CRS type 3 before and after treatment with BM-MSC.

2. Materials and Methods:

2.1 Animals used for induction of CRS-3:

For this investigation, Kasr Al Ainy Animal House at the Kasr Al Ainy Faculty of Medicine in Egypt supplied Wistar rats. Biochemistry department committee, scientific committee, and faculty committee at Kasr Al Ainy Medical Faculty authorized the study in accordance with National Research Council guidelines for animal care and use of laboratory animals. There were a total of eighteen (6-8) month-old male albino rats weighing (230–300 g) used as an animal model in this study. A 12/12 h light/dark cycle alternated between housing rats in individual cages at a constant temperature (22–24°C) and providing them with free access to food and water.

Experimental rats were divided into the following groups:

(1) Control group: included six normal healthy control rats injected with normal saline.

(2) Without treatment Group: included six rats with experimental model of cardiorenal syndrome type 3 (CRS-3).

(3) With treatment Group: included six rats injected with mesenchymal stem cells after 48 hours renal injury (CRS-3).

To induce Cardiac Hypertrophy (in vivo), rats were ligated with minimal changes to their right renal artery. A mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) intramuscularly was used to anaesthetize the animals. Plastic tube (22G cannula tube, 0.6 mm outer diameter) was positioned on top of the right renal artery (along it) and then secured around the artery with silk suture after the abdomen had been opened up. After 30 minutes, the tube was removed from beneath the knot, allowing the artery to expand to the outside diameter of the tube.

Absorbent suture material (Coated VICRYL®) was used to seal the abdominal wall and skin. Surgery was done under complete aseptic condition. Rest and recovery: The animal was continuously monitored over its whole postoperative care period until it was awake and stable with regular breathing patterns and rates. Antibiotics, analgesics, and a topical antiseptic spray were given to animals following surgery.

Rats of group (1) were kept in optimum condition for 14 days and were sacrificed on the 15th day following surgery, supposed to undergo Sham operation. Rats of group (2) were kept in optimum condition for 48 hours and were sacrificed on the 3rd day following surgery. Rats of group (3) were injected intravenous with BM-MSCs at a dose of 1×10^6 cell/ml after 48 hours and were kept in optimum condition for 14 days, then they were sacrificed on the 15th day following surgery.

At the planned-time, blood was collected from each rat, for assessment of serum levels of urea and creatinine. Then, animals were sacrificed by cervical dislocation. The kidneys

and hearts were excised and divided into two parts: First part was fixed overnight in paraformaldehyde in PBS at 4 °C for histopathological examination by hematoxylin and eosin (HE)) to display the histological details, with Masson trichrome (MT) to demonstrate the collagen fibers and with periodic acid schiff (PAS) to demonstrate glycogen content, second part was used for assessment of FGF23 and Klotho genes expression in cardiac and renal tissue by quantitative real time PCR.

2.2 Preparation of stem cell "Isolation and culture of bone marrow mesenchymal stem cells":

According to [7], Bone marrow was harvested by flushing tibias and femurs of eight weeks old albino rats (150 to 200 gm body weight) with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells were incubated at 37° C in 5% humidified CO2 for 12~14 days, until formation of large colonies. The culture was washed with phosphate buffer saline (PBS). After centrifugation, the cells were re-suspended with serum-supplemented medium. Mononuclear cells were cultured in high-glucose DMEM (GIBCO) with 2mmol/L L-glutamine, 10% FBS and 1% penicillin streptomycin-amphotericin B mixture. Non-adherent, dead as well as floating cells were removed after 3 days of incubation. And subsequently at every three to four days till the adherent cells reached 70% to 80% confluence. Cells were harvested with 0.25% trypsin–EDTA (GIBCO) and after washing, the cells were counted and tested for viability by adding 0.2% trypan blue. The BMMSCs suspended in Dulbecco's modified Eagle's medium (DMEM) with viability higher than 95% were rapidly injected intravenous in rats at a dose of 1×10^6 cell/ml. MSCs were identified by their adherence to the plastic surface soft tissue culture flasks when maintained in standard culture conditions.

2.3 RNA extraction from kidney and heart tissues:

Isolation of intact RNA from kidney and heart tissues was done using RNeasy 96 Mini Kits from QIAGEN. The RNeasy 96 Kit represents a new technology for high-throughput RNA preparation. All steps of the RNeasy 96 protocol for isolation of total RNA were performed at room temperature. Generally, DNase digestion was not required since the RNeasy 96 silica-membrane technology efficiently removes most of the DNA without DNase treatment.

2.4 SYBR Green Real - time PCR detection:

Real - time PCR quantification was done using TransScript SYBR Green One step RT-PCR Super Mix (ThermoFisher) Real-time qPCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA). One-

step reagents are used to directly amplify RNA samples on real time PCR instrument.

The reverse transcription (RT) and qPCR steps are both conducted in the same reaction well using primers mentioned in table 1.

Table (1): Sequence of the primers.

Klotho (Kl)	Forward 5'– **GGCTCTTGCTGCTCCGTTTG** –3'
	Reverse 5'– **GAAGCAGAGCTCGGCGTAAT** –3'
Fibroblast growth factor 23 (Fgf23)	Forward 5'– **ACAGCTACAGCCAGGAACAG** -3'
	Reverse 5'– **CCGGGCTGAAGTGATACGAT**-3'

2.5 Determination of serum creatinine and urea levels:

Urea concentration was determined in serum using the reagent kits purchased from BioMed Diagnostic, Egypt. It is determined by measuring the absorbance of spectrophotometer at 340 nm. Creatinine concentration was determined in serum using the reagent kits purchased from BioMed. It is determined by measuring the absorbance between (490-500) nm.

2.6 Histopathological Examination:

Serial 5-µm sections of the previously fixed kidney and heart sections were stained, and then examined histopathologically.

2.7 Statistical methodology

Data were coded and entered using the statistical package SPSS version 22. Data were summarized using mean and standard deviation. Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons post hoc test when comparing more than 2 groups. Correlations between quantitative variables were done using Pearson correlation coefficient.

3. Results :

A. Kidney markers:

Table (2): Comparison between the studied groups regarding the renal FGF23 level.

Items	Control group	Without ttt Group	With ttt Group	P-value
FGF23	1.03±0.02	6.00±0.62	2.09±0.26	<0.001*

*P-value is significant

This table showed that there was a significant difference between the three groups regarding the renal FGF23 level. The highest level was in cases without treatment group followed by cases with treatment group, then lastly control group.

Table (3): Comparison between the studied groups regarding the renal KOLTHO level.

Items	Control group	Without ttt group	With ttt group	P-value
KOLTHO	1.01±0.01	0.25±0.04	0.73±0.07	<0.001*

*P-value is significant

This table showed that there was a significant difference between the three groups regarding the renal KOLTHO level. The highest level was in cases control group followed by cases with treatment group, then lastly without treatment group.

B. Heart markers:

Table (4): Comparison between the studied groups regarding the cardiac FGF23 level.

Items	Control group	Without ttt group	With ttt group	P-value
FGF23	1.03±0.01	4.67±0.31	1.85±0.19	<0.001*

*P-value is significant

This table showed that there was a significant difference between the three groups regarding the cardiac FGF23 level. The highest level was in cases without treatment group followed by cases with treatment group, then lastly control group.

Table (5): Comparison between the studied groups regarding the cardiac KOLTHO level.

Items	Control group	Without ttt group	With ttt group	P-value
KOLTHO	1.02±0.01	0.41±0.08	0.83±0.06	<0.001*

*P-value is significant

This table showed that there was a significant difference between the three groups regarding the cardiac KOLTHO level. The highest level was in control group followed by cases with treatment group, then lastly cases without treatment group.

C. Creatinine and Urea

There was a significant difference in each of the 3 groups regarding comparison between creatinine levels at 4 hours, 24 hours and at 14 days. Creatinine levels differ significantly between controls and without ttt and between without ttt and with ttt. There was no statistically significant difference between controls and with ttt (the level in ttt group was normalized) after 14 days.

Within the same group, the creatinine level increased significantly in cases without and with ttt from 4 hours to 24 hours, whereas the levels decreased from 24 hours to 14 days.

Within the control group, there were fluctuations in the creatinine level at 4 hours, 24 hours, and 14 days within normal range.
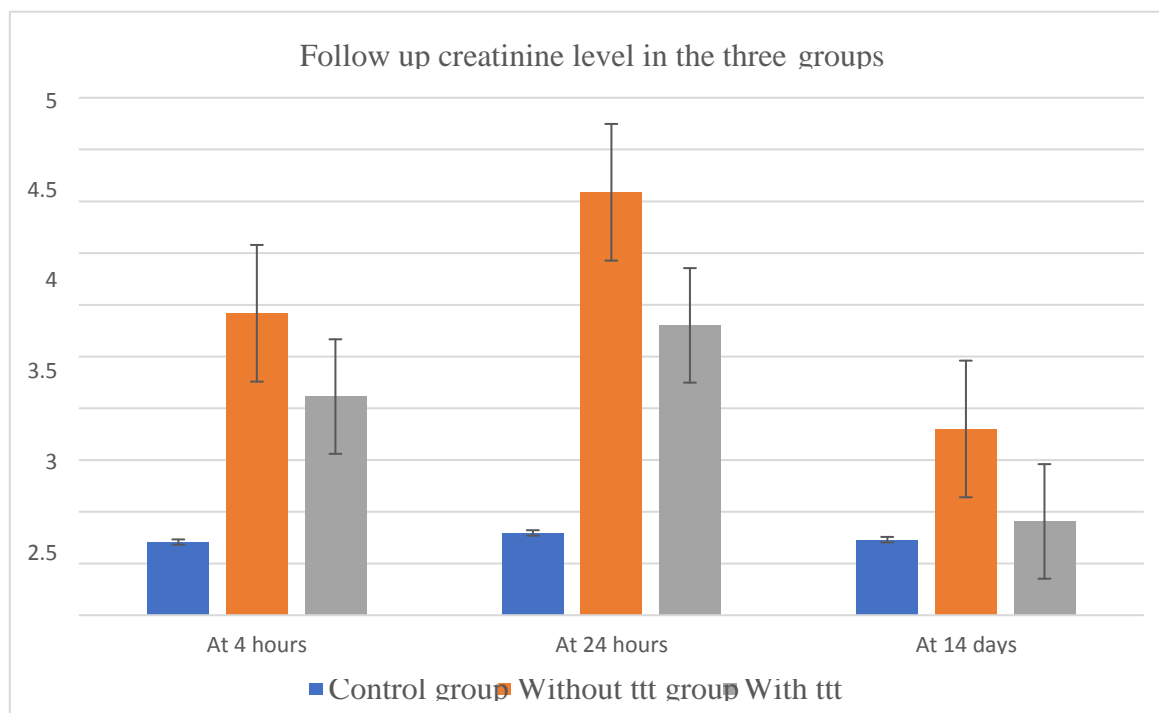

Figure (1)*: Comparison between the studied groups regarding the creatinine level at different times.

There was a statistically significant difference between the 3 studied groups regarding the urea level at 4 hours, and 24 hours, whereas urea levels were normalized at 14 days. Within the same group, urea levels decreased significantly in cases with ttt from 4 hours to 24 hours and 14 days. In without ttt group, there was a significant increase in the urea level at 24 hours than at 4 hours, and there was a significant decrease in the urea level at 14 days than at 24 hours, indicating the process of healing. These differences are within normal range.

Within the control group, there were fluctuations in the urea level at 4 hours, 24 hours, and 14 days within normal range.
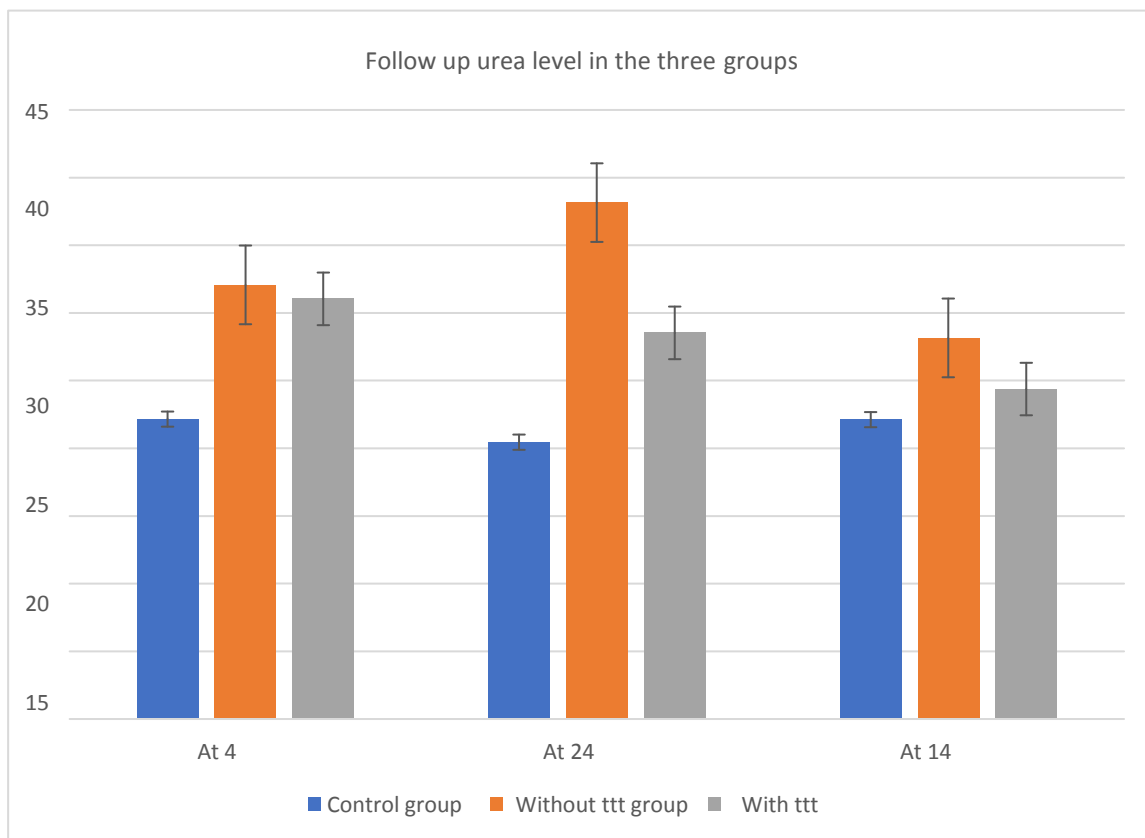

Figure (2): Comparison between the studied groups regarding the urea level at different times.
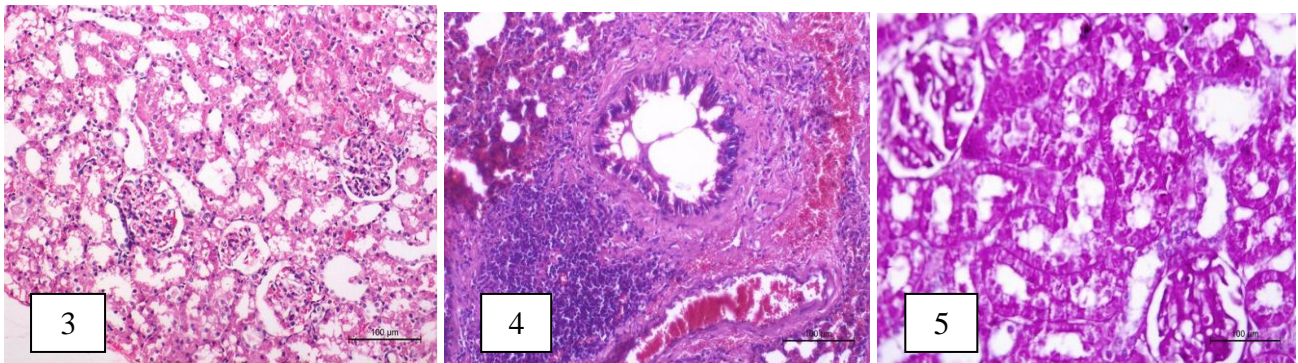
D. Histopathological results

1- Kidney sections:

Histopathological examination of Kidney tissues of the control group showed regular renal glomerular structure and normal tubules with cuboidal epithelium (fig. 3).

Histopathology of Kidney tissues of the ischemic injury group showed induced diffuse tubular lesions in the form of denudation of the renal tubular cells with loss of brush border and flattening of tubular cells

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with intratubular hyaline and cellular cast formation, hydropic changes, coagulative necrosis of some renal tubular epithelial cells and, although it showed interstitial nephritis characterized by mononuclear infiltration, mild degree of fibroplasia and glomerulonephritis (fig. 4).

Histopathology of treated Kidney tissues with mesenchymal cells showed limited clear cell changes with no obvious fibrosis (fig. 5).
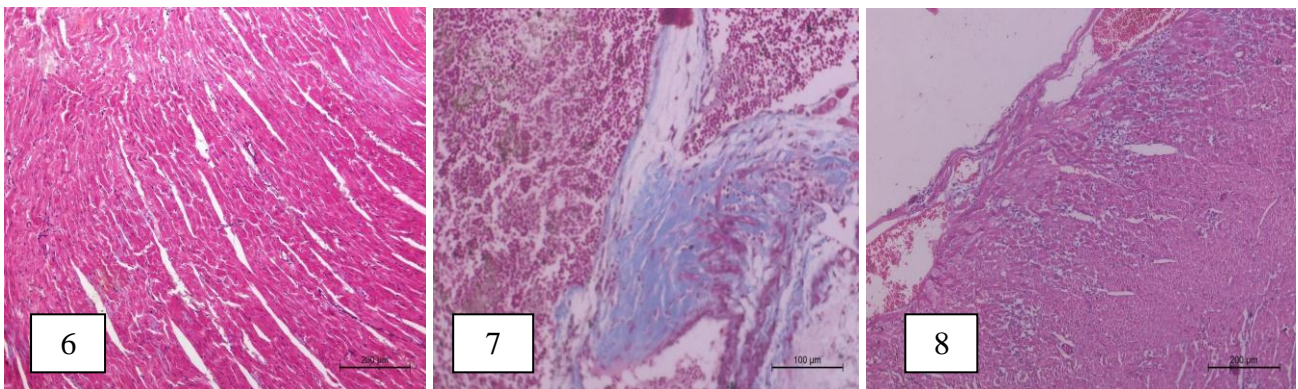

2-Heart sections:

Histopathological examination of Heart tissues of the control group showed Normal myocardial fibers with central nuclei and regular endocardial structure (fig. 6).

Histopathology of Heart tissues of the ischemic injury group showed congestion of subendocardial blood vessels, congestion and perivascular edema of coronaries. Hemorrhages were present within myocardial tissue, mononuclear cells infiltration associated with fibrosis (fig. 7).

Histopathology of treated Heart tissues with mesenchymal cells showed congestion and Hemorrhages in some areas with no myocardial changes or replacement with fibrous tissue (fig. 8).

4. Discussion:

This study aims to evaluate the efficacy of bone marrow-derived mesenchymal stem cells (BMMSCs) in the prevention of fibrosis following induction of cardiorenal syndrome type 3 in rats, and in addition, to assess their effect on FGF23 and Klotho genes, renal functions and histopathological features.

In our work, there was a significant increase in FGF23 level expression in CRS type 3 non-treated group compared to the normal control in both renal and cardiac tissues. The results of the present work agreed with [8], who found that FGF23 was present in cardiac myocytes of patients with ischemic cardiomyopathy, myocarditis, and dilated cardiomyopathy, and noticed that adult rat cardiac myocytes clearly expressed Fgf23 on the mRNA level, which was induced by Oncostatin M (OSM) treatment in vitro. The above-mentioned finding was supported by [9], who showed that in a folic acid nephropathy mouse model, plasma FGF23 levels were increased in mice with AKI as early as 4 to 6 hours after induction of renal injury. Both intact and C-terminal FGF23 levels were increased, the latter as high as 20- to 40-fold over baseline. Similarly, [10] investigated myocardial tissue of 5/6 nephrectomized (Nx) rats, which was a well-established model of experimental uremia. Indeed, cardiac Fgf23 gene expression was upregulated in myocardial tissue of 5/6Nx rats compared with sham operated animals.

In consistence with our study, [11] used a unilateral ureteral obstruction (UUO) rat model and showed increased Fgf23 mRNA expression in the obstructed kidneys. In that study, circulating iFGF23 levels increased and remained increased for up to 10 days after UUO. They further showed that removal of the 5/6 nephrectomy remnant in a CKD model, or removal of the obstructed kidney in a UUO model, did not attenuate the increase in circulating iFGF23 levels, suggesting that the production of FGF23 by the kidneys does not measurably affect circulating levels of FGF23. Accordingly, [12] measured plasma cFGF23 and iFGF23 in two large cohorts of critically ill adults patients with AKI requiring renal replacement therapy (RRT) who enrolled in the Acute renal failure Trial Network study (n = 817), and a general cohort of critically ill patients with and without AKI who enrolled in the Validating Acute Lung Injury biomarkers for Diagnosis study (n = 710). In both cohorts, patients in the highest versus lowest quartiles of cFGF23 and iFGF23 had a significantly increased risk of 60-day mortality after multivariable adjustment.

Surgical methods to induce CKD associated with cardiovascular changes are often accompanied by a high mortality and require well-trained surgeons. Thus, efforts have been made to establish non-surgical techniques

for CKD induction. A study of [13] was achieved in mice via a single injection of cisplatin and subsequent feeding of a high-phosphate diet. CKD was confirmed by high plasma levels of FGF23. The mice developed LVH and cardiac fibrosis. This model resembles the transition from acute kidney injury to chronic renal failure and thus displays a promising approach to study underlying mechanisms in humans. Data presented in the current study demonstrated a significant decrease in KLOTHO gene expression in CRS non-treated group compared to the normal control in both renal and cardiac tissues. This is in line with the observation of [14], who reported in a preclinical and clinical study, that in rodents with IRI-induced AKI, the levels of Klotho were reduced in the kidneys, urine and blood. Moreover, they also showed that a decrease in this protein level occurred before the reduction of other early biomarkers for kidney injury.

Another study of [15] have shown a downregulation of Klotho, followed by fibrosis, in rodent models with unilateral uretral obstruction (UUO), a model for CKD development. These results ware in accordance with [16], who showed that urinary klotho levels were significantly lower in patients with prerenal versus intrinsic AKI and postulated that assessment of urinary klotho levels might help differentiate these two entities. In addition, [17] examined kidney biopsy samples from patients with AKI from acute tubular necrosis or acute tubulointerstitial nephritis. They classified patients into three categories according to their renal klotho protein levels (low, medium, or high) and found that patients in the low klotho group had higher peak Serum Creatinine values and required RRT more frequently compared with patients in the medium or high klotho groups.

Renal and soluble klotho levels decrease in both animal and human models of AKI, and klotho has many important pleiotropic actions such as inhibition of apoptosis and cell senescence, antifibrosis and up-regulation of autophagy in kidney tubular cells. Thus, counteracting the decrease in klotho expression that occurs in AKI represents a promising strategy to prevent/ ameliorate AKI and related outcomes [18].

In treated group with BMMSCs FGF23 was markedly inhibited, and KLOTHO level was upregulated with little significant difference between treated group and normal control. These results agreed with [19], who investigated the protective effects of adipose-tissue-derived mesenchymal stem cells (ATMSCs) transplantation in Streptozotocin-Induced Diabetic Nephropathy. Interestingly, the researchers evaluated the higher levels of Klotho after the intervention proposed.

In the present study, the use of BM-MSCs was found to be capable of improvement of the

histological indices of injury in the renal and cardiac tissues. Histopathological data were analyzed for renal and cardiac tissues of rats and the results were reported. Myocardial fibers and glomerular architecture were normal in the control animals. In the CRS group, Hemorrhages were seen in the cardiac tissue and mononuclear cells were shown to be infiltrating the tissue along with fibrosis, concerning renal tissues, tubulointerstitial lesions (inflammatory cell infiltration and tubular dilation), coagulative necrosis, and a minor degree of fibroplasia were seen. Results agreed with [20], whose study was to apply adenine-containing diet, which is metabolized to 2,8-dihydroxyadenine, and which in turn precipitates as crystals in the renal proximal tubular epithelium causing inflammation and fibrosis, a non-surgical techniques for CKD induction. Indeed, mice treated with adenine developed symptoms of the cardiorenal syndrome, in particular cardiac hypertrophy, impaired cardiac function, as well as increased fibrosis.

Our results showed no cardiac alterations or fibrous tissue replacement in the tissues treated with mesenchymal cells, although there was congestion and hemorrhages in several places. There were very little alterations in the cellular morphology and no evidence of fibrosis in the kidney tissues treated with mesenchymal cells. The results of the current work are in accordance to [19], who investigated the protective effects of ADMSC transplantation in Streptozotocin-Induced Diabetic Nephropathy, which were further injected with adipose-tissue-derived mesenchymal stem cells (AT-MSCs). It was found that these cells resulted in a reduction of typical histological alterations in the animals treated, such as tubulointerstitial fibrosis and glomerular hypertrophy. Furthermore, the authors observed a decrease in apoptosis in renal tissue, as seen by a restoration of Bax and Bcl-2 to normal levels.

In concurrence with the present study, [21] indicated that transferring adipose-derived MSCs (A-MSCs) into a mouse model of unilateral I/R injury-induced AKICKD transition alleviated the fibrosis and atrophy of renal tissues 28 days after the severe event of AKI. These results go parallel with [22], who has suggested that MSCs treatment improves glomerular filtration, renal function, and alleviates oxidative stress-induced cell senescence and inflammation and increases the proliferation of kidney cells in an ischemia/reperfusion injury (IRI)-induced acute kidney injury model. Analogously, [23] conducted a study using allogeneic human BM-MSCs in two doses (2×10^7 and 1×10^8) administered via trans endocardial injection to patients with ischemic cardiomyopathy. Twelve months after MSC

administration, no serious treatment-related adverse events were observed. The allogeneic MSC therapy improved cardiac function, with scar size reduction in both groups and an increase in ejection fraction only in the group that received the higher dose. In a separate non-randomized and placebo-free phase I clinical trial, [24] treated patients receiving peritoneal dialysis with autologous ASCs at a dose of $1.2 \pm 0.1 \times 10^6$ cells/kg. While hematological and systemic biochemical parameters were stable over 24 weeks, a significant change in the ASC group was decreased body mass index (BMI), which probably resulted from the decrease in the degree of edema due to the increased ultrafiltration rate.

To closely examine the protective mechanism of MSC therapy, [25] investigated the role of intravenously infused autologous ASCs at doses of 1×10^5 and 2.5×10^5 for the treatment of atherosclerotic renovascular disease. They found that ASC infusions were well tolerated by the patients. Three months after cell transplantation, increased cortical perfusion and renal blood flow were reported. Increased kidney perfusion was accompanied by decreased fractional tissue hypoxia and stabilization of the glomerular filtration rate.

Despite the experimental success of MSCs in the treatment of renal diseases in animal models and the results from a limited number of human clinical trials demonstrating the safety and feasibility of MSC-based kidney therapy, the efficacy of the studies remains controversial. [26] conducted a phase II randomized placebo-controlled trial to determine the safety and efficacy of allogeneic MSCs in reducing the recovery time from AKI after cardiac surgery. Intra-aortic administration of MSCs at a dose of 2×10^6 cells/kg body weight was safe and well tolerated but did not markedly improve renal function or patient mortality. Further, MSC transplantation did not decrease the time to recovery of kidney function. Similarly, [27] conducted another clinical study in which autologous BM-MSCs at a dose of 2×10^6 cells/kg body weight were given to patients with autosomal-dominant polycystic kidney disease revealed no cell-related adverse effects at 12 months. Concomitantly, MSC infusion did not induce any significant changes in estimated glomerular filtration rate (eGFR) or reductions in serum creatinine compared to baseline in all patients.

Serum levels of urea and creatinine expression were demonstrated as a clinical marker in the diagnosis and monitoring of CRS. They rose between 4 and 24 hours in diseased group compared with control group. In the present study, the use of BM-MSCs was found to be capable of ameliorating renal dysfunction, as demonstrated by improvement of serum creatinine and urea levels. Similar previous

MSC-based clinical trials of [28] on Diabetic kidney disease (DKD) patients reported significant improvements in renal function, with decreased levels of serum creatinine, blood urea nitrogen (BUN), albuminuria and kidney hypertrophy. Moreover, the treatment showed the sustained reduction of blood glucose levels and insulin requirements. According to some studies, this occurs because MSCs and MSC-derived EVs reach the injured pancreas and restore glucose homeostasis through the preservation of β-cell function and the promotion of its proliferation.

5. Conclusion and Recommendations

The current thesis represents a unique work in studying the effect of applying MSCs in evaluation of Klotho and FGF23 genes. These represent a step towards proper diagnosis of CRS. However, further studies and testing are required to determine the potential use of MSCs for cell therapies and regenerative medicine.

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