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Research Article

Therapeutic Potential of Mesenchymal Stem Cell Conditioned Media on Creatinine Levels, Tubular Injury, and Proliferation in Ischaemic Reperfusion Kidney Injury Mice Models

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Abstract

Background: Ischemia/reperfusion (I/R) is a key factor in the pathophysiology of acute kidney injury (AKI), which can lead to chronic renal failure. The annual incidence of AKI is approximately 2000-3000 patients per million of the population. Despite various approaches attempted to combat AKI, both the general public and scientists remain unsatisfied. Conditioned medium from mesenchymal stem cells (CM-MSCs), containing growth factors, holds potential as an alternative therapy for AKI.

Objective: This study aimed to investigate whether CM-MSCs could reduce serum creatinine levels, repair injured tubular cells, and enhance proliferation of tubular epithelial cells in a mice model of renal I/R.

Methods: A pure experimental study was conducted using a completely randomized design with 20 Swiss male mice, aged 3-4 months and weighing 25-35 grams, classified into 4 groups, each comprising 5 mice. Periodic Acid Schiff (PAS) staining was employed to assess renal tubular injury, and tubular injury scores were recorded. Immunohistochemical staining with PCNA antibody was used to observe cell proliferation. Statistical analysis was performed using STATA 12.

Results: The statistical analysis revealed that treatment with 0.1 cc and 0.2 cc CM-MSCs did not significantly decrease serum creatinine levels when compared to the IR group. However, treatment with 0.1 cc and 0.2 cc CM-MSCs resulted in a significant reduction in tubular epithelial cell injury scores when compared to both the IR and SO groups. Conversely, treatment with 0.1 cc and 0.2 cc CM-MSCs did not significantly decrease the proliferation of renal tubular epithelial cells when compared to the IR group.

Conclusion: The administration of CM-MSCs in the mice model had a significant impact on serum creatinine levels, tubular epithelial cell injury, and proliferation of renal tubular epithelial cells (demonstrated by PCNA expression) during renal IRI. CM-MSCs show potential as an alternative therapy for AKI, although further comprehensive and multiperspective research is necessary to advance their development in regenerative medicine.

Keywords: CM-MSCs, IRI, PCNA, AKI, Regenerative Medicine.

1. Introduction

Acute kidney injury (AKI) was characterized by ischaemic/reperfusion related to excalation mortality, length of hospitalization, and acceleration toward chronic kidney disease. In Indonesia, there are 499.800 citizen that suffer from renal failure diseases in 2013 p[1]. According to the 7th Report of Indonesian Renal Registry 2014, there was 56% renal sufferers who belonging to productive age below 55 years old [2].

In AKI, the microvascular/endothelial injury is happening, tubular epithelial cells injury, and inflammation activation until disrupted cellular structures towards cells death. Severe ischaemic causes ions exchanges, swelling cells, no-reflow phenomenon, until cell death. Conditioned media - mesenchymal stem cells (CM-MSCs) can inhibit inflammation, so vasoactive cytokines decreases, NF-KB doesn't active, chronic kidney failure (CKD) doesn't happen. CM-MSCs accelerate regeneration, so tubular (epithelial) injury and hypoxia doesn't exist, fibrosis interstitial doesn't happen, and inhibit CKD. CM-MSCs inhibit activation of inflammation and thrombogenic cascade, so disruption of cellular structures, necrosis, and apoptosis don't happen, cell death doesn't exist (figure 1) [3, 4]. Any approaches will give no enlightenment in AKI management recently.

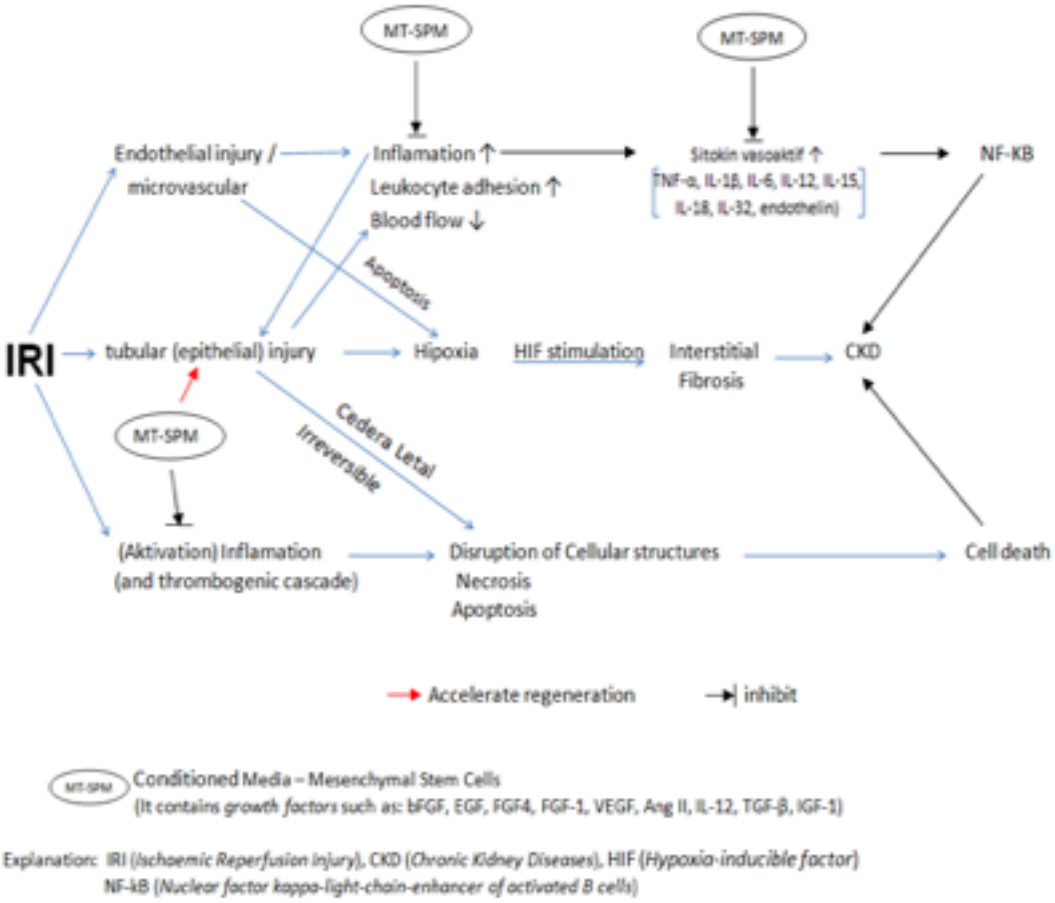


Figure 1: Theoretical Framework of this Research

Mesenchymal stem cells conditioned medium (MSCs–CM) contain growth factors and leukemia inhibitory factor which have important roles in various regeneration and repair processes [5, 6]. Conferment MSCs–CM inhibit inflammation, prevent descent of blood flow, cytokines doesn't be released, cellular structural doesn't experience disturbance, hamper apoptosis [7, 8, 3]. These are scientific reasons to do this research, i.e. to observe the potential of MSCs–CM as an adjunctive or supportive therapy in AKI management. The aims of this research are to explore the effects of MSCs–CM on creatinine level, injury, and proliferation of renal tubular in ischaemic reperfusion kidney injury mice models.

2. Material and Methods

This research was a pure experimental with completely randomized design, using 20 Swiss male mice, 3-4 months, 25–

35 gram, maintained in metabolic cage and provided with water and rat's food of ad libitum. They were classified into 4 groups; Sham operation (SO, n=5), Ischaemic-Reperfusion (IR, n=5), IR + MSC-CM 0.1 cc ip injection (IRMT1, n=5), and IR + MSC-CM 0.2 cc ip injection (IRMT2, n=5). All mice were terminated on 7th day. This research had accepted legal permission with No.Certificate 00106/04/LPPT/II/2017 from Ethical Clearance Commitee for pra-clinical research LPPT. PAS (Periodic Acid Schiff) staining to assess renal tubular injury with tubular injury scores, immunohistochemical staining with PCNA antibody to observe proliferation. Analysis of statistical results used STATA 12.

Blood serum was obtained from retroorbital vein for creatinine measurement. Tubular injury score measurement used Periodic Acid-Schifft (PAS) staining, examined with light microscope (Olympus CX22®) and captured by Optilab software with 400x magnification at the corticomedulary junction area as many as 15 fields per kidney. Scoring divided into 4 category, they were: 0-4 (0=normal; 1=tubular injury <25%; 2=tubular injury involve 25-50%; 3=tubular injury involve 50-75%; 4=tubular injury involve >75%). The assessment included renal tubular dilatation, loss of brush border of proximal tubules, depletion of tubular epitelial cell and the accumulation of intraluminal cast.

Data were analyzed using STATA 12 software; Shapiro Wilk test for normality and Levene test for homogeneity [9]. Multiple comparisons among the groups were done by one way ANOVA and followed by pos hoc LSD test, if data normality distributed. If data abnormally distributed, Kruskall Wallis test and pos hoc Mann Whitney test were used. $p<0.05$ was used to determined the level of significance.

3. Results and Discussion

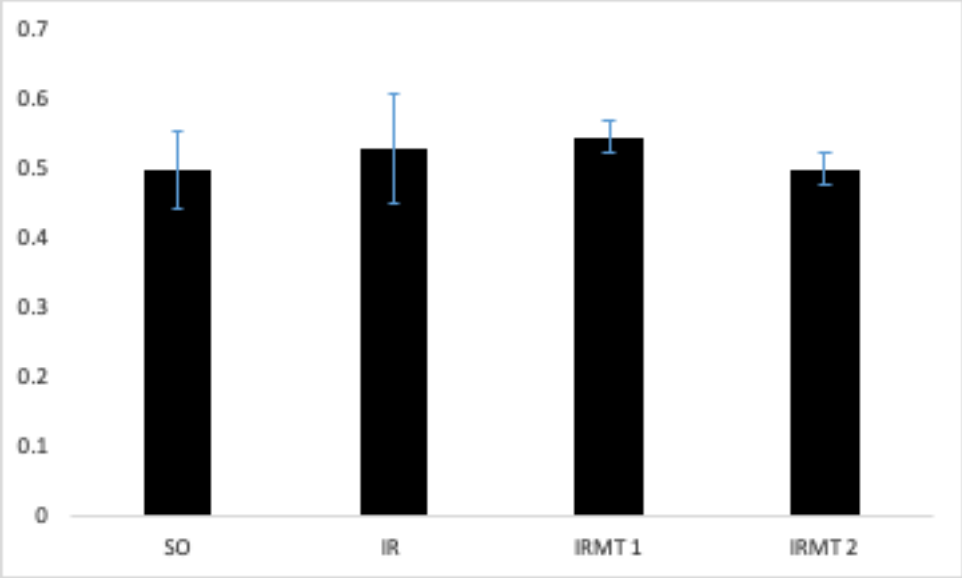
3.1 Creatinine Serum Level

Table 1: Description of the Result of Creatinine Serum Level

No	Variable	Mean±SD	Shapiro-Wilk
1	SO	0,494±0,064	0,354
2	IR	0,526±0,089	0,253
3	IR-MT 1	0,544±0,027	0,196
4	IR-MT 2	0,498±0,026	0,501

Mean values±SD, median (minimum – maximum), Shapiro Wilk test.
Explanation: SO (Sham Operation), IR (Ischemic Reperfusion), IR-MT1 (Ischemic Reperfusion – MSCs-CM 0.1 cc), IR-MT2 (Ischemic Reperfusion – MSCs-CM 0.2 cc).

The table 1 shows the details about the results of creatinine serum level. The graphic 1 shows that there is no real difference among groups. It may be caused by overlapping of standard error values among groups. The result of Shapiro-Wilk test is used to ascertain assumption of normality on each groups of treatment, if p-value more than 0.05, it means data distribution of creatinine serum result has similarity with normal distribution. This result means that ANOVA test can be used to assess creatinine data.



Graphic 1: Bar graphic of creatinine test result on each groups with line-saw of standard error. Explanation: SO (*Sham Operation*), IR (*Ischemic Reperfusion*), IR-MT1 (*Ischemic Reperfusion* – MSCs-CM 0.1 cc), IR-MT2 (*Ischemic Reperfusion* – MSCs-CM 0.2 cc).

Based on ANOVA result on table 2 (on supplement), it can be concluded that there is no difference of means among groups with p-value 0,512. This result was reinforced by the result of LSD test on table 3 (on supplement) that show the result of LSD test on each two groups. The result of LSD shows that there is no difference the means of creatinine level on each groups. Based on this result, it can be concluded that treatment (IR-MT1 and IR-MT2) can't lower level of creatinine serum significantly if compared with IR group.

Table 2: Result Anova Test; Variable Creatinine

Variable	Mean	95% interval confidence	p-value ANOVA
SO	0.494	0,463 – 0,556	0,512
IR	0.526	0,415 – 0,636	
IR-MT1	0.544	0,510 – 0,577	
IR-MT2	0.498	0,466 – 0,530	

Table 3: Result *post hoc* LSD Test Creatinine

	SO	IR	IR-MT1
IR	0,030*		
	1,000**		
IR-MT1	0,048	0,018	
	1,000	1,000	
IR-MT2	0,002	-0,028	-0,046
	1,000	1,000	1,000

3.2 Renal Tubular Epithelial Cells Injury

Renal tubular epithelial cells injury is one of hallmarks IRI on AKI. On this research, it will be shown about renal tubular epithelial cells injury through scientific image.

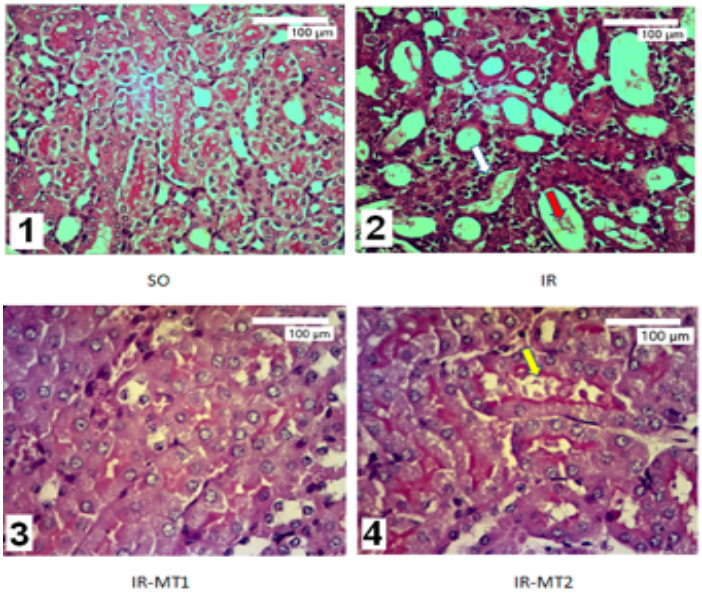


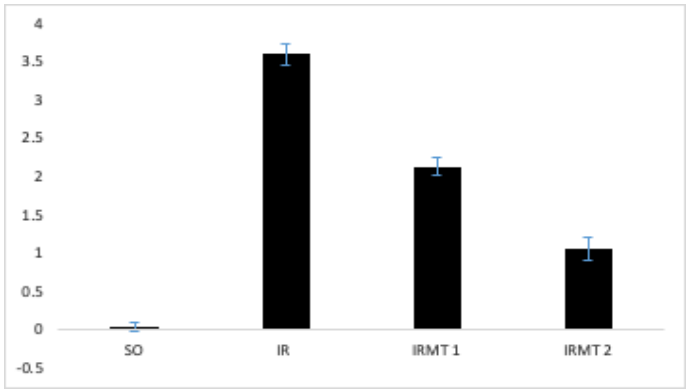
Figure2: Kidney microscopically with PAS staining, using light microscope, magnification 400x, scale 100 µm. White arrow shows infiltration of inflammation cells. Red arrow shows *intraluminal cast*. Yellow arrow shows brush border.

Table 4. Description of Renal Tubular Injury Score

No.	Variabel	Mean±SD	Shapiro-Wilk
1	SO	0,038±0,058	0,359
2	IR	3,60±0,158	0,133
3	IR-MT 1	2,132±0,140	0,072
4	IR-MT 2	1,066±0,174	0,852

Mean value±SD, median (minimum – maximum), Shapiro-Wilk test. Explanation: SO (*Sham Operation*), IR (*Ischemic Reperfusion*), IR-MT1 (*Ischemic Reperfusion* – MSCs-CM 0.1 cc), IR-MT2 (*Ischemic Reperfusion* – MSCs-CM 0.2 cc).

Table 4 reveals the details data of treatment towards results of renal tubular injury score. The result of Shapiro-Wilk test is used to ascertain assumption of normality on each groups of treatment, and p-value is 0.001. It means that the results data distribution of renal tubular injury score has similarity with normal distribution. This result shows that ANOVA test can be used to assess renal tubular injury score.



Graphic 2: Bar graphic the test result of tubular injury score on each groups with line-saw of standard error.

(**p-value* = 0,001) Explanation: SO (*Sham Operation*), IR (*Ischemic Reperfusion*), IR-MT1 (*Ischemic Reperfusion* – MSCs-CM 0.1 cc), IR-MT2 (*Ischemic Reperfusion* – MSCs-CM 0.2 cc).

Based on ANOVA result on table 5 (on supplement), it was shown that means of renal tubular injury score among treatment groups with p-value = 0,001. Analysis was continued with LSD test that is shown on table 6 (on supplement). The result of LSD test generally shows significance between two groups (p-value = 0,001). Based on this result, it can be concluded that treatment group IR-MT1 and IR-MT2 could decrease value of renal tubular injury score significantly compared with IR and SO group.

*Difference mean of row – mean of column; **P-value

Table 5. Result ANOVA test; variable Tubular Injury Score

Variable	Mean	95% interval confidence	p-value ANOVA
SO	0,038	(-0,016) – 0,092	0,001
IR	3,6	3,452 – 3,748	
IRMT 1	2,132	2,001 – 2,263	
IRMT 2	1,066	0,903 – 1,229	

Table 6. Result *post hoc* LSD Test; Tubular Injury Score

	SO	IR	IRMT1
IR	3,562*		
	0,001**		
IRMT1	2,094	-1,468	
	0,001	0,001	
IRMT2	1,028	-2,534	-1,066
	0,001	0,001	0,001

* Difference mean of row – mean of column; **P-value

3.3 Proliferation of Renal Tubular Epithelial Cells

The quantity of proliferation of renal tubular epithelial cells is shown through positive interpretation of IHC staining using PCNA antibody. PCNA antibody is a marker for proliferation.

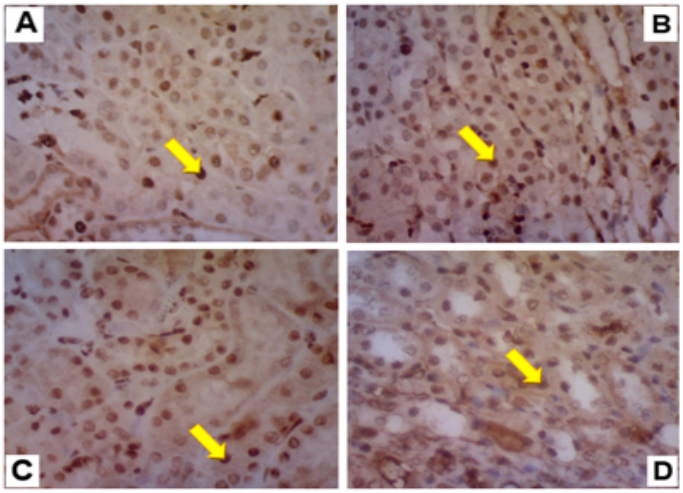


Figure 3:

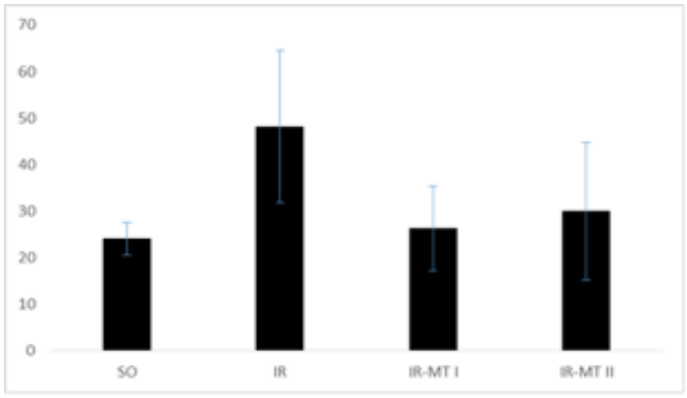
Microscopic image of kidney on 7th day with IHC staining using PCNA antibody, seen using light microscope, magnification 400x, scale 100 µm. Yellow arrows are PCNA expression. Explanation: In A group (SO), PCNA is expressed on nucleus, fewer than B group. Even though, treatment 0.1 and 0.2 cc MSCs-CM lower PCNA expression on C group (IRMT1) and D group (IRMT2).

Table 7. Description of PCNA Result

No.	Variabel	Mean±SD	Shapiro-Wilk
1	SO	24,107±3,524	0,212
2	IR	48,222±16,382	0,875
3	IR-MT 1	26,328±9,080	0,715
4	IR-MT 2	29,991±14,845	0,454

- Mean value±SD, median (minimum – maximum), and Shapiro Wilk test.
- Explanation: SO (*Sham Operation*), IR (Ischemic Reperfusion),
- IR-MT1 (*Ischemic Reperfusion* – MSCs-CM 0.1 cc),
- IR-MT2 (*Ischemic Reperfusion* – MSCs-CM 0.2 cc).

Table 7 shows data treatment on PCNA. Graphic 3 shows big difference among IR group, IR-MT1 group, and IR-MT2 group. However, standard error from three treatment groups are still overlapping. The result of Shapiro-Wilk test is used for observing normality assumption on each treatment groups. It shows p-value more than 0.05. It means that distribution of data PCNA test result has similarity with normal distribution. This results show us that ANOVA test can be used for PCNA data.



Graphic 3. Bar Graphic the result of PCNA test results on each treatment group with line-saw of standard error. Explanation: SO (*Sham Operation*), IR *(Ischemic Reperfusion*), IR-MT1 (*Ischemic Reperfusion* – MSCs-CM 0.1 cc), IR-MT2 (*Ischemic Reperfusion* – MSCs-CM 0.2 cc).

Based on ANOVA result on table 8 (on supplement), there is no difference data distribution among four treatment groups with p-value = 0,024. Result from LSD test on table 9 (on supplement) shows the same result, i.e.: there is no difference of data distribution on each groups as compared between two groups. Based on that result, it can be concluded that statistically treatment groups (IR-MT1 and IR¬-MT2) couldn't decrease PCNA value significantly if compared with IR group.

Table 8. Result ANOVA test; variable PCNA

Variable	Mean	95% Interval Confidence	p-value ANOVA
SO	24,107	20,809 – 27,405	0,024
IR	48,222	32,888 – 63,556	
IRMT 1	26,328	17,829 – 34,828	
IRMT 2	29,991	16,095 – 43,886	

Table 9. Result *post hoc* LSD Test; variable PCNA

	SO	IR	IRMT1
IR	24,115*		
	0,037**		
IRMT1	2,221	-21,894	
	1,000	0,067	
IRMT2	5,884	-18,231	3,663
	1,000	0,178	1,000

* Difference mean of row – mean of column; **P-value

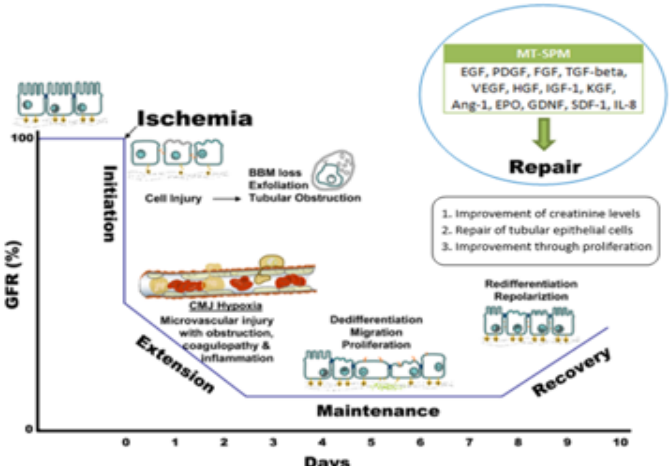
4. Discussion

Normal creatinine level on mice ranges from 0.1-0.4 mg/dL (mean 0.3 mg/dL) [10, 11]. Based on this parameter, value of creatinine on this research somewhat increased, i.e.: mean 0.496 (SO group), mean 0.526 (IR group), mean 0.544 (IR-MT1 group), mean 0.498 (IR-MT2 group). It appropriates with Wei and Dong analysis (2012); parameter of renal ischaemic reperfusion can be monitored from many level. One way is based on its severity of renal injury, i.e. reduction renal function which is detected through increasing creatinine serum and BUN (blood urea nitrogen) [12].

As maintenance phase on 7th day, serum creatinine level is relatively normal (borderline). On SO group, the average of creatinine serum level is 0.494. On IR group, the average of creatinine serum level is 0.526. On IR-MT1 group, the average of creatinine serum level is 0.544. On IR-MT2 group, the average of creatinine serum level is 0.498 which relatively similar with SO group, i.e. 0.494. The result of this research is appropriate with Kim et al, (2010) that stated creatinine plasma level on mice with bilateral renal ischemic treatment during 30 minutes tend to be normal on 8 days later [13].

In this research, MSCs-CM has a role in repair processes, such as emendation of creatinine level, tubular epithelial cells, and tubular proliferation (table 10) because of having various growth factors. Mesenchymal stem cells conditioned media contain various growth factors (such as fibroblast growth factor, etc.) and leukemia inhibitory factor. All of these components are important for early renal development, inducted on mature renal after ischemic injury. They play important roles in various regeneration and repair mechanisms [5, 6].

Table 10. Various phases on ischemic AKI



- There is an intimate relation between clinical phases and cellular phases of AKI.
- Including temporal effects of organ functions, representated through GFR.
- Treatment of MSCs-CM (contains various growth factors) induces repair process [14, 15, 7].

There is correlation between MSCs-CM with renal tubular epithelial cell injury. In this research, treatment 0.1 cc MSCs-CM could normalize renal tubular epithelial cell injury. On control group (IR), we hadn't given MSCs-CM injection, the average of renal tubular epithelial cell injury score was 3.6 whereas after giving treatment 0.1 cc MSCs-CM, the average of renal tubular epithelial cell injury score became 2.132. This score decreased until 1.066 while giving 0.2 cc MSCs-CM (table 5). Therefore, treatment 0.1 cc and 0.2 cc MSCs-CM decreased tubular epithelial cell injury in mice with renal I/R injury. This research was appropriated with Xing et al (2014). Xing and colleagues revealed that mesenchymal stem cells can improve renal function, increase survival and proliferation of parenchymal cells and decrease significantly apoptotic cells [16].

There are intimate relation among MSCs-CM, ischemic-reperfusion, and proliferation of renal tubular epithelial cell. Renal ischemic-reperfusion treatment can increase proliferation of renal tubular epithelial cell. This research proved average value renal tubular epithelial cell proliferation (shown through PCNA expression) increase from 24.107 (in SO group) to 47.040 (in IR group). Treatment 0.1 cc MSCs-CM tended to normalize proliferation of renal tubular epithelial cell. The average of renal tubular epithelial cell proliferation showed 26.200 (IR-MT1 group), which was nearly similar with average of renal tubular epithelial cell proliferation on SO group, i.e. 24.107. Treatment 0.2 cc MSCs-CM decrease average of renal tubular epithelial cell proliferation became 30.947 from 47.040 on IR group (table 8). The result of this research was different from Herrera et al (2004) which us-

ing mesenchymal stem cells. Mesenchymal stem cells (MSCs) increase tubular proliferation, characterized by rise of positive PCNA (proliferating cell nuclear antigen) cells. Effect of intramuscular-injected MSCs infusion was observed from tubular regeneration and recovery of C57/BL6 mice from acute renal failure [17]. The result of this research was also different from Chen et al (2015) because of effects MSCs-CM on increasing proliferation and migration.18 PCNA is known as marker of proliferation. Chen et al (2015) had proven that activation JNK and P38, but not ERK, are needed for proliferation and migration AEC (alveolar epithelial cells) and SAEC (small airway epithelial cells). Pretreatment with AEC or SAEC with SP600125, JNK1 inhibitor, or SB200358, P38 inhibitor, significantly reduces cell migration and proliferation. A protein array including type 1 TGF-beta receptor, type 2 TGF-beta receptor, Ras-related C3 botulinum toxin substrate 1, and Ras-related C3 botulinum toxin substrate 2 that influence migration and proliferation AEC and SAEC were detected on MSCs-CM [18].

Proliferation has related with AKI. Witzgall et al (1994) show rise proliferation on contralateral renal after ipsilateral IRI. Subpopulation from tubular cells (scattered tubular cells, STCs) became abundant as response toward AKI and had an important role in regenerative process. Excalation of phenotype induction of STCs in a little but significant from proximal tubular cells in contralateral renal was observed during recovery phase after unilateral ischaemic AKI [19]. This research showed that treatment MSCs-CM statistically didn't have an impact on proliferation of renal tubular epithelial cells (assessed through PCNA expression) on mice with renal I/R injury. This was happened because of two things. First, shortness of half-life growth factors. Second, complexity, variety, quantity, quality of growth factors and another particles or substances that contained within MSCs-CM. Growth factors contributes in regeneration process of injured tissue organs, with focus on proliferation. PDGF (Platelet-derived growth factor) has an important role in connective tissues, glial, and another cells; EGF (Epidermal growth factor) for epithelial cells, glial, and mesenchym; IGF-I (Insulin-like growth factor 1) and IGF-II for various cells [20]. Placental growth factor (PlGF) is a member of VEGF family, increases VEGF activity in vitro and in vivo [21]. Keratinocyte growth factor (KGF) impede epithelial cells induced by oxidative stress [22]. Nerve growth factor (NGF) increase neuritis outgrowth. BDNF (brain-derived neurotropic factor) is neuroprotective, increase cells' viability, reduce astroglial scar formation and several growth factors, including HEGF (Human Epidermal Growth Factor), FGF-7 (Fibroblast Growth Factor 7), EGF (epidermal growth factor), and HGF (Hepatocyte growth factor) increase liver regeneration [23, 24].

The explanation about mechanism of cell proliferation which is caused by MSCs-CM can be explored herein. Macrophage colony stimulating factor receptor atau MCSFR21 increases myeloid progenitor, mononuclear phagocytes, development and growth of placental trophoblast growth [25]. PDGF-R (Platelet-derived growth factor receptors) can become interactive together with various signals molecules or integrin [21]. This causes cell proliferation, cell motility, cell differentiation, or survival through apoptosis inhibition [26].

There is two hypotheses in connection with process of renal tubular epithelial proliferation after acute injury. First, progenitor cells or stem cells create novel epithel. Second, differentiated epithelial cells experience dedifferentiate process, reenter cell cycle, and produce novel epithelial cells through self-duplication process. Both of these two theories have supporters and evidence-based [27]. Human proximal tubular cells which expresses vimentin, CD24, and CD133 had been identified. These putative progenitor cells were found in parietal epithel and also spread in all proximal tubular. If isolated ex vivo, these cells can shape spheres and develop clonally. Those cells repair AKI and contribute to epithelial lineage on experimental model [28-31].

5. Conclusion

The administration of mesenchymal stem cells-conditioned medium (MSCs-CM) demonstrated promising outcomes in the context of acute kidney injury induced by renal ischemia/reperfusion (IRI). Notably, the use of MSCs-CM resulted in a notable reduction in serum creatinine levels, particularly evident with the administration of 0.2 cc MSCs-CM, although statistical significance was not achieved. Moreover, the treatment exhibited a significant and favorable impact on the reduction of renal tubular epithelial cell injury in mice subjected to renal IRI. However, it should be noted that the administration of MSCs-CM did not yield a significant increase in the proliferation of renal tubular epithelial cells in the same mice model of renal IRI. These findings collectively highlight the potential of MSCs-CM as a valuable therapeutic approach for mitigating renal injury; nevertheless, further research is warranted to explore and optimize its regenerative effects comprehensively and holistically.

Limitation and Recommendation

This research still has several limitations and areas for improvement, highlighting the need for further investigations into cellular-molecular and cellular-genetic mechanisms, while upholding the utmost research ethics and obtaining informed consent when involving human subjects. Future studies are encouraged to explore various aspects, including diverse dosage regimens, administration routes, and intervals (e.g., days 1, 5, 7, 10, etc.). Additionally, a comparative analysis of CM-MSCs administration should be conducted, either alone or in combination with optogenetics, exosome delivery, or omics-based methodologies for precise measurement. Expanding this research to encompass gene expression profiling and assessments of apoptosis parameters and fibrosis percentage, utilizing diverse strains or varieties of mice, would also be advantageous. Furthermore, employing pharmacological interventions for AKI and evaluating pharmacokinetic and pharmacodynamics profiles are essential components of future investigations. Finally, conducting

comparisons between the effectiveness of CM-MSCs and other cell types such as Mesenchymal stem cells, adult renal stem/progenitor cells, Renal progenitors derived from iPSCs, and Nrf2, known as the master regulator of genes encoding antioxidants with anti-inflammatory effects, should be pursued as well.

Acknowledgement

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