Study on Mitochondrial Transplantation Therapy in the Treatment of Acute Kidney Injury-Induced Oxidative Injury in Renal Tubular Epithelial Cell

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Received December 29, 2024
Accepted July 07, 2025
Electronic access July 31, 2025

Acute kidney injury (AKI) is an inflammatory kidney disease that may lead to kidney failure if untreated. AKI is a widespread disease with limited treatments against it after being diagnosed. AKI is often caused by elevated levels of Reactive Oxygen Species (ROS), which damages the kidney cells, especially their mitochondria. Damaged mitochondria causes several negative impacts to the cell, one of which is an increased level of ROS, causing necrosis and apoptosis. Thus, mitochondria are one of the factors which a treatment can be applied to reduce cell damage in AKI. Specifically, we believe that mitochondrial transplantation may be a treatment towards AKI since it provides healthy mitochondria to the damaged cells, replacing the damaged mitochondria and halting further damage. Nevertheless, mitochondrial transplantation is considered a novel treatment towards multiple inflammatory-related diseases, and there are no direct studies showing its treatment against AKI; therefore, our study aims to discover the effect of mitochondrial transplantation. In this study, we use H_2O_2a type of ROSto induce AKI in NRK52E cells in vitro. Then, with isolated healthy mitochondria, we treat a group of cells and compare its result. The result presents a significant treatment by mitochondria towards cells damaged by H_2O_2. Specifically, Hypoxia-inducible factor-1 reduces in cells treated with mitochondria. Moreover, ROS reduced in production and generation; antioxidant-related proteins such as nuclear factor erythroid 2related factor 2 (Nrf2), silent information regulator 1 (Sirt1), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PCG-1), and superoxide dismutase 2 (SOD2) increased; and apoptosis-related proteins such as Caspase3 reduced in the group of cells treated with mitochondria. In conclusion, this study demonstrates mitochondrial transplantation as a promising future treatment towards AKI.

Introduction

Acute kidney injury (AKI) is a pressing public health concern which affects approximately 13.3 million patients each year and leads to 1.7 million deaths[1]). The condition poses a considerable burden on healthcare systems and patients alike. AKI not only results from various factors such as dehydration, and medication toxicity, but also a systemic inflammatory response syndrome with severe tubular cell injury (Figure 1)[2]),[3].

Reactive oxygen species (ROS) is one of the main factors that cause tubular cell injury during systemic inflammatory response syndrome (Figure 2)[4],[5]. ROS production is largely ascribed to inflammation since ROS is one of the mechanisms used by the immune system to eliminate pathogens (Figure 3)[6]

ROS damages both pathogenic and non-pathogenic cells by breaking DNA strands in cells, a process that induces apoptosis[7]. While ROS eliminates pathogens, it also damages the DNA of the organisms cells. Among ROS, H_2O_2 is one of the most

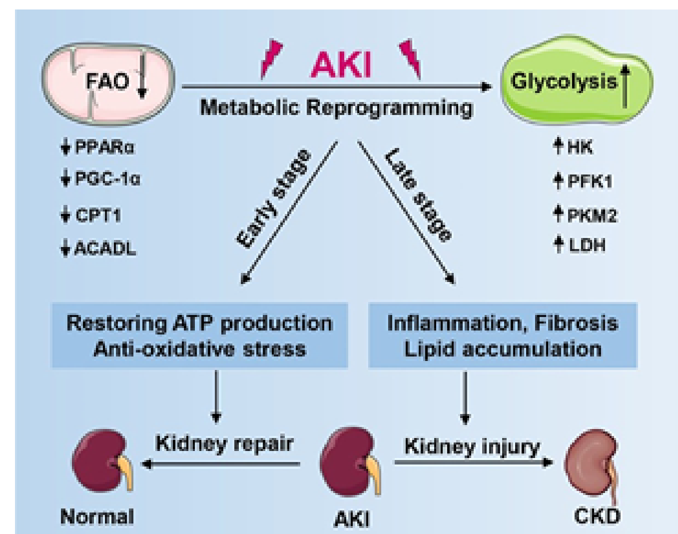

Fig. 1 Early Stage of AKI Is Related to Oxidative Stress[2]

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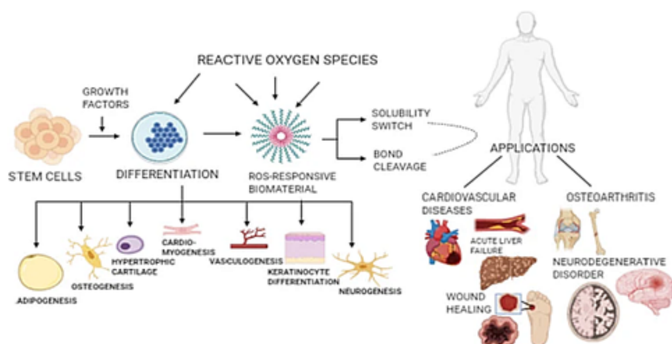

Fig. 2 ROS Is a Factor That Injures Tubular Cell in Systemic Inflammatory Response[4]

stable; therefore, H_2O_2 is considered to contribute the most ROS damage[8].

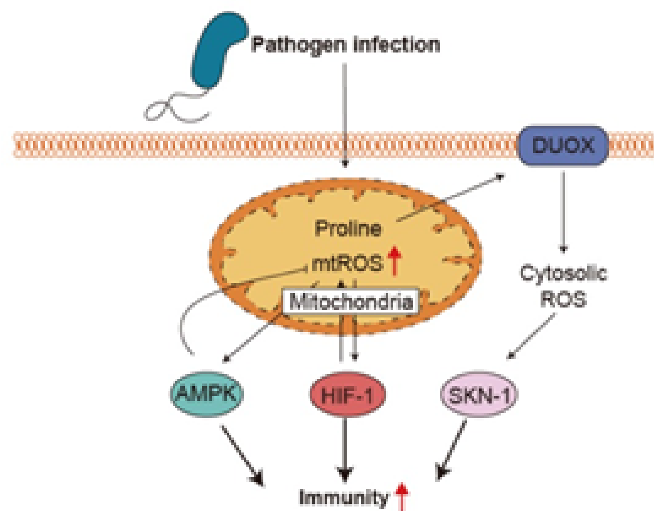

Fig. 3 Reactive Oxygen Species Enhance Immunity[6]

In order to balance the oxidative stress caused by ROS, cells produce proteins involved in countering the oxidative stress, one of which is silent information regulator 1 (Sirt1)[9]. Some findings suggest the correlation between the Sirt1 and the resistance to oxidative stress of cultured renal medullary interstitial cells (Figure 4)[10]. The findings also mentioned that in mice with AKI, the lack of Sirt1 exacerbates renal apoptosis and fibrosis[11,12]. Sirt1 is also related to several other proteins, some of them are nuclear factor erythroid 2related factor 2 (Nrf2) and heme oxygenase-1 (HO-1). Nrf2 is involved in detoxifying ROS and exhibits elevated levels during high oxidative stress in active cells (Figure 5)[13]. Therefore, the Nrf2/HO-1 pathway can be used as an index for the amount of active cells.

Mitochondria are especially susceptible to ROS. Mitochondrial damage can cause insufficient production of ATP and cell damage from built-up oxygen, sugar, and fat molecules in the

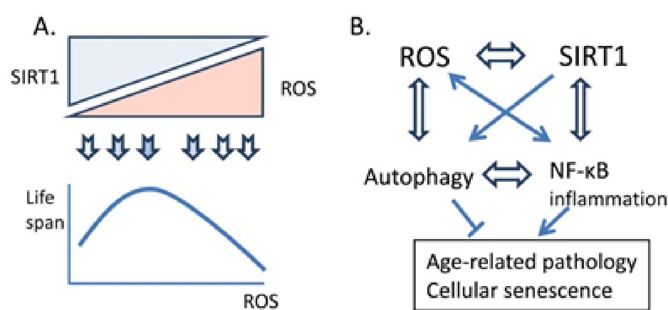

Fig. 4 Sirt1 Plays an Important Role in ROS[10]

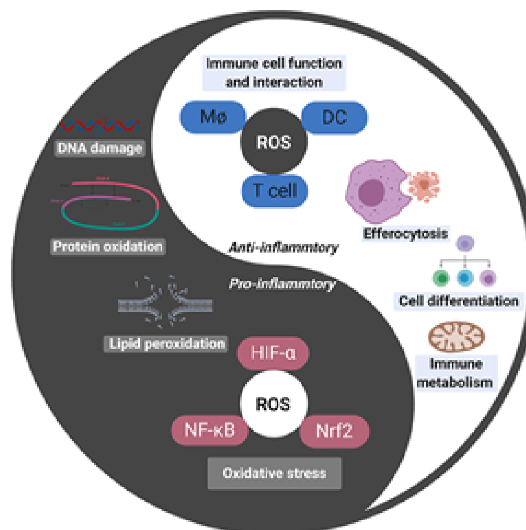

Fig. 5 Oxidative Stress and Signal Molecule of ROS[13]

cell (Figure 6)[14]. Furthermore, damaged mitochondria also produce ROS, which ultimately leads to cell necrosis and apoptosis.

Current clinical therapies are typically supportive in terms of retarding the damage caused by AKI, such therapies include providing fluids challenge and dialysis (Figure 7)[15]. Nevertheless, these therapies have no substantial impact on damaged cells. Previous studies frequently observed Mitochondrial damage in AKI[16].

Mitochondrial damage causes insufficient production of ATP and damage from built-up oxygen, sugar, and fat molecules in the cell (Figure 8)[17]. Furthermore, damaged mitochondria also produce ROS[18]. ROS ultimately leads to cell necrosis and apoptosis[19].

Increasingly, studies have pointed out that mitochondrial transplantation therapy can use the phenomenon of mitochondrial transfer to replace damaged mitochondria in cells and further rescue cells (Figure 9)[20–22].

Mitochondrial transplantation is studied in different disease models, Hayashida, K et al. discovered that mitochondrial transplantation recovers neural function[23]. McCully et al. im-

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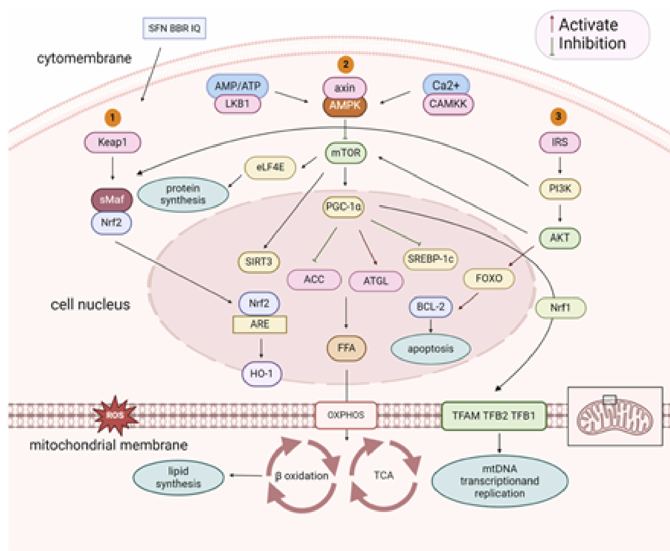

Fig. 6 ROS-induced Mitochondrial Dynamics-related Signaling Pathways [14]

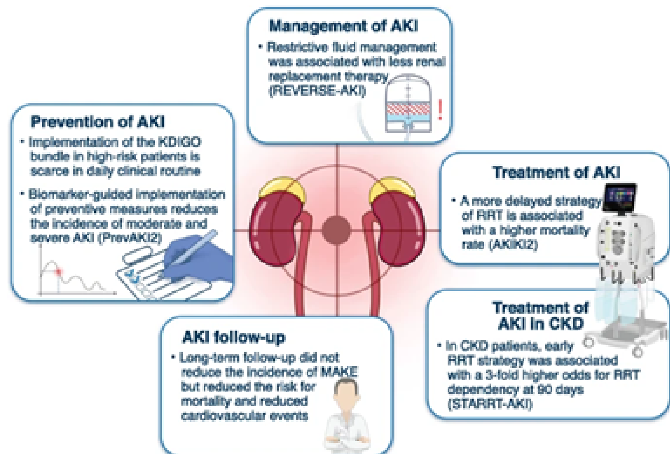

Fig. 7 Current Clinical Therapies of AKI [15]

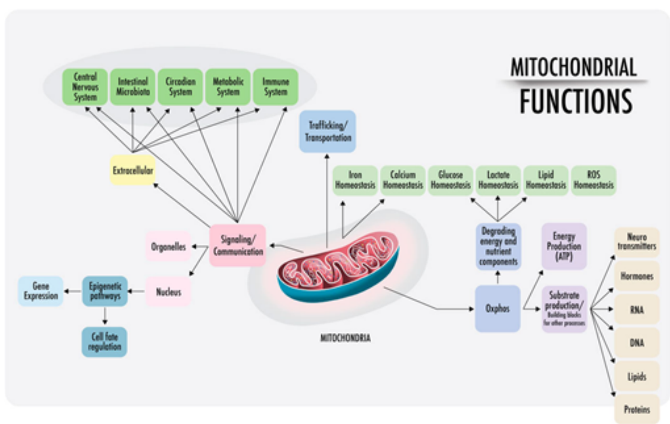

Fig. 8 Mitochondrial Functions [17]

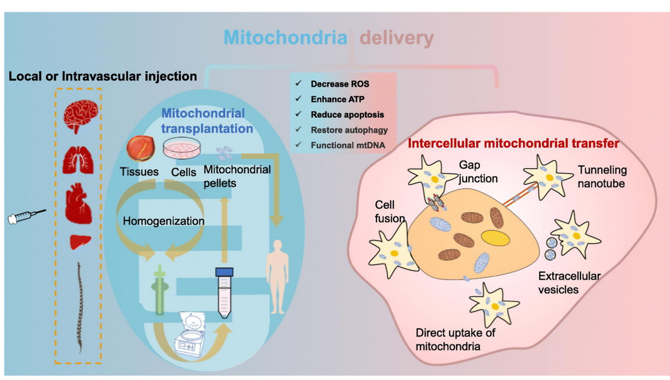

Fig. 9 Benefits of Mitochondrial Transplantation Surgery [22]

plemented mitochondrial transplantation for the rescue of cell viability and cell function following ischemia-reperfusion injury [24,25]. Emani et al. implemented mitochondrial transplantation in clinical trials on patients with ischemia-reperfusion injury [26]. In addition, studies implemented mitochondrial transplantation on curing cancer, Tsai et al. shows that mitochondrial transplantation reduces gastric cancer tumor growth in vivo [27]. Furthermore, according to the study of Andrea Rossi et. al., mitochondrial transplantation mitigates damage in an in vitro model of renal tubular injury and in an ex vivo model of DCD renal transplantation, proving the ability of mitochondrial transplantation to reduce kidney damage in clinical conditions [28].

However, there is currently no research related to the effect of mitochondrial transplantation on AKI. Thus, we hypothesise that mitochondrial transplantation is able to recover the function of mitochondria and suppress the ROS level in cells to reduce cell damage. We used a cell model in our study to prove our hypothesis. According to the study of Kenji Kasuno et. al., H_2O_2 can be used for a factor to induce AKI in cell models [29]. Therefore, we used a similar method to conduct our study. Our experiment design uses H_2O_2 to stimulate cells and observe whether providing healthy mitochondria to cells reduces ROS and restores mitochondrial activity in cells, hence saving the damaged cells (Figure 10). Goals of the Study

- To demonstrate whether H_2O_2 induces cell-death
  1. The cells are treated with different concentrations of H_2O_2 and CCK8 assay is used to analyze cell viability.

- To demonstrate whether mitochondrial transplantation recover the oxidative stress by H_2O_2 treatment
  1. After mitochondrial transplantation, the anti-oxidative capacity of the cell is analyzed by flow cytometry to observe oxidation-related markers (mitoSOX, H2DCFDA, DHE).
  2. The level of oxidative stress after mitochondrial transplantation is analyzed by western blot to observe HIF-1

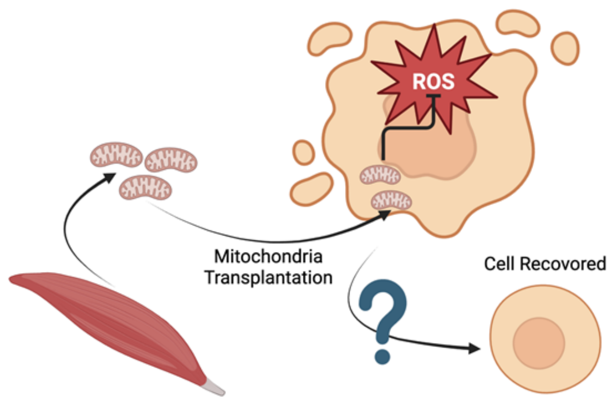

Fig. 10 Mitochondrial Transplantation Reduces Cellular ROS and Recovers Cell Injury

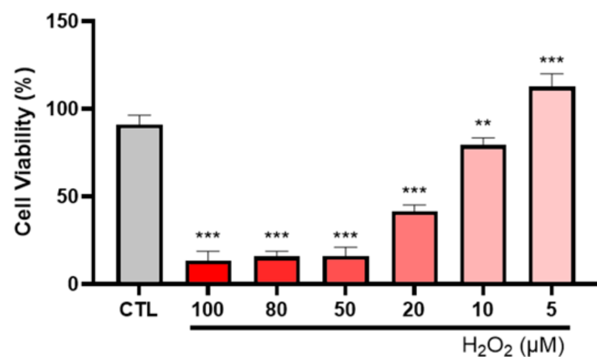

Fig. 11 H_2O_2 Reduced Cell Viability in a Dose-dependent Manner. The cell viability was analyzed by cck8 on NRK52E cells after being treated with H_2O_2 (5, 10, 20, 50, 80, and 100 μM) for 24 hours. Data are shown as the mean $\pm SEM; n \geq 6$ independent experiments, one-way ANOVA: $^{*}P_{Bonferroni} < 0.008, ^{**}P_{Bonferroni} < 0.001, ^{***}P_{Bonferroni} < 0.0001$.

expression.
3. After mitochondrial transplantation, the anti-oxidative capacity of the cell is analyzed by western blot to observe oxidation-related markers (Nrf2, Sirt1, PCG-1, SOD2).

- To demonstrate whether mitochondrial transplantation recover the damage by H_2O_2 treatment
1. After mitochondrial transplantation, the cell-death is analyzed by flow cytometry to observe apoptosis markers (Caspase3 activity).
2. After mitochondrial transplantation, the cell-death is analyzed by western blot to observe apoptosis markers (Bax, Bcl2, Caspase3).
3. After mitochondrial transplantation, the cell viability is analyzed by CCK8 assay.

Result

H_2O_2 Induces Cell-death

In our study, we use H_2O_2 to simulate the AKI in vitro model. Our results suggest H_2O_2 induced cell damage in different concentrations (Figure 11). Our data show the concentration of IC_{50} is 20 μM. Thus, the experiment is conducted under 20 μM of H_2O_2 treatment.

Mitochondrial Transplantation Effectively Recovers ROS on NRK52E Cells

Present study shows that AKI is an inflammatory disease. Hence, our study uses mitochondria transportation to observe if it recovers the oxidative stress by H_2O_2 treatment. First, we observe if mitochondrial transplantation reduces mitochondrial superoxide production. The cell is co-cultured with or without mitochondria under H_2O_2 treatment for 24 hours. We used mitoSOX Red to analyze the superoxide production in mitochondria. We observed accumulation of superoxide in the group treated with H_2O_2 and decreased superoxide level in the group with both mitochondria and H_2O_2. The data show mitochondrial transplantation reduces superoxide production effectively (Figure 12). Next, we used H2DCFDA and DHE dye to reveal that H_2O_2 increases ROS level and mitochondria reduces ROS production (Figure 13 and 14). The results above show that mitochondrial transplantation has a significant effect on decreasing cell oxidative stress.

Due to the correlation between AKI and hypoxia, We observe hypoxia-inducible factor 1-alpha (HIF-1α) expression after mitochondrial transplantation. Our data show H_2O_2 induces HIF-1α expression, which is reduced after mitochondrial transplantation (Figure 15). The result shows the potential for mitochondrial transplantation to recover the injury caused by AKI. Furthermore, we observed the antioxidative ability of mitochondrial transplantation. Our data show antioxidant-related proteins such as Nrf2, Sirt1, PGC-1α, Superoxide dismutase 2 (SOD2) are inhibited by H_2O_2 treatment. Those proteins increased in expression after mitochondrial transplantation (Figure 16). According to Figure 15 and 16, the groups with mitochondria show that mitochondrial transplantation effectively recovers the increase in inflammatory factors induced by H_2O_2 and defences the inflammatory response by upregulating the antioxidant related protein, proving that mitochondrial transplantation has the ability to counteract the oxidative stress. In summary, our study proved the potential of AKI treatment by mitochondrial transplantation.

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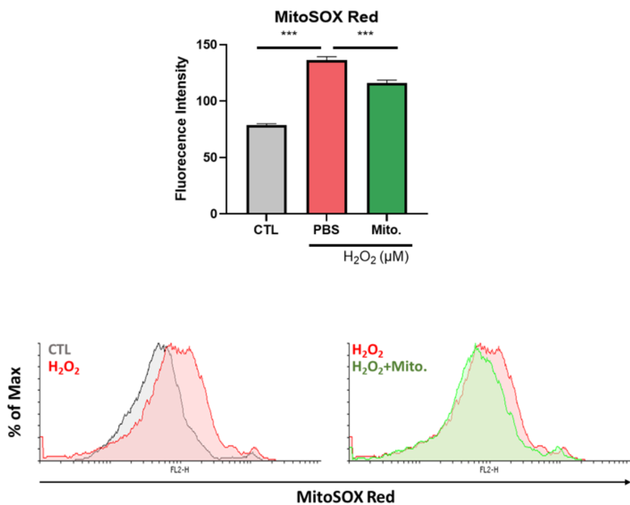

Fig. 12 Mitochondria Recovered the Damage Caused by the Superoxide Production Induced by H_2O_2 Treatment. (A) The superoxide production was analyzed by mitoSOX Red staining through the flow cytometry on NRK52E cells after being treated with or without mitochondria under H_2O_2 (20 μM) for 24 hours. (B) The quantification of the superoxide production was analyzed by mitoSOX Red staining through the flow cytometry on NRK52E cells after being treated with or without mitochondria under H_2O_2 (20 μM) for 24 hours. Data are shown as the mean ± SEM; $n \geq 3$ independent experiments, one-way ANOVA: $^{*}P_{\text{Bonferroni}} < 0.016$, $^{**}P_{\text{Bonferroni}} < 0.003$, $^{***}P_{\text{Bonferroni}} < 0.0003$.

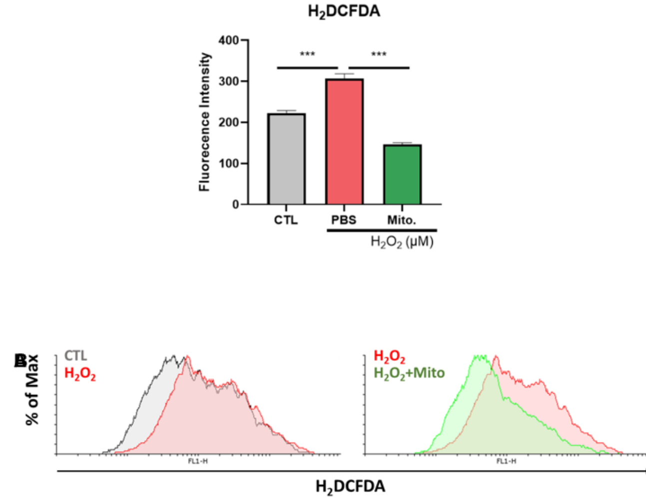

Fig. 13 Mitochondria Recovered the Damage Caused by the ROS Production Induced by H_2O_2 Treatment. (A) The ROS production was analyzed by H2DCFDA staining through the flow cytometry on NRK52E cells after being treated with or without mitochondria under H_2O_2 (20 μM) for 24 hours. (B) The quantification of the ROS production was analyzed by H2DCFDA staining through the flow cytometry on NRK52E cells after being treated with or without mitochondria under H_2O_2 (20 μM) for 24 hours. Data are shown as the mean ± SEM; $n \geq 3$ independent experiments, one-way ANOVA: $^{*}P_{\text{Bonferroni}} < 0.016$, $^{**}P_{\text{Bonferroni}} < 0.003$, $^{***}P_{\text{Bonferroni}} < 0.0003$.

Mitochondrial Transplantation Recovers the Cell Death of NRK52E Cells

Furthermore, our research aims to know whether mitochondria are able to recover the cells damaged by H_2O_2. We utilized flow cytometry to analyze the activity of Caspase3 (Figure. 17). Figure 16 shows that the fluorescence density of the H_2O_2 group increases compared to the control group and the group with mitochondria decreases in fluorescence density compared to the H_2O_2 group. The result demonstrates the ability for mitochondria to reduce the Caspase3 activity effectively. In addition, we utilize western blot to analyze the apoptosis-related protein expression. The result shows a significant increase in Bax expression after H_2O_2 treatment, and mitochondrial transplantation inhibited Bax expression. On the contrary, the result shows decrease in BCL2 expression after H_2O_2 treatment, and mitochondrial transplantation increased Bax expression. Bax and BCL2 are known to play important roles in the process of apoptosis. We prove that mitochondria inhibits H_2O_2 induced cell apoptosis. Similarly, mitochondria inhibits Cleaved Caspase-3 (c-Caspase3) expression, which aligns with the result of flow cytometry (Figure 18). According to Figure 17 and 18, mitochondrial transplantation prevents cell apoptosis in acute inflammation, rescuing the cell from death. Finally, the result of CCK8 shows that mitochondrial transplantation increases the cell viability under H_2O_2 treatment (Figure 19). In summary, our study proves that mitochondrial transplantation may recover the cell damage from AKI.

Mitochondrial Transplantation Increase the ATP Concentration on NRK52E Cells

Finally, our study analyses the ATP concentration in cells with mitochondrial transplantation. The data shows that the cells treated with H_2O_2 are significantly lower than control and there is a significant increase in ATP concentration in cells with mitochondria transplantation, proving mitochondrial transplantation recovers the quantity of ATP decreased by H_2O_2. Since mitochondrial activity directly affects the production of ATP in cells, the result proves that mitochondrial transplantation increases mitochondrial activity, presenting the method as having a rescuing effect towards mitochondrial disability in AKI and decrease in ATP quantity.

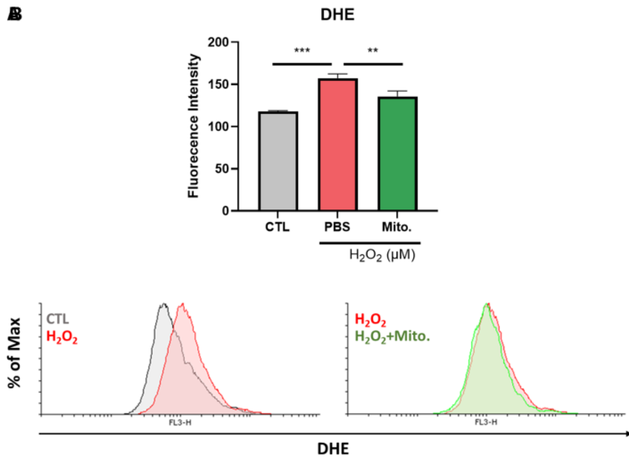

Fig. 14 Mitochondria Recovered the Damage Caused by the ROS Generation Induced by H_2O_2 Treatment. (A) The ROS generation was analyzed by DHE staining through the flow cytometry on NRK52E cells after being treated with or without mitochondria under H_2O_2 (20 μM) for 24 hours. (B) The quantification of the ROS generation was analyzed by DHE staining through the flow cytometry on NRK52E cells after being treated with or without mitochondria under H_2O_2 (20 μM) for 24 hours. Data are shown as the mean ± SEM; $n \geq 3$ independent experiments, one-way ANOVA: $^{*}P_{\text{Bonferroni}} < 0.016$, $^{**}P_{\text{Bonferroni}} < 0.003$, $^{***}P_{\text{Bonferroni}} < 0.0003$.

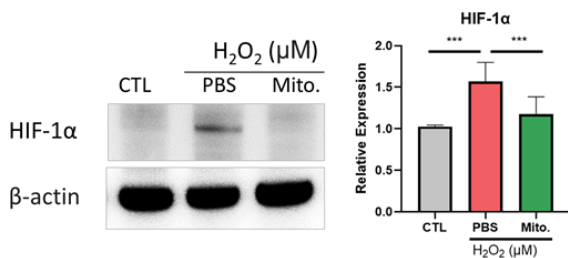

Fig. 15 Mitochondria Inhibited the Expression of HIF-1α Induced by H_2O_2 Treatment. (A) The protein expression of HIF-1 was analyzed by western blot on NRK52E cells after being treated with or without mitochondria under H_2O_2 (20 μM) for 24 hours. (B) The quantification of the protein expression of HIF-1 was analyzed by western blot on NRK52E cells after being treated with or without mitochondria under H_2O_2 (20 μM) for 24 hours. Data are shown as the mean ± SEM; $n \geq 6$ independent experiments, one-way ANOVA: $^{*}P_{\text{Bonferroni}} < 0.008$, $^{**}P_{\text{Bonferroni}} < 0.001$, $^{***}P_{\text{Bonferroni}} < 0.0001$.

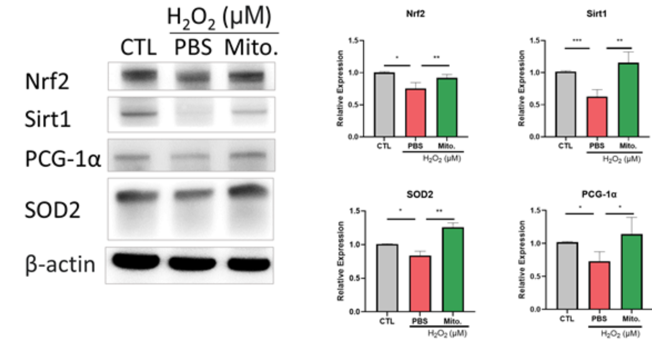

Fig. 16 Mitochondria Inhibited the Expression of Antioxidant-related Protein Induced by H_2O_2 Treatment. (A) The protein expression of Nrf2, Sirt1, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PCG-1α), and SOD2 were analyzed by western blot on NRK52E cells after being treated with or without mitochondria under H_2O_2 (20 μM) for 24 hours. (B) The quantification of the protein expression of Nrf2, Sirt1, PCG-1α, and SOD2 were analyzed by western blot on NRK52E cells after being treated with or without mitochondria under H_2O_2 (20 μM) for 24 hours. Data are shown as the mean ± SEM; $n \geq 6$ independent experiments, one-way ANOVA: $^{*}P_{\text{Bonferroni}} < 0.008$, $^{**}P_{\text{Bonferroni}} < 0.001$, $^{***}P_{\text{Bonferroni}} < 0.0001$.

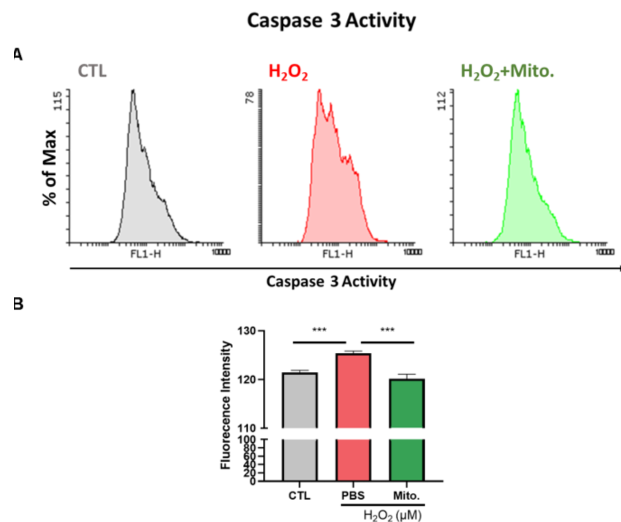

Fig. 17 Mitochondria Recovered the Damage Caused by the Caspase3 Activity Induced by H_2O_2 Treatment. (A) The Caspase3 Activity was analyzed by flow cytometry on NRK52E cells after being treated with or without mitochondria under H_2O_2 (20 μM) for 24 hours. (B) The quantification of the Caspase3 Activity was analyzed by flow cytometry on NRK52E cells after being treated with or without mitochondria under H_2O_2 (20 μM) for 24 hours. Data are shown as the mean ± SEM; $n \geq 6$ independent experiments, one-way ANOVA: $^{*}P_{\text{Bonferroni}} < 0.008$, $^{**}P_{\text{Bonferroni}} < 0.001$, $^{***}P_{\text{Bonferroni}} < 0.0001$.

Discussion

Mitochondrial transplantation for the prospective treatment of AKI

Acute kidney injury (AKI) is an inflammation related disease. AKI refers to rapid decline in renal function with various causes. Patients worldwide experience AKI in hospitals [30], and the long-term prognosis is poor [31]. Therefore, elucidating the repair mechanism is necessary [32]. Currently, the clinical therapy for AKI is typically supportive [33], and there is no effective method to quickly rescue kidney injury Therefore, it is urgent to find

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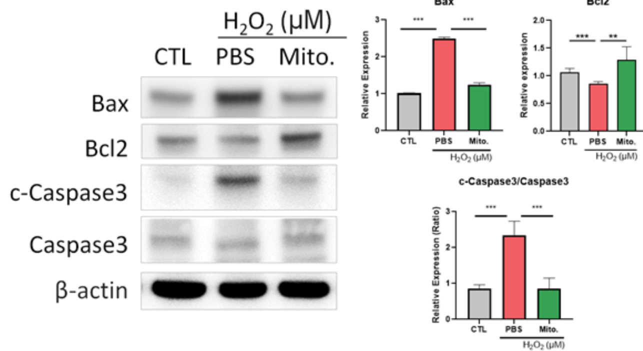

Fig. 18 Mitochondria Inhibited the Expression of Apoptosis-related Protein Induced by H_2O_2 Treatment. (A) The protein expression ratio of Bax/Bcl2 and c-Caspase3/Caspase3 were analyzed by western blot on NRK52E cells after being treated with or without mitochondria under H_2O_2 (20 μM) for 24 hours. (B) The quantification of the protein expression ratio of Bax/Bcl2 and c-Caspase3/Caspase3 were analyzed by western blot on NRK52E cells after being treated with or without mitochondria under H_2O_2 (20 μM) for 24 hours. Data are shown as the mean $\pm$ SEM; n $\geq$ 6 independent experiments, one-way ANOVA: $*P_{\text{Bonferroni}} < 0.008$, $**P_{\text{Bonferroni}} < 0.001$, $***P_{\text{Bonferroni}} < 0.0001$.

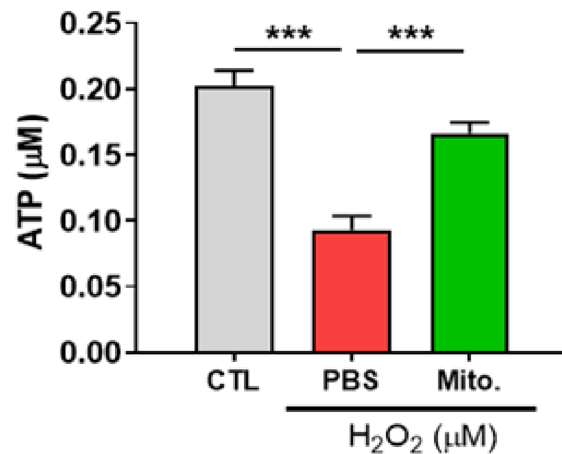

Fig. 20 Mitochondria Increase the ATP concentration under H_2O_2 Treatment. The ATP concentration is analyzed by the $ATPlite^{TM}$ luminescence kit on NRK52E cells after being treated with H_2O_2 for 24 hours. Data are shown as the mean $\pm$ SEM; $n \geq 6$ independent experiments, one-way ANOVA: $*P_{\text{Bonferroni}} < 0.008$, $**P_{\text{Bonferroni}} < 0.001$, $***P_{\text{Bonferroni}} < 0.0001$.

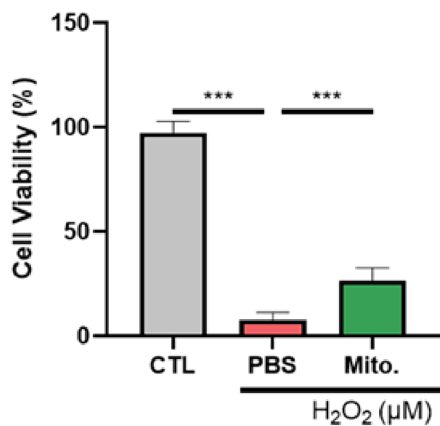

Fig. 19 Mitochondria Recovered the Cell Viability Reduced under H_2O_2 Treatment. The cell viability is analyzed by cck8 on NRK52E cells after being treated with H_2O_2 for 24 hours. Data are shown as the mean $\pm$ SEM; $n \geq 6$ independent experiments, one-way ANOVA: $*P_{\text{Bonferroni}} < 0.008$, $**P_{\text{Bonferroni}} < 0.001$, $***P_{\text{Bonferroni}} < 0.0001$.

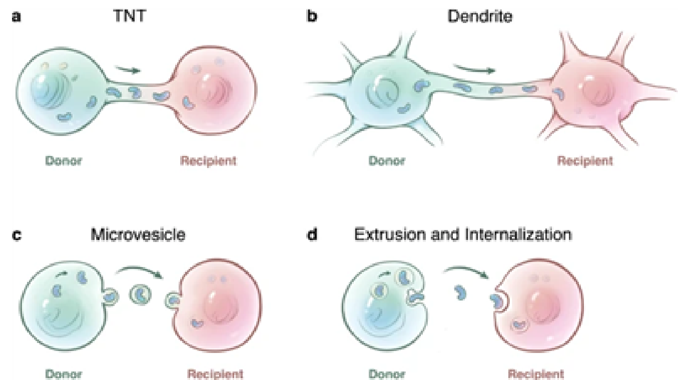

Fig. 21 Routes of Mitochondrial Transfer from Donor Cells to Recipient Cells [34]

suitable treatment options. Increasingly, studies have identified the phenomenon of mitochondrial transfer via tunneling nanotubes, dendrites, microvesicles, and extrusion and internalization (Figure 21) [34].

Many studies point out that by treating the damaged cells with mitochondria, the cell viability increased and cell damage decreased [25,26]. Related studies demonstrate methods to increase the efficiency in mitochondrial treatment in vitro, in addition to directly extracting mitochondria and co-culture with cells (Figure 22) [35].

Since no current research is related to the effect of mitochondrial transplantation on AKI, the purpose of our study is to observe if mitochondrial transplantation reduces ROS and restores mitochondrial activity in cells, hence rescuing the damaged cells. To achieve our research goal, this study conducted a series of experiments, the result of which will be discussed in the following section.

H_2O_2 simulates the cell damage caused by AKI

Our study is based on the study of Ishihara et al. [36], which utilized H_2O_2 as the medicine to induce AKI. The result of our cell

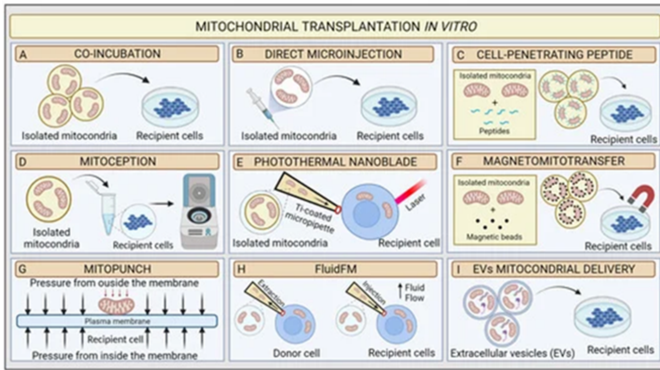

Fig. 22 Schematic Illustration of Mitochondrial Transplantation Methods in Vitro[35]

viability experiment presents the significance of H_2O_2s effect in decreasing NRK52E cell survival rate. The result proves the reliability of our experiment. To demonstrate the effect of mitochondria treatment, we choose IC50 of the medicine as the concentration in the following experiments.

Mitochondrial transplantation regulates HIF-1 to alleviate the inflammatory reaction caused by AKI

Hypoxia has long been recognized as an important factor in the pathogenesis of acute kidney injury[37]. Hypoxia-inducible factor (HIF) is the master transcription factor that regulates adaptive responses against hypoxia. Many studies prove that HIF-1 is highly expressed in the early stage after AKI[32]. In addition, its activation protects the kidney against AKI via various mechanisms, such as apoptosis suppression[38], autophagy activation[39], and interstitial macrophage-dependent inflammatory response modulation[40]. Therefore, our study analyzes HIF-1, whose expression increased in the cell treated by H_2O_2 and decreased in the cell transplanted with mitochondria in our study. The result illustrates how mitochondrial transplantation can recover the inflammation caused by AKI.

Mitochondrial transplantation increases the cells resistance against oxidative stress

Oxidative stress plays a crucial role in the complex web of processes behind AKI as it is evident in its circuitous and interdependent pathways, which both provoke and harm the organ's delicate responses[41]. Mitochondrial dysfunction is one of the important indicators of AKI. Our research analyzed how mitochondrial transplantation affected the accumulation of superoxides and the generation of ROS. Our result proved the ability for mitochondrial transplantation to reduce the accumulation of superoxides and the generation of ROS induced by H_2O_2, illustrating the potential for mitochondrial transplantation to treat AKI. Hypoxia is another crucial factor that contributes to AKI. Hypoxia is not only related to Ischemia-Reperfusion injury, but also related to toxins, sepsis, and ureteral obstruction[32,38].

Mitochondrial transplantation treats AKI though Sirt1-Nrf2 mediated pathway

Nuclear factor erythroid 2 related factor 2 (Nrf2) is a regulator of redox balance showing improvement to kidney disease by reducing ROS. Past research found the use of compounds activated by Nrf-2 reduces ROS effectively, thereby halting the progression of various types of AKI (Figure 23)[42]. Our study proves the ability of mitochondrial transplantation to restore the reduced Nrf2 expression caused by H_2O_2, displaying the ability of mitochondrial transplantation to protect NRK52E. Sirt1 is a

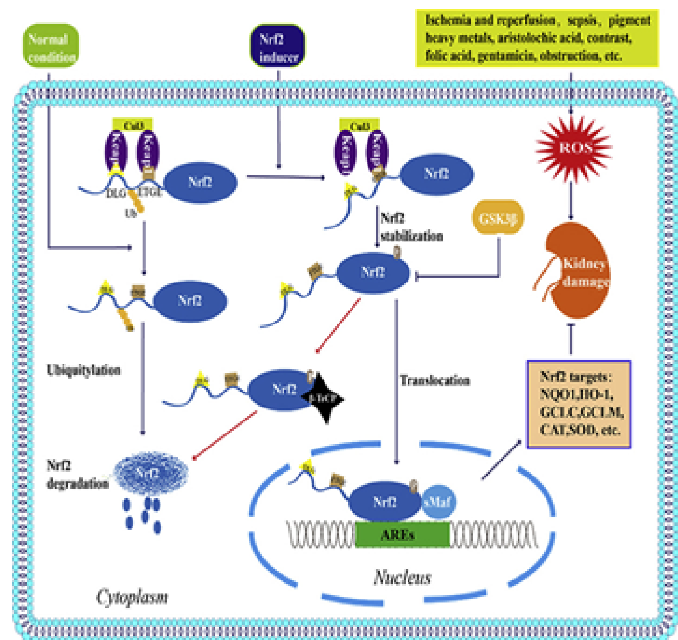

Fig. 23 The Nrf2 Protects the Cell during AKI[42]

member of Class III HDACs, and it is a protein encoded by the SIRT1 gene in humans. Multiple results show that increased Sirt1 expression reduces the ROS and damage of renal cells[43–45]. The study of Shi S et al. points out Melatonin can attenuate oxidative stress in AKI by activating the SIRT1/Nrf2/HO-1 pathway[46,47], proving Sirt1 is an important biomarker of treatment of AKI (Figure 24) .Our study observed the restoration of Sirt1 expression after mitochondrial transplantation, demonstrating mitochondrial transplantation may recover cell damage caused by AKI though Sirt1-Nrf2 mediated pathway.

Endogenous antioxidant enzymes substantially reduced ROS through catalysis ROS through catalysis. SODs, divided into cytosolic CuZn-SOD (SOD1), mitochondrial Mn-SOD (SOD2), and extracellular (SOD3) SOD enzymes, seem to be the first line of defense in ROS elimination. SODs can be rapidly activated in

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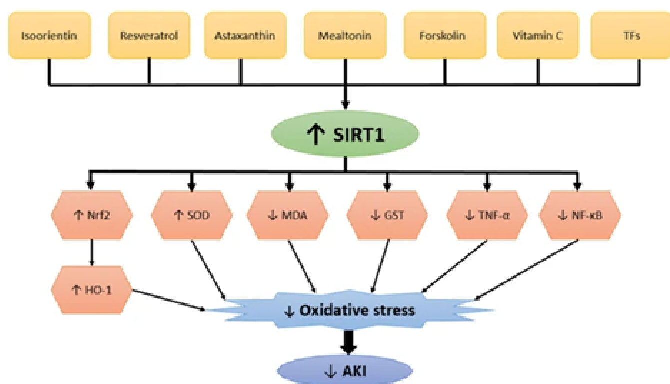

Fig. 24 Mediation of SIRT1 and Its Mechanisms in Reducing Oxidative Stress during AKI[47]

some situations and catalyze superoxide into molecular oxygen and H_2O_2 (Figure 25). The catalyzation converts ROS into less reactive species[48,49].

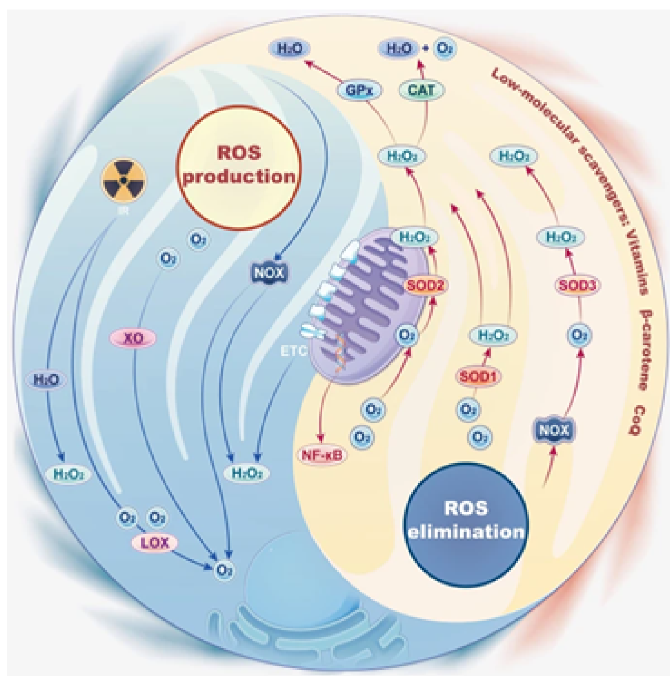

Fig. 25 Production and Elimination of ROS[49]

According to the study, we discovered that SOD2 expression increases after mitochondrial transplantation. We conclude that mitochondrial transplantation reduces H_2O_2 induced ROS and recovers cell damage caused by the inflammation.

Mitochondrial transplantation inhibits the apoptosis caused by AKI

AKI is often caused by transient ischemia from hypovolemia, hypotension, or heart failure, which are considered to be the cause of nearly one-third of patients requiring acute renal replacement therapy. Necrosis is considered as an important cause of ischemic AKI[50]. Modern research believes that cell apoptosis causes ischemic renal dysfunction[51]. Apoptosis is a type of programmed cell death, meaning the cell death is an ordered process, which, once the process has started, continues automatically according to the established program, mainly occurring in development and cell renewal. Pothana Saikumar and his colleagues found that cell death may be caused by apoptosis and necrosis, which are caused by ischemia in experimental models of renal injury both in vivo and in vitro. They prove that in hypoxic renal cells, Bax and Bak work together to permeabilize proteins such as cytochrome c. When caspase is inhibited, inflammation is reduced, along with the cascading parenchymal injury that is associated with inflammation (Figure 26)[51,52]. In our study, we proved that mitochondrial transplantation effectively decreased H_2O_2 induced Caspase3 activation. Furthermore, The result of western blot also presents a decrease in the ratio of Bax/Bcl2 and c-Caspase3. Moreover, we used CCK8 analysis to analyze cell viability, and the result shows mitochondrial transplantation is able to effectively recover the decrease in cell viability caused by H_2O_2. Finally, many studies show that mitochondrial disability and the decrease in ATP production is related to AKI[14,53,54]. These studies see mitochondrial dysfunction as a target to treating AKI. Our study shows that mitochondrial transplantation significantly increases the ATP concentration after cell damage, further proving mitochondrial transplantation being a new strategy in treating AKI. All results above prove that mitochondrial transplantation has the ability to protect the cell from cell death caused by oxidative stress.

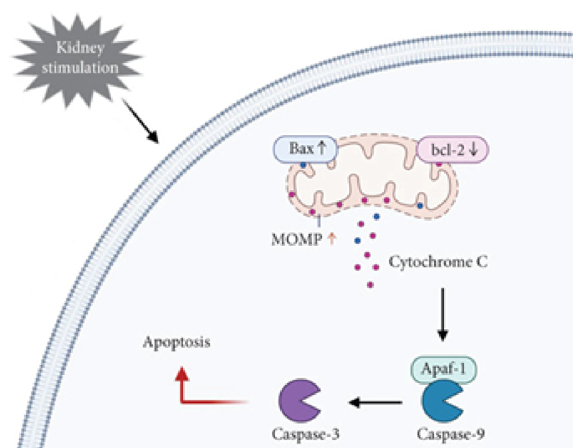

Fig. 26 AKI in Apoptosis[52]

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Summary

Since ROS production is associated with apoptosis and is observed in several AKI models, it may be reasonable to explore drugs that inhibit or scavenge ROS as potential strategies for managing organ injury. Various antioxidants have been successfully used to prevent ischemic, septic, or toxic AKI in animal models[50]. However, these interventions are currently limited to prevention, rather than treatment of renal injury. Mitochondrial transplantation is one of the main topics that is being researched around the globe in many inflammation related diseases and has proven the ability to prevent cell and tissue damage, despite this, there was no AKI-related studies that implement mitochondrial transplantation as the treatment. Our study discovered that mitochondrial transplantation is a promising candidate for clinical use in AKI treatment. In the future, we hope to find a suitable animal model to conduct mitochondrial transplantation and conduct a more in-depth discovery in the protein regulatory pathway in the cell to achieve more verification before clinical application.

Conclusion

Our study provides insights into the protective potential of mitochondrial transplantation. mitochondrial transplantation exhibits antioxidant, anti-inflammatory, and antiapoptotic effects both in vitro in the context of AKI. In addition, mitochondrial transplantation recovers the ATP quantity reduced by cell damage. These beneficial effects can be attributed to activation of the antioxidative protein such as Nrf2, Sirt1, PCG-1, and SOD2. They indicate that mitochondrial transplantation may represent a promising therapeutic option for treatment of AKI (Figure 26).

The results of our study demonstrate that mitochondrial transplantation is a promising therapeutic approach for treating AKI, potentially overcoming the limitations of conventional supportive treatments, whose effectiveness often varies based on the patients condition. Specifically, the protective effect of mitochondrial transplantation on cell damage makes an important contribution to slowing down the course of the disease and improving the survival rate of patients. To achieve this, in vivo trials will be an important future work. In addition to animal models, several transcription factors in this study can also serve as important research targets. In-depth exploration of the molecular mechanisms induced by mitochondrial transplantation is also one of the important future work.

Limitations

In this research, we investigated the importance of mitochondrial transplantation in treating AKI. However, some limitations occur in the quantification of mitochondrial activity and the storage of mitochondria.

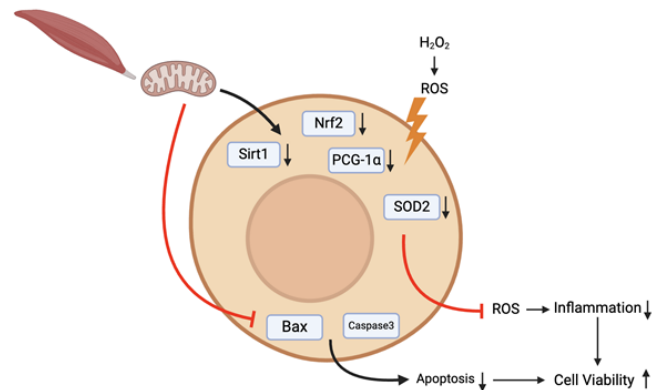

Fig. 27 Extra-mitochondria Reduces ROS, Apoptosis, and Inflammation by Mediated Antioxidative Protein

First of all, each cell has a different number of mitochondria, each with a different mitochondrial activity. Although some current methods such as flow cytometry can give some data, the data is limited in the accuracy and consistency due to differences in individual mitochondrion. We used protein quantification to the mitochondria used in this experiment to resolve the inconsistency in mitochondria used in this experiment. The mitochondria are extracted directly from the living muscle tissue to exclude the inconsistency in mitochondrial activity.

On the other hand, freezing and chemical preservation of mitochondria are unable to sustain its activity and function because freezing preservation of mitochondria damages the structure of mitochondria and chemical preservation of mitochondria decreases its activity. In this experiment, the mitochondria are extracted directly from the living muscle tissue to negate the limitation in its storage. Despite the limitation in storage of mitochondria may be resolved in the future, currently, these limitations exist in the methods used in this research.

Method

Cell Culture and Reagent

Rattus norvegicus (rat) Cell type kidney epithelial-like cells (NRK-52E) was cultured using Dulbecco's Modified Eagle Medium (Gibco, Waltham, MA, USA) medium with 5% fetal bovine serum (Gibco, Waltham, MA, USA) under 5% CO2 at 37°. The cell line is maintained using trypLE reagent (Gibco, Waltham, MA, USA) under 5% CO2 at 37° for 5 minutes, and the reaction is terminated by medium. Stock solutions of Hydrogen Peroxide (H_2O_2) 10M solution (Sigma-Aldrich, St. Louis, MO, USA) are prepared and dissolved in culture medium before treatment. Control group: culture medium with PBS, H_2O_2 group: culture medium with H_2O_2 solution, H_2O_2+mito group: culture medium with H_2O_2 solution and mitochondria in PBS.

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Mitochondria Isolation

The muscle tissue of the rat is obtained while the rat is under anesthesia via Isoflurane (Sigma-Aldrich, St. Louis, MO, USA). The muscle tissue is washed with PBS and disrupted with a dounce homogenizer while on ice. The tissue is centrifuged at 1,000 rcf for 3 minutes at 4°. The supernatant is discarded. The pellet is suspended in 800 μL of BSA/Reagent A Solution. The tube is vortexed at medium speed for 5 seconds and incubated on ice for exactly 2 minutes. 10 μL of Mitochondria Isolation Reagent B is added to the sample, which is vortexed at maximum speed for 5 seconds. The tube is incubated on ice for 5 minutes and vortexed at maximum speed every minute. 800 μL of Mitochondria Isolation Reagent C is added to the tube, which is inverted several times to mix and centrifuged at 700 rcf for 10 minutes at 4°. The supernatant is transferred to a new tube and centrifuged at 3,000 rcf for 15 minutes at $4°C$. The supernatant is discarded, and 500 μL of Wash Buffer is added to the mitochondrial pellet. The tube is centrifuged at 12,000 rcf for 5 minutes, and the supernatant is discarded. Mitochondria are maintained on ice before treatment. Fresh mitochondria are isolated for every experiment at a protein concentration of 25 micrograms per milliliter.

Cell viability analysis

NRK-52E cells are seeded in 96-well dishes in quadruplicate at 8000 cells/well and cultured for 24 hours before treatment. For dose-dependent tests, cells are treated with different concentrations of H_2O_2 (100, 80, 50, 20, 10, 5 μM) for 24 hours. For mitochondria treatment, cells are treated under 20 μM of H_2O_2 with or without mitochondria for 24 hours. After treatment, 10 μL of Cell Counting Kit-8 (SigmaAldrich, St. Louis, MO, USA) reagent is added to each well. The reagent reacted for 90 minutes, and the absorbance of the reagent is measured at 450 nm using a microplate reader. The cell viability is calculated with the following formula.

$$\textbf{Cell viability (\%)} = \frac{(\text{OD}_{\text{sample}} - \text{OD}_{\text{Blank}})}{(\text{OD}_{\text{Control}} - \text{OD}_{\text{Blank}})} \times 100\%$$

Mitochondrial Superoxide (MitoSOX) Analysis

NRK-52E cells are seeded in 6-well dishes in quadruplicate at 1x105 cells/well for 24 hours before treatment. Cells are treated under 20 μM of H_2O_2 with or without mitochondria for 24 hours. After the treatment, the cells are harvested with triPLE and washed with cold PBS. Cells are stained using 5 μM of MitoSOX Mitochondrial Superoxide Indicators (Invitrogen, Waltham, MA, USA) under $37°C$ for 15 minutes. The sample is centrifuged at 2000 rpm for 10 minutes. The sample is washed with cold PBS and analyzed through flow cytometry.

Intracellular Reactive Oxygen Species Analysis

NRK-52E cells are seeded in 6-well dishes in quadruplicate at 1x105 cells/well for 24 hours before treatment. Cells are treated under 20 μM of H_2O_2 with or without mitochondria for 24 hours. After the treatment, the cells are harvested with triPLE and washed with cold PBS. Cells are stained using 10 μM of H2DCFDA (MedChemExpress, Monmouth Junction, NJ, USA) under $37°C$ for 25 minutes. The sample is centrifuged at 2000 rpm for 10 minutes. The sample is washed with cold PBS and analyzed through flow cytometry.

Superoxide and Hydrogen Peroxide Analysis

NRK-52E cells are seeded in 6-well dishes in quadruplicate at 1x105 cells/well for 24 hours before treatment. Cells are treated under 20 μM of H_2O_2 with or without mitochondria for 24 hours. After the treatment, the cells are harvested with triPLE and washed with cold PBS. Cells are stained using 10 μM of Dihydroethidium (Hydroethidine) (Invitrogen, Waltham, MA, USA) under $37°C$ for 15 minutes. The sample is centrifuged at 2000 rpm for 10 minutes. The sample is washed with cold PBS and analyzed through flow cytometry.

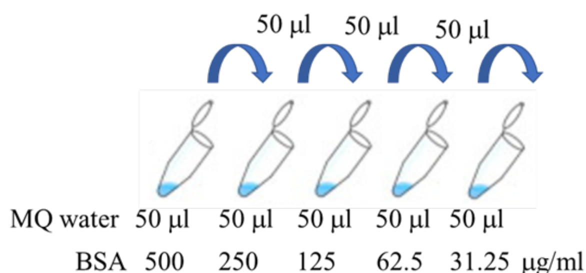
Caspase Activity Analysis

NRK-52E cells are seeded in 6-well dishes in quadruplicate at 1x105 cells/well for 24 hours before treatment. Cells are treated under 20 μM of H_2O_2 with or without mitochondria for 24 hours. After the treatment, the cells are harvested with triPLE and washed with cold PBS. Cells are suspended in 300 μL of PBS, stained with 1 μL of FITC-DEVD-FMK, and mixed. Cells are incubated in darkness under $37°C$ for 30 minutes. The sample is centrifuged at 3000 rpm for 5 minutes, and the supernatant is discarded. The sample is washed with Wash Buffer and analyzed through flow cytometry.

Western Blot Analysis

- Protein Preparation
  NRK-52E cells are seeded in 10 cm dishes in quadruplicate at 1x106 cells/well for 24 hours before treatment. Cells are treated under 20 μM of H_2O_2 with or without mitochondria for 24 hours. Cells are harvested with Cell Scraper and collected into a tube. Cells are washed and dispersed with PBS. Cells are centrifuged at 1500 rpm for 5 minutes, and the supernatant is discarded. The tube containing the cells are added and washed with a solution of lysis buffer containing 1% protease inhibitor and 1% phosphatase inhibitor. The sample is left at rest at $4°C$ for 5 minutes. The sample is centrifuged at 15,300 rpm for 30 minutes, and the supernatant is collected as the protein sample.

- Protein Quantification
  The standard is set up using stage-diluted BSA (500, 250, 125, 62.5, and 31.25 μg/mL). The quantification is done with Bradford protein assay and ELISA reader. The quantity is determined with the absorbance of the blue complex measured at a wavelength of 595 nm.



- Protein Electrophoresis
  20 μg of protein sample is isolated for electrophoresis. The sample is mixed with 4x Laemmli Sample Buffer at 3 to 1 ratio and heated in water at 95°C for 5 minutes. The SDS page is inserted into the electrophoresis tank, which is filled with a MES buffer. The protein marker is loaded into the wells of the SDS page, which is electrophoresed at 165 volts for 50 minutes.

- Transfer
  The PVDF transfer membrane is activated with methanol then assembled with the electrophoresed SDS page in the transfer buffer. 100 volts of electricity is applied for 90 minutes to transfer the protein onto the PVDF transfer membrane. The membrane is submerged in a solution of blocking buffer for an hour before being submerged into a solution of the primary antibody. The membrane is submerged in the solution of the primary antibody under 4°C overnight, allowing the primary antibody to attach to the proteins on the membrane.

PBST is utilized to wash out the unbonded primary antibodies from the PVDF transfer membrane. The washing is repeated 3 times, each wash lasting 10 minutes. The washed PVDF transfer membrane is submerged under the solution of secondary antibodies for an hour under room temperature. PBST is utilized to wash out the unbonded secondary antibodies from the PVDF transfer membrane. The washing is repeated 3 times, each wash lasting 10 minutes. The washed PVDF transfer membrane is dyed with ECL.

ATPlite luminescence assay

- Sample preparation NRK-52E cells are seeded in 6-well dishes in quadruplicate at 1x105 cells/well for 24 hours before treatment. Cells are treated under 20 μM of H2O2 with or without mitochondria for 24 hours. After the treatment, the cells are harvested with triPLE and washed with cold PBS.

- ATP Standard Solution Preparation Use M.Q. water to dissolve ATP in the kit to 10 mM. Then, use M.Q. water to stair dilute the sample (200100502512.56.253.1250 mM).

- Analysis Add cells into 96 well white plates at the concentration of 1x104 cells/50 μL well (n=8). Add ATP standard solution into cell-free well (n=2). Add 50 μL of mammalian cell lysis solution into every well. Use a shaker to mix the sample at 700 rpm for 5 minutes.

  Remove the sample from the shaker and add 10 μL ATP control into the standard solution. Use a shaker to mix the sample at 700 rpm for 5 minutes. Then, add 50 μL of substrate solution to the well. Use a shaker to mix the sample at 700 rpm for 5 minutes.

  Remove the sample from the shaker and put it in a dark room for 10 minutes. Then, collect the luminescence data

Table 1 Primary Antibody

Primary antibody	KDa	Source	Recommended Dilutions	Catalog No.
BCL-2	28	Rabbit	0.73611	#2876
c-Caspase	17, 19	Rabbit	0.73611	#9661
nrf2	110	Rabbit	0.73611	A3577
sirt1	90	Rabbit	0.73611	#2310
Bax	20	Rabbit	0.73611	ab182734
Caspase3	17, 19, 35	Rabbit	0.73611	#14220
PCG-1	91	Goat	0.73611	NB-100-60955
HIF-1	120	Rabbit	0.73611	#14179
SOD2	22	Rabbit	0.73611	#13141
-actin	46	Mouse	3.51389	#MAB1501

Table 2 Secondary Antibody

Secondary antibody	Recommended Dilutions	Catalog No.
ECL Anti-Rabbit IgG antibody, Peroxidase (HRP)-conjugated	3.513888889	Cytiva NA934-1ML
ECL Anti-mouse IgG antibody, Peroxidase (HRP)-conjugated	1:10000	Cytiva NA931-1ML
Peroxidase AffiniPure Donkey Anti-Goat IgG (H+L)	1:10000	705-035-003

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and calculate the concentration of ATP.

Statistical Analysis

All data were analyzed by GraphPad Prism version 10. All graphs in figures were presented as means ± standard deviation (SD). Statistical analysis was performed one-way ANOVA analysis to compare data between each group. The experiment is conducted independently with more than 3 trials. All analyses with statistical significance were $P < 0.05$. Statistical results were labeled in each figure as $^{*}P < 0.05, ^{**}P < 0.01, ^{***}P < 0.001$.

Acknowledgments

This would not have been made possible with the support of Hsin-Yi Tsai.

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