Exogenous biological renal support improves kidney function in rhabdomyolysis-induced acute kidney injury in mice

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Research

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Abstract

Background

Rhabdomyolysis (RM) is a clinical syndrome characterised by the breakdown of skeletal muscle fibres and release of their contents into the circulation. Myoglobin-induced acute kidney injury (AKI) is one of the most severe complications of RM. Based on our previous study, exogenous biological renal support alleviates renal ischaemia-reperfusion injury (IRI) in elderly mice. This study aimed to determine whether exogenous biological renal support promoted renal recovery from RM-induced AKI and to preliminarily explore the mechanisms involved.

Methods

A parabiosis animal model was established to investigate effects of exogenous biological renal support on RM-induced AKI. Male wild-type C57BL/6 mice and C57BL/6-TgN (ACTb-EGFP) transgenic mice were used to determine whether shared circulation was established among parabiotic pairs 3 weeks after parabiosis surgery. Mice were divided into three groups: the control group (sterile saline injected); RM group (glycerol (8 mL/kg) injected); and parabiosis + RM group (three weeks after the parabiosis model was established, the recipient mouse was injected with glycerol). Blood samples and kidney tissue were collected for further processing 48 hours after RM induction. Bioinformatics analysis was conducted with Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, functional enrichment analysis and clustering analysis.

Results

At 48 hours after the procedure, all mice survived. Exogenous biological renal support attenuated the histological and functional deterioration in RM-induced AKI in mice. Bioinformatics analysis identified key pathways and proteins involved in this process. We further demonstrated that exogenous biological renal support ameliorated kidney injury through multiple pathways, including suppressing the complement system; attenuating oxidative stress, inflammation, and apoptosis; and increasing proliferation.

Conclusions

Exogenous biological renal support provided by parabiosis can improve renal function in RM-induced AKI by suppressing the complement system; decreasing oxidative stress, inflammation, and apoptosis; and promoting tubular cell proliferation. Our study provides new ideas for effectively preventing and treating RM-induced AKI and provides basic research evidence for the use of bioartificial kidneys to treat RM-induced AKI.

Background

Rhabdomyolysis (RM) is a clinical syndrome characterised by the breakdown of skeletal muscle fibres and release of their contents into the circulation [1]. It can be caused by many reasons, such as trauma [2,
3], heat exposure [4], marathons [5], drugs [6] and viruses (such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)) [7–9]. Myoglobin-induced acute kidney injury (AKI) is one of the most severe complications of RM [10]. Myoglobin and its oxygen-carrying moiety haem play a key role in the development of AKI. AKI can cause renal tubular obstruction, renal vasoconstriction, and the direct activation of oxidative stress, lipid peroxidation and macrophages, which injure proximal tubular cells [11–13].

Renal replacement therapy (RRT) is often used in the treatment of RM-induced AKI, in which a high permeability membrane eliminates circulating myoglobin. However, RRT is a non-specific treatment method that removes blood solutes, and it cannot regulate inflammation or promote the repair of damaged renal tubules. Moreover, current research has shown that RRT does not significantly improve the mortality and renal repair of AKI patients, while artificial biological reduces the mortality of patients with AKI [14–16]. Parabiosis models in mice produce a shared circulatory system; mice then share circulating antigens and are free of adverse immune reactions [17, 18]. This model has been used in several physiological studies, such as kidney hypertension [19], the migration of haematopoietic stem cells [20], neurodegenerative disease [21], and lymphocyte trafficking [22]. Our previous study used a parabiosis model to show that exogenous biological renal support may attenuate inflammation and apoptosis and increase proliferation in an ischaemia-reperfusion injury (IRI) mouse model [16, 23].

In this study, we established a parabiosis model in mice and then used glycerol injections to induce AKI. To study the therapeutic effects of exogenous biological renal support on RM-induced AKI, proteomic analysis was used to screen and study changes in protein expression and key pathways and verify the exogenous biological renal effect on the complement system, oxidative stress, inflammation, apoptosis, and renal tubular epithelial cell proliferation. We aimed to determine whether exogenous biological renal support promoted renal recovery from RM-induced AKI and preliminarily explore the mechanisms involved.

**Materials And Methods**

**Experimental animals**

All animal protocols were approved by the Animal Ethics Committee of the Chinese PLA General Hospital and Military Medical College. Eight- to twelve-week-old male C57BL/6 mice were obtained from the Si Bei Fu Laboratory Animal Company (Beijing, China). Male C57BL/6-TgN (ACTb-EGFP) transgenic mice expressing GFP were obtained from the Model Animal Research Center of Chinese Nanjing University (Nanjing, China). Animals were housed in a temperature-controlled room (22°C±1°C) with a 12-hour light-dark cycle and were given free access to food and water.

Parabiosis was performed based on the methods developed by Donskoy and Goldschneider [22]. The shared circulation created between the two mice was verified in our previously study [23, 24]. Briefly, after anaesthetisation (intraperitoneal injection of 1% pentobarbital sodium at a dose of 30 mg/kg) and sterilisation, the skin and subcutaneous tissue of two mice were cut to expose the subcutaneous muscle.
For each pair, the chest and back muscles were isolated in the donor mouse and sutured to the chest and back muscles of the recipient mouse. Then, the edges of the skin were sutured. The details of this procedure are described in Figure S1.

After 3 weeks of parabiosis, the RM model was induced as previously reported.[25] The mice were deprived of water for 24 hours and then administered diluted glycerol (50% v/v in sterile saline) in each hindlimb muscle at a dose of 8 mL/kg following mild sedation with pentobarbital. Blood samples and kidney tissue were collected for further processing 48 hours after the induction of RM.

The mice were divided into three groups: the sham group with sterile saline administration; the RM group with glycerol administration; and the parabiosis + RM group. Three weeks after the parabiosis model was established, the recipient mouse was administered glycerol, and this mouse was defined as the P_RM_R. The other mouse in the parabiosis model supplied exogenous biological renal support and was defined as the P_RM_S.

**Verification of the establishment of shared circulation in parabiotic mice**

GFP-expressing mice and wild-type mice were used for parabiosis. The successful establishment of shared blood circulation was proven by three methods from our previous studies [23]: (1) a peripheral blood smear test for GFP detection; (2) flow cytometry for the measurement of the GFP+ cell ratio; and (3) small animal in vivo imaging. For further details on the methods, see the supplementary section (Figure S2).

**Serum biochemistry analysis**

Serum samples were collected and centrifuged at 3000 rpm for 10 minutes and stored at -80°C before analysis. Serum creatinine (Cr) and blood urea nitrogen (BUN) were analysed with an autoanalyser (Cobas 8000; Roche, Mannheim, Germany).

**Histopathologic examination and scoring**

All tissue sections were independently evaluated by two investigators (XDG and QH) in a blinded manner. Mouse kidneys were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at a thickness of 3 μm and stained with periodic acid–Schiff (PAS). Renal tubular injury was scored by counting the percentage of tubules that displayed cellular necrosis, the loss of the brush border, cast formation and tubule dilatation. Zero represents normal histology and 1 to 5 represent ≤10%, ≤25%, ≤50%, ≤75%, and >75%, respectively [23].

**Measurements of SOD, GSH, MDA, CK and PC contents in kidney samples**

A 10% homogenate was prepared from the kidney and centrifuged at 730 g at 4°C for 15 minutes to obtain the supernatant. Total superoxide dismutase (SOD), glutathione peroxidase (GSH), malondialdehyde (MDA), creatine kinase (CK) and protein carbonyl (PC) contents were measured with
commercial kits according to the manufacturer’s instructions (Beijing Jinenlai Biochemistry Co., Beijing, China).

**Measurements of MYO, CK, NGAL, TNF-α, SAA1 and SAA2 contents in serum samples**

Mouse plasma was centrifuged at 730 g at 4°C for 10 minutes. Then, the upper serum layer was collected. The contents of MYO, CK, NGAL, TNF-α, SAA1 and SAA2 were measured with commercial kits according to the manufacturer’s instructions (Jinenlai Biochemistry Co., Beijing, China).

**Western blot analysis**

Frozen kidney tissues (100 mg) and 1 mL of RIPA buffer (Thermo Fisher Scientific, Inc.) were homogenised at 12000 g at 4°C for 30 minutes to obtain the supernatant. Equal amounts of proteins were obtained from each sample and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes and probed with primary antibodies against the following proteins at 4°C overnight: beta-actin (0061R, 1:2000; Beijing Biosynthesis Biotechnology Co., Beijing, China), cleaved caspase-3 (9664; 1:500; Cell Signaling Technology, Boston, USA), Bcl2 (2876; 1:1000; Cell Signaling Technology), cyclin D1 (60186; 1:1000; Proteintech), and cyclin E1 (11554; 1:1000; Proteintech). The blots were probed with horseradish peroxidase-conjugated IgG (sc-2096 and sc-2963, 1:1000; Santa Cruz Biotechnology, USA). Immunoreactive bands were visualised by enhanced chemiluminescence, and blot signals were analysed by the image analysis software ImageJ 1.52.

**TUNEL staining**

Kidney tissue was fixed in 10% formalin overnight, dehydrated, embedded in paraffin, cut into 3-μm-thick sections, and placed on a numbered polylysine-coated glass slide. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling (TUNEL)-positive cells, which were stained brown, were counted under 200x magnification. Nuclei were stained with haematoxylin to observe the characteristics of TUNEL-positive cells. Six to eight fields per section and two to three sections per kidney were examined in each experiment. We calculated the percentage of TUNEL-positive cells relative to the total number of renal tubular cells as the apoptosis rate. The TUNEL assay was performed according to the manufacturer’s instructions (Merck Millipore, Billerica, MA, USA).

**Immunohistochemistry**

Immunohistochemical staining for the detection of CD3 and proliferating cell nuclear antigen (PCNA) in renal tissue was performed on formaldehyde-fixed and paraffin-embedded tissues using the avidin-biotin-immunoperoxidase method. As in our previous study [16, 23], we performed antigen retrieval from sections by microwaving them for 10 minutes in 10 mM sodium citrate buffer (pH 6.0), incubating them in 3% hydrogen peroxide for 30 minutes, and placing them in 1.5% normal goat serum for 40 minutes. Then, the sections were incubated in anti-CD3 (5690, 1:100; Abcam) or anti-PCNA antibodies (Abcam, 18197, 1:1000) overnight at 4°C, followed by immersion in biotin-conjugated goat anti-rabbit IgG for 40
minutes and finally in streptavidin-conjugated peroxidase for 30 minutes. The sections were observed under a microscope.

**Immunofluorescence**

Immunofluorescence staining for the detection of complement 3 (C3) in renal tissue was performed on formaldehyde-fixed and paraffin-embedded tissues. Slides were air-dried, fixed with methanol/acetone for 10 minutes, and treated with a FITC-conjugated anti-C3 antibody (21337; 1:500; Proteintech) at room temperature for 1 hour. Nuclei were counterstained by DAPI. Five fields on each of three slides per animal were randomly selected for visualisation, and analysis was performed using ImageJ software.

**Proteomic sample extraction and LC-MS/MS analysis**

Peripheral blood and kidney tissue were collected and digested with trypsin. After isobaric tags for relative and absolute quantitation (iTRAQ) labelling, the tryptic peptides were fractionated with high pH reverse-phase high performance liquid chromatography (HPLC) using a Thermo BetaSil C18 column (5-μm particles, 10-mm ID, and 250-mm length), and then the peptides were subjected to a nanospray ionisation (NSI) source followed by tandem mass spectrometry (MS/MS) in a Q ExactiveTM Plus (Thermo) coupled online to an ultra performance liquid chromatography (UPLC) platform. The resulting MS/MS data were processed using the MaxQuant search engine (v.1.5.2.8). The details of those procedures are provided in the supplementary materials (Supplementary File).

**Bioinformatics analysis**

Bioinformatics analysis was conducted with Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, functional enrichment analysis and clustering analysis. The details of the software and analysis parameters are provided in the supplementary materials (Supplementary File). A 1.3-fold up- or downregulation was chosen to identify significant protein over- or underexpression, respectively, with a P value lower than 0.05.

**Statistical analyses**

All data were analysed using R 3.6.1 software. Data are expressed as the mean and standard deviation (SD). Statistical significance was determined using two-way analysis of variance (ANOVA) or Student’s t test. Statistical graphs were produced with GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). A threshold of P < 0.05 was defined as statistically significant.

**Results**

**Verification of cross-circulation in the parabiosis model**

Male wild-type C57BL/6 mice and C57BL/6-Tg (ACTb-EGFP) transgenic mice were used for parabiosis, and the establishment of shared circulation was confirmed among parabiotic pairs 3 weeks after the
parabiosis surgery. The three sets of results consistently demonstrated that 3 weeks after the parabiosis surgery, the donor and recipient mice had established shared blood circulation. The results are shown in Figure S2.

**Exogenous biological renal support attenuates histological and functional deterioration in RM-induced AKI in mice**

At 48 hours following the procedure, all mice survived. Glycerol administration resulted in significant increases in serum creatinine (Scr) and BUN levels in the RM group compared with the parabiosis + RM and sham groups. Sham mice did not show any significant tubular damage. RM mice showed the loss of tubular brush borders, cast formation, tubular dilatation and tubular necrosis, accompanied by an increase in the acute tubular injury score. Parabiosis + RM mice had significantly improved renal histological injury (Figure 1).

**Proteomic analysis**

We first analysed the proteome quality of three replicates. Principal component analysis (PCA) and Pearson correlation coefficient analysis suggested that the proteome quality was suitable for subsequent analysis (Figure S3).

In the kidney tissue sample, 5887 identified proteins were found in the proteome, among which 5136 proteins were quantified. In the serum sample, 1102 identified proteins were found in the proteome, among which 962 proteins were quantified. The upregulated and downregulated proteins in different groups are shown in Figure S4.

To further understand the functions and features of the identified and quantified proteins, they were classified into four categories, namely, Gene Ontology (Figure 2), subcellular localisation (Figure 2), pathway (Figure 3 and Figure 4) and domain (Figure S5 and Figure S6).

The number of differentially expressed proteins in each subcellular location was determined according to the subcellular location annotation of the identified proteins (Figure 2). Exogenous biological renal support led to an increase in proteins associated with extracellular, nuclear, cytoplasmic, mitochondrial and plasma membrane localisation in kidney tissue compared with those in the RM group. Meanwhile, exogenous biological renal support induced an increase in proteins associated with extracellular, cytoplasmic, nuclear, mitochondrial and plasma membrane localisation in serum compared with those in the RM group (Figure 2).

Analysis of the functional enrichment of pathways showed that exogenous biological renal support ameliorated kidney injury through multiple pathways, including suppressing the complement system (mmu04610 Complement and coagulation cascades), attenuating oxidative stress (mmu04146 Peroxisome and mmu04014 Ras signalling pathway), attenuating inflammation (mmu04020 Calcium signalling pathway and mmu04610 Complement and coagulation cascades), attenuating apoptosis (mmu04020 Calcium signalling pathway, mmu04217 Necroptosis) and increasing proliferation.
(mmu04330 Notch signalling pathway, mmu04340 Hedgehog signalling pathway, mmmu04151 PI3K–Akt signalling pathway, and mmu04110 Cell cycle). The top 30 upregulated and downregulated proteins are shown in Table S1.

Exogenous biological renal support suppressed complement activation in RM-induced AKI in mice

Staining with anti-C3 antibody revealed the expected weak signal in the parabiosis + RM group compared with that in the RM group at 48 hours after RM-induced AKI in mice. These results indicate that complement system activation was suppressed by exogenous biological renal support provided by parabiosis (Figure 5).

Exogenous biological renal support decreased oxidative stress in RM-induced AKI in mice

Compared with the RM group, the parabiosis + RM group showed higher SOD and GSH antioxidant enzymatic activities at 48 hours after RM-induced AKI in mice. In addition, the MDA and PC contents in the parabiosis + RM group were lower than those in the RM group. This indicated that there was less lipid and protein damage (Figure 6).

Exogenous biological renal support decreased inflammation in RM-induced AKI in mice

Expression levels of TNF-α, SAA1, SAA2 and NGAL and the number of renal CD3-positive cells were significantly lower in the parabiosis + RM group (Figure 7). These results indicate that the renal inflammatory level induced by RM may have been reduced by exogenous biological renal support provided by parabiosis.

Exogenous biological renal support decreased apoptosis in RM-induced AKI in mice

Renal tissue expression levels of cleaved caspase-3 and the percentage of TUNEL-positive tubular cells were significantly decreased in the parabiosis + RM group. The level of Bcl-2 was significantly increased in the parabiosis + RM group. These results indicate that exogenous biological renal support provided by parabiosis may have alleviated the apoptosis level in RM-induced AKI (Figure 8).

Exogenous biological renal support promotes tubular cell proliferation in RM-induced kidney injury

At 48 hours following RM-induced AKI in mice, the expression of cyclin D1 and cyclin E1 and the percentage of PCNA-positive cells were higher in the parabiosis + RM group than in the RM group. These findings indicate that exogenous biological renal support provided by parabiosis can significantly increase tubular cell proliferation in RM-induced AKI in mice (Figure 9).

Discussion

In the parabiosis animal model, muscles and subcutaneous tissues of two mice are sutured together during surgery to form a shared blood circulatory system between the mice. Due to this shared blood circulatory system, blood cells and soluble factors are exchanged between the mice. Therefore, the use of
parabiosis animal models can provide exogenous biological renal support for AKI mice. Our previous research results showed that exogenous biological renal support from young mice can improve renal tissue inflammation, autophagy and apoptosis and promote dedifferentiation and proliferation in aged mice after IRI model establishment [16, 23]. We also found that the expression of various inflammatory factors in renal tissue was upregulated, which may have been related to the dedifferentiation, proliferation and repair of damaged renal tissue cells [16].

A previous study demonstrated that there is a mean exchange flow of 0.66% of the circulating blood volume among parabiotic mice per hour. This exchange flow is equivalent to a mean daily exchange of 8% of the circulating volume [22]. Our previous observational study showed that GFP-positive cells rarely entered the kidneys of wild-type mice that were parabiosed with GFP mice [23]. These observations suggest that the physiological benefits afforded to the RM-induced AKI mice were unlikely to have resulted from exogenously transferred blood cells but were more likely attributable to the non-injured mice partially assisting in the water excretion, metabolism, transfer of bioactive molecules, and endocrine function of the injured mice.

The causes of RM include trauma, heat exposure, marathons, drugs such as statins, infection and hymenopteran stings [6]. An animal model using intramuscular glycerol injection with consequent myoglobinuria is closely related to the human syndrome of RM. RM-induced AKI is a major related adverse event and has been shown to be closely related to oxidative stress, renal inflammation, apoptosis, and proliferation [13, 26, 27]. In our study, the Scr and BUN levels were significantly increased in the RM group compared with the parabiosis + RM and sham groups. Sham mice did not show any significant tubular damage. RM mice showed the loss of tubular brush borders, cast formation, tubular dilatation and tubular necrosis, accompanied by an increase in the acute tubular injury score. Parabiosis + RM mice showed significantly improved renal histological injury. To analyse the role of exogenous biological renal support, we used proteomic analysis to identify key proteins and key pathways. The results showed that exogenous biological renal support led to an increase in proteins associated with extracellular, nuclear, cytoplasmic, mitochondrial and plasma membrane localisation in kidney tissue and serum compared with those in the RM group. The analysis of the functional enrichment of pathways identified the suppression of complement system activation; attenuation of oxidative stress, inflammation, and apoptosis; and increased proliferation. Furthermore, we further confirmed these effects with in vivo experiments.

Tubular changes are mainly sublethal 24 hours after the onset of RM [28, 29]. By 72 hours, the glycerol-treated mice developed AKI and exhibited significantly increased Scr and BUN levels and significant morphological changes [25]. In this study, to observe the effect of exogenous biological renal support, we collected specimens at 48 hours after glycerol injection.

Myoglobin and CK are the main products of the breakdown of skeletal muscle fibres in a glycerol-induced RM model [30]. Myoglobin-induced renal toxicity plays a key role in RM-induced AKI by activating the complement system and increasing oxidative stress, inflammation, endothelial dysfunction,
vasoconstriction, and apoptosis [10-12]. The clinical symptoms of RM are characterised by elevated serum CK and myoglobin [1]. Our results show that levels of CK and myoglobin were not significantly different between the RM and P_RM_R groups (Figure S7). The reason may be that, on the one hand, the blood exchange rate in parabiotic mice is not high (only 8% is exchanged every day) and the amount of blood exchanged at 48 hours after RM is established is limited; on the other hand, previous research has indicated that a number of biological systems are activated following muscle extract infusion and that these systems may be more important than the nephrotoxicity of myoglobin in the pathogenesis of renal injury [31]. Our research results showed that in addition to removing myoglobin, exogenous biological renal support alleviates AKI by suppressing complement system activation; decreasing oxidative stress, inflammation, and apoptosis; and promoting tubular cell proliferation. In addition, proteomic analysis showed that metabolic signalling pathways were involved in this process, and metabolic reprogramming may be an important part of kidney regeneration and repair. Therefore, further study is needed to explore this process. Furthermore, exogenous biological renal support has multifaceted effects on the recovery of renal function. The effect of key pathways or proteins cannot be independently verified with a parabiosis model, and we need to further examine a single mouse model of RM.

**Conclusion**

In summary, we demonstrated that exogenous biological renal support supplied by parabiosis can improve renal function in RM-induced AKI by suppressing complement system activation; decreasing oxidative stress, inflammation, and apoptosis; and promoting tubular cell proliferation. Our study provides new ideas for effectively preventing and treating RM-induced AKI and provides basic research evidence for the use of bioartificial kidneys to treat RM-induced AKI.

**Abbreviation**

AKI, acute kidney injury; GO, Gene ontology; KEGG, Kyoto encyclopedia of genes and genomes; RM, rhabdomyolysis; P_RM_R, the mouse in the parabiosis model administered with glycerol. P_RM_S, the other mouse in the parabiosis model supply the exogenous biological renal support.

**Declarations**

**Ethical Approval and Consent to Participate**

All animal protocols were approved by the Animal Ethics Committee of the Chinese PLA General Hospital and Military Medical College.

**Consent for publication**

Not applicable.

**Availability of data and material**
The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are provided in the paper and its Supporting Information files.

**Competing interests**

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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**Authors' contributions**

Designed the research: CL, QH, XMC, XFS. Performed the research: CL, KC, XDG, ZM, QH, DL, YQW, YZ. Methodology: QH, ZM, FHZ, GYC, XFS. Analyzed the data: CL, KC, XDG, ZM, QH, GYC, XMC, FHZ. Wrote the manuscript: CL, KC, XDG, ZM, XFS. All authors revised the manuscript draft and approved the final version for submission.

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Supplementary Materials

Figure S1. Parabiosis procedure.

The procedures of parabiotic surgery are described as follows (Figure S1): a. Anesthesia and skin preparation; Make semicircular marks at the lateral abdomen of both donor and recipient; b and c. Separate skin and subcutaneous tissue, then cut the marked skin to expose subcutaneous muscle; d, e and f. Isolate the chest muscle flap of donor mice, and then suture to the chest of the recipient mice; and abdominal muscles of two mice were connected by 3-points intermittent suture; g and h. Suture the outer skin of two mice. The successful surgery relies on the establishment of a shared blood circulation, which requires a minimum 1-1.5cm² area of isolated chest and abdomen muscle flap.
**Figure S2. Confirmation of shared blood circulation after parabiosis**

(1) Peripheral blood smear test for GFP detection: established isochronic (3 months) parabiosis of C57BL/6 GFP mouse (donor) and C57BL/6 wild-type mouse (receptor); three weeks after parabiosis, venous blood from the donor and recipient were taken for the smear test; and the area of green fluorescent protein was observed under a fluorescent microscope.

(a-d) Fluorescence microscopy of peripheral blood smears. (a) No green fluorescent protein (GFP) positive blood cells were observed in the peripheral blood smear from the wild-type mouse. (b) GFP-positive blood cells in the peripheral blood smear from the enhanced GFP (EGFP) transgenic mouse. (c-d) Three weeks after parabiosis was established between the EGFP transgenic mouse and the wild-type mouse, GFP-positive blood cells were observed in the peripheral blood smear of the (c) EGFP transgenic mouse and the (d) wild-type mouse.

(2) Flow cytometry for the measurement of GFP⁺ cell ratio: established the same animal model as (1), prepared leukocyte blood cells from the venous blood of the donor and recipient, and the ratio of GFP⁺ cells in the red blood cells of donor and recipient mice was measured by flow cytometry (CYTOMICS FC 500; Beckman Coulter Inc., USA), FlowJo Software, version 7.6 (Tree Star Inc., Ashland, OR), was used for data analysis.

(e-h) Flow cytometry measurements. (e) The wild-type mice had a negligible amount of GFP-positive cells in the peripheral blood. (f) The majority of peripheral blood cells in the EGFP mice were GFP positive. In the parabiosis model, the amounts of GFP-positive cells in the peripheral blood of the (g) EGFP transgenic mouse and (h) wild-type mouse were similar.

(3) Small animal in vivo imaging: established isochronic (3 months) parabiosis of C57BL/6 wild-type mouse (donor) and C57BL/6 wild-type mouse (receptor). Two weeks after the parabiosis, DiR (100 ml, 16 ml/ml of PBS) were injected into the caudal vein of the donor mouse. Thirty minutes after injection, the distribution of DiR in the donor and recipient mice was traced using a small animal in vivo imaging system (IVIS Spectrum, US Caliper Life Sciences).

(i-n) Using a small animal in vivo imaging system, we observed the body surface positions of fluorescent dyes in mice. (i) Wild-type mouse injected with phosphate-buffered saline. (g) Wild-type mouse injected with 1,1,3,3-tetramethylindotricarbocyanine iodide (DiR) fluorescent dye. (k,i) Parabiosis model. (k) After 30 minutes to 2 hours, the distribution of DiR was observed at the spleen's body surface position in the other mouse. (i) DiR fluorescent dye was injected into 1 mouse of the parabiosis model. (m,n) Parabiosis model. (m) After 30 minutes to 2 hours, the distribution of DiR was observed at the liver's body surface position in the other mouse. (n) DiR fluorescent dye was injected into 1 mouse of the parabiosis model.

**Figure S3.** Principal Component Analysis (PCA) analysis.
Figure S4. Differentially expressed protein statistics.

Figure S5. Enrichment of protein domain analysis of kidney tissue.

Figure S6. Enrichment of protein domain analysis of serum.

Figure S7. The level of creatine kinase (CK) and myoglobin (MYO) expression.

Table S1. The different protein of P_RM_R group versus RM group at 48 hours. RM, rhabdomyolysis; P_RM_R, the mouse in the parabiosis model administered with glycerol.

Figures

Exogenous biological renal support attenuates the histological and functional deterioration in RM-induced AKI. a, Serum creatinine (Scr) levels in the 4 groups. b, Blood urea nitrogen (BUN) levels in the 4 groups. c, Renal tubular injury score. d, Representative photographs of kidney sections with periodic acid–Schiff (200× magnification). Values are presented as means±SD. Scr, Serum creatinine; BUN, blood urea nitrogen; RM, rhabdomyolysis; P_RM_R, the mouse in the parabiosis model administered with glycerol. P_RM_S, the other mouse in the parabiosis model supply the exogenous biological renal support. *P<0.05 versus the sham group; #P<0.05 versus the RM group; $P<0.05 versus the P_RM_R group.
Figure 2

Gene ontology and subcellular localization of kidney tissue and serum. RM, rhabdomyolysis; P_RM_R, the mouse in the parabiosis model administered with glycerol. P_RM_S, the other mouse in the parabiosis model supply the exogenous biological renal support.
Figure 3

KEGG pathway enrichment of kidney tissue. RM, rhabdomyolysis; P_RM_R, the mouse in the parabiosis model administered with glycerol. P_RM_S, the other mouse in the parabiosis model supply the exogenous biological renal support.
Figure 4

KEGG pathway enrichment of serum. RM, rhabdomyolysis; P_RM_R, the mouse in the parabiosis model administered with glycerol. P_RM_S, the other mouse in the parabiosis model supply the exogenous biological renal support.
Figure 5

Immunofluorescence staining for C3. Exogenous biological renal support decreases C3 deposited in RM-induced AKI. C3 was deposited along the renal tubular basement membrane in the RM and P_RM_R groups, but not in the sham and P_RM_S groups. RM, rhabdomyolysis; P_RM_R, the mouse in the parabiosis model administered with glycerol. P_RM_S, the other mouse in the parabiosis model supplying the exogenous biological renal support. *P < 0.05 versus the sham group; #P < 0.05 versus the RM group; $P < 0.05 versus the P_RM_R group.
Figure 6

Exogenous biological renal support decreased oxidative stress in RM-induced AKI. MDA, Malondialdehyde; SOD, superoxide dismutase; GSH, Glutathione peroxidase; PC, Protein carbonyl. RM, rhabdomyolysis; P_RM_R, the mouse in the parabiosis model administered with glycerol. P_RM_S, the other mouse in the parabiosis model supply the exogenous biological renal support. *P < 0.05 versus the sham group; #P < 0.05 versus the RM group; $P < 0.05 versus the P_RM_R group.
Figure 7

Exogenous biological renal support decreased inflammation in RM-induced AKI. a-d, the levels of TNF-α, NGAL, SAA1 and SAA2 were measured by ELISA. e, Representative images of renal CD3 expression in four groups. f, Comparison of CD3-positive cells per field. TNF-α, tumor necrosis factor-α; NGAL, Neutrophil gelatinase-associated lipid carrier protein; SAA, serum amyloid A protein; RM, rhabdomyolysis; P_RM_R, the mouse in the parabiosis model administered with glycerol. P_RM_S, the other mouse in the parabiosis model supply the exogenous biological renal support. *P < 0.05 versus the sham group; #P < 0.05 versus the RM group; $P < 0.05 versus the P_RM_R group.
Figure 8

Exogenous biological renal support decreased apoptosis in RM-induced AKI. a, the levels of Bcl2 and cleaved caspase-3 were measured by Western blot. b-c, Quantitative analyses of the band densities of Bcl2 and cleaved caspase-3 protein expression. d, The percentage of TUNEL-positive tubular cells. e, TUNEL staining. RM, rhabdomyolysis; P_RM_R, the mouse in the parabiosis model administered with glycerol. P_RM_S, the other mouse in the parabiosis model supply the exogenous biological renal support. *P<0.05 versus the sham group; #P<0.05 versus the RM group; $P<0.05 versus the P_RM_R group.
Figure 9

Exogenous biological renal support promotes tubular cell proliferation in RM-induced AKI. a, the levels of cyclin D1 and cyclin E1 were measured by Western blot. b-c, Quantitative analyses of the band densities of cyclin D1 and cyclin E1 protein expression. d, The percentage of PCNA-positive tubular cells. e, PCNA staining. RM, rhabdomyolysis; P_RM_R, the mouse in the parabiosis model administered with glycerol. P_RM_S, the other mouse in the parabiosis model supply the exogenous biological renal support. *P<0.05 versus the sham group; #P<0.05 versus the RM group; $P<0.05 versus the P_RM_R group.

Supplementary Files

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- FigureS3.pdf
- FigureS4.pdf
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