A young blood environment decreases aging of senile mice kidneys

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Abstract:

Whether changes in internal body environment affect kidney aging remains unclear. Specifically, it is unknown whether transplanted kidneys from older donors recover from tissue damage after placement in younger recipients. In this study, a parabiosis animal model was established to investigate the effects of a young internal body environment on aged kidneys. The animals were divided into six groups: young (Ycon) and old (Ocon) control groups, isochronic youth-youth group (Y-IP), elderly-elderly group (O-IP) and heterochronic youth (Y-HP) elderly (O-HP) group. After parabiosis, tubule and interstitial tissue scores in the O-HP group were significantly lower than in the Ocon and O-IP groups. The expression of aging-related protein p16 and SA-β-gal in the O-HP group was significantly reduced compared with the Ocon and O-IP groups. Autophagy factor Atg5 and LC3BII were significantly upregulated, while the expression of the autophagic degradation marker (P62) was significantly downregulated in the O-HP group compared with the Ocon and O-IP groups. With the same comparison, the positive cells of TUNEL staining and the expression of IL-6 and IL-1β were significantly reduced, while the total/cleaved caspase-3 and total/pNF-κB were significantly increased in the O-HP group. The results demonstrated that a young blood environment significantly reduces kidney aging. These findings provide new evidence supporting an increase in the upper age limit for human kidney transplantation donors.

Key words: senescence; parabiosis; rejuvenation; blood environment
Introduction

Despite continual development of modern medical technology, we continue to face serious challenges from chronic diseases and aging societies. Determining how to effectively slow down aging and make aged organs "young again (rejuvenation)" has been a research focus in recent years.

In 2005, Conboy et al. conducted an experiment using a heterochronic parabiosis mouse model. Results from that study revealed that the dysfunction of aging-related stem cells originated mainly from changes in their microenvironment rather than the inherent functional changes of stem cells [1]. Since then, more aging-related research has established a parabiosis animal model with different ages. These studies involved the circulatory system [2,3], nervous system [4,5], muscle tissue [6] and endocrine system [7]. The research findings consistently revealed that the young blood environment can variably promote tissue proliferation, reduce tissue damage and restore organ function. However, no research has investigated the effect of the internal body environment on aged kidneys using a parabiosis animal model.

The kidney is the typical target organ of body aging. Indeed, renal senescence promotes the development of chronic kidney disease (CKD) in elderly patients. A national epidemiological survey of chronic kidney diseases in China revealed that the average age of patients who suffered from chronic kidney disease (CKD) at phase 3 or higher was 63.6 years [8]. The National Health and Nutrition Examination Survey (NHANES) also found that elderly people aged 70 or older are at a high risk of CKDs [9]. In addition, data from the US Renal Data System (USRDS) indicated that starting from age 45, end-stage renal disease (ESRD) morbidity increased annually and reached a peak at age 75 [10]. Although kidney transplantation is the best treatment for ESRD, a lack of donors, particularly from younger age groups, is the main
bottleneck. Many older donors are willing to donate a kidney; however, it is unclear whether kidney tissue damage and renal function loss in aged kidneys can be restored after transplantation into young recipients. Therefore, in-depth study on the pathogenesis of kidney aging, particularly the effects of blood environmental changes on the aged kidney, is necessary.

Previous studies have shown that the autophagy, apoptosis and inflammation levels of cells are closely related to cell senescence. A significant decrease in autophagy levels of aged kidney cells, whereby they cannot effectively remove damaged proteins and damaged organelles (such as mitochondria), results in the accumulation of harmful substances and eventually leads to apoptosis or cell death [11]. In addition, by removing damaged proteins and organelles, autophagy can reduce cell inflammation and reduce cell apoptosis. After autophagy levels are down regulated, cell inflammation and apoptosis levels can increase and cause degeneration [12].

We hypothesized that by regulating autophagy, apoptosis and inflammation levels in a young blood environment, we can reduce kidney aging and restore tissue damage in aged kidneys. Hence, we investigated the effects and mechanisms of young blood environment on kidney aging by establishing isochronic and heterochronic parabiosis animal models, thus providing new basic research evidence on the feasibility of kidney transplantation from older donors into young recipients.
Materials and Methods

Experimental Animals

Three-month-old and 22-month-old male C57BL/6 mice (specific pathogen-free, SPF) were provided by the Experimental Animal Center of Chinese PLA General Hospital; 3-month-old male Actb-GFP/C57/BL6 homozygous mice were provided by the Nanjing Institute of Animal Models. The animals were given free access to food and water. All experiments were approved by Chinese PLA General Hospital’s Committee on Animal Protection and Utilization.

The animals were divided into two groups: control and parabiosis groups. The control group was further divided into two subgroups: young and elderly control groups. The parabiosis group was further divided into three groups: isochronic youth-youth group, elderly-elderly group and heterochronic youth-elderly group. All mice were wild mice. Blood and kidney tissue samples were collected 5 weeks after the parabiosis operation and at the same time for the control groups (meaning the control mice were sacrificed at the same age as the parabiosis groups).

Parabiosis

Surgical procedures that followed the methods of previous researchers [13,14] were applied to connect two mice and establish a shared blood circulation; the parabiotic surgery details are described in the Supplementary section and Figure S1.

To verify the establishment of shared circulation in parabiotic mice, we used transgenic mice with fluorescent marker (GFP-expressing C57BL/6) and wild mice (C57BL/6) for parabiosis. The successful establishment of shared blood circulation was proven by three methods: (1) peripheral blood smear test for GFP detection; (2) flow cytometry for the measurement of GFP+ cell ratio; and (3) small animal in vivo imaging. For further details on the methods, see the Supplementary section.
Blood biochemistry analysis

A Hitachi 7150 automatic biochemistry analyzer (PLA General Hospital Biochemistry Department) was used to measure mouse serum creatinine and urea nitrogen levels.

Pathological scores

Using the double-blind method, the pathological data were analyzed by two independent professional and trained pathologists holding a Certificate of Pathological Technical qualification and the Certificate of Physician Practice. All group samples were evaluated and, after scoring 10 randomly selected fields from each sample, the average scores were calculated.

The NIH semi-quantitative scoring method was applied to evaluate the tubulointerstitial lesions. The four main indicators were (1) tubular epithelial cell degeneration; (2) tubular atrophy; (3) interstitial inflammatory cell infiltration; and (4) interstitial fibrosis. Scoring was based on the percentage of lesions mentioned above in the renal interstitial area: 0% was counted as 0 point, 0–25% was counted as 1 point, 25–50% was counted as 2 points, 50–75% was counted as 3 points, and >75% was counted as 4 points.

Calculation of glomerular area

Glomerular area was measured by DotSlide software as follows: 20 glomeruli were randomly selected from each section, and the glomerular area was obtained with irregular continuous closed measurement by DotSlide software. Each section was evaluated independently by two investigators and the average of the values was considered in the analysis.
**TUNEL staining**

Paraffin-embedded tissue sections were prepared according to the TUNEL Apoptosis Assay Kit (Roche, Shanghai, China) and operated strictly according to the procedure. After performing the apoptosis detection test, the slides were carefully evaluated using a light microscope. Positive staining in the apoptosis detection assay was represented by a dark brown (DAB) signal.

**Senescence-associated β-gal staining**

Tissue sections (4 μm) of the frozen kidney were warmed for 5–10 minutes, and stained with a frozen section senescence-specific β-galactosidase *in situ* staining kit (GMS10012.3; Genmed Scientifics Inc., Wilmington, DE, USA). Cells with a blue precipitate in the cytoplasm were defined as positive. The details of the methods see the Supplementary section.

**Western blot**

A 60–100 μg measure of protein was added into the 10–15% SDS-polyacrylamide gel. Then, the protein was transferred onto a nitrocellulose membrane and covered with 1×CASEIN at room temperature for 1 hour. The necessary antibodies were added to the membrane and incubated at 4°C overnight (detailed information on the antibodies is shown in the Supplementary section). Next, the horseradish peroxidase-labeled goat anti-rabbit or goat anti-mouse IgG (Santa Cruz Biotechnology) was diluted at 1:1000 against specific primary antibodies. Immunoreactivity bands were determined by enhanced chemiluminescence using Quantity One (Bio-Rad Laboratories, Hercules, CA, USA) software for semi-quantitative analysis.
**Statistical analysis**

All data are presented as mean±SD. For assessing statistical significance, one-way ANOVA was used for comparisons of quantitative data among groups, and post hoc Dunnett-t tests was used to evaluate inter-group differences. The Chi-squared test was used to compare the difference in numeration data. P values < 0.05 were considered statistically significant. All statistical analyses were performed using SPSS v18.0 (SPSS, Chicago, IL, USA) software.

**Results**

**Survival rate and confirmation of shared blood circulation after parabiosis**

A total of 36 pairs of mouse parabioses were completed, including isochronic parabioses (IP) and heterochronic parabioses (HP); all of these animals came from wild male C57BL/6 mice. There were 10 pairs of young mice IP (Young-Young, Y-IP); 11 pairs of old mice IP (Old-Old, O-IP); and 15 pairs of young mice HP (Y-HP) and old mice HP (O-HP). Observation of the survival rate during the 5 weeks after the parabiosis operation revealed that the survival rate in the HP group was significantly lower than in the IP group (53.3% vs. Y-IP: 80%, O-IP: 72.7%), and of the 7 pairs of dead animals in heterochronic parabioses, there were 5 pairs of failed parabioses caused by death of young mice, accounting for 71.4%. However, no significant difference in mortality was observed between isochronic parabiosis groups (p=0.696).

Of the two failed pairs of Y-IP group after parabiosis, the donor mouse in one pair died, while in another pair, both the donor and the recipient mice died. As for the O-IP group, of three failed pairs, the donor mouse in one pair died, the recipient mouse in another pair died, and in the third pair, both the donor and recipient mice died. A total of 24 pairs of parabioses were obtained 5 weeks after the operation, and there were 8 pairs in each group (Y-IP, O-IP and HP groups).
Young C57BL/6 and GFP-expressing C57BL/6 transgenic mice were used to determine whether a shared circulation was established among the parabiotic pairs 2 weeks after the parabiosis operation. The three sets of results consistently demonstrated that 2 weeks after parabiosis operation, the donor and recipient mice had established an interflow of blood circulation. The results are shown in the Supplementary section and in Figure S2.

**Animal age and blood environment had no effect on levels of creatinine and BUN in the mice**

Five weeks after the parabiosis operation, peripheral blood of animals from different groups was taken to measure creatinine and urea nitrogen levels. There was no significant difference in the levels of creatinine and BUN among the youth control group (Ycon), old control group (Ocon), individual young animals of Y-IP, individual old animals of O-IP, and young/old individual animals of HP (Figure S3). These results indicate that raised animal age and the blood environment do not have significant effects on levels of creatinine and BUN in mice.

**A young blood environment retarded age-related renal tissue damage**

Glomerular area was measured by DotSlide software; the results showed that glomerular area in aged mice was larger than that of young mice, regardless of parabiotic group (heterochronic or isochronic) or control group. A young blood environment had no effect on the glomerular area of old mice (Fig S4). In addition, no other differences were found in glomeruli between young and old mice.

For the Ocon group, tubular epithelial cell degeneration, necrosis and shedding of tubular cells, tube formation, renal interstitial fibrosis and other tissue damages were observed; while there was almost no pathological manifestation described above in the Ycon group. Following heterochronic parabiosis, renal tubular degeneration and
the numbers of tubule cell shedding due to necrosis significantly decreased; the areas of renal interstitial fibrosis significantly reduced in the O-HP group compared with O-IP and Ocon groups (Figures 1A, B and C), but were still higher than those of the Y-con, Y-IP and Y-HP groups. At the same time, the degree of interstitial fibrosis slightly increased in the Y-HP group, but the difference was not significant ($P>0.05$). Pathological manifestations in the Y-IP group were similar to that in the Ycon group, and manifestations in the O-IP group were similar to that in the Ocon group (Figures 1A, B and C). These results indicate that after heterochronic parabiosis, a young blood environment retarded age-related renal tissue damage in old mice.

**A young blood environment reduced the expression of aging-associated markers in aged kidneys**

Five weeks after the parabiosis operation, kidney tissues were taken to detect senescence-associated β-galactosidase (SA-β-gal). The presence of blue precipitates in the cytoplasm of cells was defined as ‘positive’. Results revealed that positive areas in the O-HP group were significantly lower than that in the Ocon and O-IP groups; and positive areas in samples from the three groups (Ycon, Y-HP and Y-IP groups) were similar, and significantly lower than that in the Ocon, O-IP and O-HP groups (Figures 2A and C). The expression of p16 protein in kidneys was significantly reduced after heterochronic parabiosis in the O-HP group, compared with the Ocon and O-IP groups, and was similar to protein expression levels in the Ycon, Y-IP and Y-HP groups (Figures 2B and D).
A young blood environment enhanced the autophagy of aged kidneys and mitigated apoptosis and inflammation

Five weeks after the heterochronic parabiosis operation, autophagy-related protein Atg5 and LC3BII levels increased in the O-HP group, and were significantly higher than that in the Ocon and O-IP groups; however, autophagy levels of the mice in isochronic parabiosis groups did not experience any significant change (Figures 3A, B and C). The expression of autophagy degradation marker ‘p62’ decreased in the O-HP group, and was significantly lower than that in the Ocon and O-IP groups but still higher than those of Y-con, Y-IP and Y-HP groups (Figures 3A and D). These results suggest that following heterochronic parabiosis, autophagic activity increased in the O-HP group.

TUNEL staining results showed that the percentage of positive cells in the O-HP group was significantly lower than the Ocon and O-IP groups, but still higher than those of Y-con, Y-IP and Y-HP groups (Figure 4A and C). At the same time, the percentage of positive cells slightly increased in the Y-HP group compared with the Y-con and Y-IP group, but the difference was not significant (P>0.05). The detection results of the total/cleaved caspase3 ratio revealed that after heterochronic parabiosis, the ratio of total/cleaved caspase3 significantly increased in the O-HP group compared with the Ocon and O-IP groups, but was still lower than those of Y-con, Y-IP and Y-HP groups (Figure 4B and D). The level of apoptosis in the Ocon and O-IP groups did not significantly change. These results suggest that following heterochronic parabiosis, the level of apoptosis decreased in the O-HP group.
In addition, inflammation-related indicators pNF-κB, IL-1β and IL-6 were tested to assess the impact of heterochronic parabiosis on inflammatory factors. Results revealed that inflammatory indicators IL-1β and IL-6 in the Ocon group were significantly higher than that in the Ycon group, while the ratio of total/p NF-κB in the Ocon group was significantly lower than that in the Ycon group (Figures 5A, B, C and D). Parabiosis operation had no effect on the expression of inflammatory proteins, and the protein expression levels of total/pNF-κB, IL-1β and IL-6 in the isochronic parabiosis groups (Y-IP and O-IP) were not different from that in the corresponding control groups (Figures 5A, B, C and D). Heterochronic parabiosis significantly reduced the level of inflammation in the O-HP group, and the protein expression levels of IL-1β and IL-6 in the O-HP group were significantly lower than that in the Ocon and O-IP groups, whereas the ratio of total/pNF-κB significantly increased in the O-HP group (Figure 5A, B, C and D).

**Discussion**

The parabiosis animal model establishes microcirculation within muscle and subcutaneous tissues between two animals after surgery. After parabiosis, a continuous exchange of blood cells and soluble factors between two animals can be achieved through blood circulation interflow. Indeed, according to the literature, about 8% of the circulating blood from each of the two animals after parabiosis can be exchanged every day and full blood exchange can be completed every 14 days [15]. In this study, we confirmed shared blood circulation between two conjoined animals 14 days after parabiosis. Our values of results indicated that the blood circulation between the two mice in the established parabiosis animal model achieved interflow, and a homogenous distribution of blood cells was reached that was consistent with the literature [16].
The blood environment has a slow and sustained influence on the organ function of animals. Moreover, several studies have shown that the effects of blood environment on organs or tissues can be observed 4–5 weeks after heterochronic parabiotic surgery [2–7]. Due to a lack of study on changes of kidneys from an altered blood environment, we chose 5 weeks as the time point according to the time of the establishment of shared blood circulation and previous studies. Through continuous observation of animal survival rates during the 5-week period after parabiosis, it was found that survival rates were higher in mouse pairs with matched age, weight and gender [17], while the survival rate of heterochronic parabiosis mice was significantly reduced. Animal death usually occurred around 2 weeks after the parabiosis operation. This timeframe can rule out the effect of anesthesia and surgery itself on the experimental animals. The main cause of death was probably associated with conjoined disease. Conjoined disease usually happens within the first 2 weeks following parabiosis, in which a shared blood circulation is established between the donor and recipient mice during this period. Even in the case of parabiosis between two highly inbred mice, the incidence of conjoined disease could be as high as 20–30% [18]. These findings are consistent with our results. We also observed that the failure rate of heterochronic parabiosis due to death of young animals in the HP was above 70%, thus suggesting that a rapidly aged blood environment might have a serious impact on organ function in young mice, and likely lead to their deaths.

A young blood environment can variably promote tissue proliferation in multiple organs, reduce tissue damage, and restore organ functions in elderly people [2–7]. However, the impact of a young blood environment on aged kidneys has not been reported. Senescence-associated renal structural changes mainly include podocytopenia, glomerulosclerosis, vascular changes (arteriolosclerosis and
thickening of the arterial intima or media), and changes in tubulointerstitial (tubular atrophy and interstitial fibrosis) \cite{19}. Our results were consistent with the previous research. However, we also found that 5 weeks after heterochronic parabiosis, and after being affected by the young blood environment, kidney tissues in elderly mice significantly changed and progression of aging-related tissue damage was significantly retarded compared with the O-IP and old control groups. The results also showed the same changes in senescence-associated protein p16 and senescence-associated β-galactosidase. These changes clearly confirmed our experiment hypothesis—i.e. rejuvenating changes in aged mice can delay the progression of aging-related tissue damage and kidney aging. Some studies of muscle and nervous system reported that the proliferation of muscle tissue is enhanced in aged parabiotic mice but simultaneously reduced in young parabiotic mice \cite{4–6}; while the pancreatic β-cell replication was not affected in the young mice after heterochronic parabiosis \cite{7}. Similarly, in studies on age-related cardiac hypertrophy, dilated cardiomyopathy or heart failure, shared blood circulation from old mice did not cause cardiac hypertrophy or dilatation in young mice \cite{2,3}. The researchers speculate that the "unfavorable" microenvironment and local cardiac senescence-related secretory phenotype (SASP) of the aged mice does not transmit to young mice through blood circulation via parabiosis. Whether the tissues or organs in young mice undergo changes may be dependent on the characteristics of specific tissues or organs and the mechanism of aging. Moreover, the age of animals in heterochronic parabiosis was also an influential factor \cite{5}. In our study, there was no significant difference between young parabiotic mice and their controls, regarding not only senescence-related indicators but also renal autophagy and inflammatory levels. The effect of
heterochronic parabiosis on kidneys of young mice may be consistent with the effect on islet and heart cells.

In research on humans, the average GFR declines from approximately 130 to 80 ml min\(^{-1}\)/1.73 m\(^2\) as subjects age from 30 to 80 years old, and the rate of decline in GFR accelerates after age 65 years [20]. Despite this, serum creatinine concentrations in healthy older people are not dissimilar to those in younger people; older patients with normal serum creatinine commonly have reduced GFR [21]. In another study, among 38 older outpatients with moderately or severely reduced GFR (GFR<60 ml min\(^{-1}\)/1.73 m\(^2\)), only 14 had serum creatinine concentrations exceeding the reference range [22].

In our study, the levels of serum creatinine and BUN in individual young and old mice of HP were not significantly different from those in young or old mice of IP, or from those in the mice of Ycon and Ocon groups. This may be because the levels of serum creatinine and BUN were not sensitive enough to reflect renal function. However, due to the short observation period associated with the parabiosis model, it cannot be ruled out that structural changes in renal tissue were not serious enough to affect renal function. It should be emphasized that the change of renal tissue caused by aging happens before renal function changes. Kidney mass loss and possible renal dysfunction mainly depend on change in the renal tubular interstitial region, rather than the glomerulus [23]. Therefore, in this study, we focused on the impact of blood environment changes on renal tubules in aged mice and explored the associated mechanisms.

Autophagy is a relatively conservative life activity. In both rodents and primates, the level of autophagy decreases with age, and life expectancy extends when autophagy is activated [24]. As time-dependent accumulation of cell damage is an
important reason for aging, autophagy activity decreased gradually with age and may be involved in many aspects of aging [25]. On the other hand, autophagy plays an important role in the fight against cellular stress, the development of innate immune response, and maintaining cell numbers. Therefore, autophagy is an essential approach for the self-protection of cells, and also a potential anti-aging factor [23]. The decreased autophagic ability of muscle satellite cells in aged mice caused the aging and depletion of stem cells of muscles; however, following the upregulation of autophagy, the cell-aging process is reversed and proliferation ability is restored [26]. Similarly, the specific knock-off autophagy-related genes (Atg5) in kidney podocytes and proximal tubular epithelial cells results in glomerular sclerosis, proteinuria and renal tubular epithelial cell shedding from necrosis [27,28]. In addition, persistent autophagy is an essential factor in maintaining the polarity of tubular epithelial cells and the structure of tubular structures [29].

In our study, with a young blood environment, the level of autophagy in aged kidney tissue was significantly enhanced, the aging damage of kidney tissue was repaired, and senescence-associated protein and markers were also decreased. These findings suggested that the level of autophagy enhanced by a young blood environment may improve tissue damage and the aging phenotype of the aged kidney.

In addition, studies have shown that autophagy can prevent apoptosis [23] and reduce inflammation [11]. Autophagy can reduce inflammation by multiple mechanisms. Cell death can lead to inflammation, and autophagy can mediate macrophages in clearing dead cells. Thus, inflammation is reduced [30]. Autophagy can also effectively control intracellular pathogenic microorganisms by enhancing the innate immune response [31]. Damaged mitochondria are needed for non-infectious substances (such as uric acid or silica) to activate NLRP3 inflammatory corpuscles
Autophagy inhibits the activity of NLRP3 inflammatory corpuscles by clearing the damaged mitochondria and mitigating the body's inflammatory response. Indeed, a young blood environment may also have a direct impact on reducing the levels of inflammation and apoptosis in kidney tissue, thereby improving the tissue damage in the aged kidney. However, these mechanisms need further study.

Renal interstitial fibrosis is one of the important features of renal aging, and it is also a common pathway by which a variety of chronic kidney diseases (CKD) progress to end-stage renal disease [33,34]. Although severe renal fibrosis cannot be reversed completely, appropriate intervention can induce regression of the processes. Previous research revealed that both angiotensin inhibitors and endogenous hydrogen sulfide production can decrease the collagen deposition and reverse kidney sclerosis in aged rodents compared with a control group [35,36].

Autophagy, apoptosis and inflammation in the body may have an impact on renal fibrosis. Indeed, a variety of inflammatory cells and inflammatory factors are involved in renal fibrosis. Moreover, several studies have shown that renal fibrosis is closely related to high expression of NF-κB [37,38]. NF-κB can directly activate fibroblasts as well as renal tubular cells to secret many cytokines, and it also exerts a positive feedback effect on angiotensin, together promoting renal fibrosis through various pathways.

In our study, the expression of pNF-κB in the old kidney was significantly decreased after heterochronic parabiosis, resulting in a reduced activation of fibroblast in old mice, which might be one of the mechanisms by which a shared blood environment decreased renal fibrosis of senile kidneys. In addition, tyrosine kinase inhibitors can effectively reduce renal interstitial cell infiltration and renal interstitial fibrosis [39]. Receptors with tyrosine kinase activity include platelet-derived growth
factor receptor (PDGFR) and stem cell factor receptor (c-KIT), and their ligands are platelet-derived growth factor (PDGF-D) and stem cell factor (SCF). Recent studies have shown that PDGF-D is significantly increased in fibrotic kidneys, and inhibition of PDGF-D expression can reduce renal fibrosis [40]. The abnormality of SCF/c-kit signaling pathway is closely related to the development of fibrosis in multiple organs [41–43].

With the above two cytokines as example, multiple profibrotic signaling pathways in the activated kidney of aged mice may be inhibited or weakened when shared with a young blood environment, achieving an alleviation of aged kidney fibrosis. On the other hand, plasminogen activator inhibitor-1 (PAI-1), which was directly induced by angiotensin, inhibits matrix degradation and is pivotal in remodeling of renal fibrosis. The level of angiotensin (can be activated by pNF-κB) and PAI-1 increased with age, and old mice parabiosed to young mice may have restored the ability of anti-fibrosis by reduce the expression of PAI-1, angiotensin and pNF-κB. Moreover, circulating blood from the young animal may directly affect the renal tissue and cell in the elderly, leading to changes in cellular biological function and renal tissue remodeling in elderly kidney.

In conclusion, through the approach of creating parabiosis and establishing a shared blood circulation, we have demonstrated for the first time that a young blood environment can not only significantly enhance the level of autophagy in elderly kidneys, but also significantly downregulate apoptosis and inflammation levels in aged kidney thereby slowing injury progression of kidney tissues and improving kidney aging. Since the effect of a young blood environment on renal function in old mice was not observed in our study, it was a defect of the research. This problem
might be solved by prolonging the observation duration, or by using other mouse strains.

This study provides new basic research evidence supporting heterochronic kidney transplantation, especially transplantation using older donors’ kidneys. It supports extending the upper age limit of renal transplant donors so that the pool of potential donors is increased. In later studies we intend to identify environmental factors in the young blood environment that play a key role in the regulation of autophagy, apoptosis and inflammation in the aged kidney and its mechanism in signal transduction.

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References


33. Boor P, Ostendorf T, Floege J. Renal fibrosis: Novel insights into mechanisms and


Figure 1 Youth blood environment slowing the progression of age-related renal tissue damage. Periodic Acid-Schiff (PAS) staining results (A) and Masson staining results (B) of renal tissue 5 weeks after parabiosis surgery. Age-related renal tissue damages in aged mice exposed to a young circulation (old heterochronics) are milder compared to old isochronic controls.

- Tubular epithelial cell degeneration
- Necrosis or shedding of tubular cell
- Interstitial fibrosis

(C) Graph representing tubulointerstitial lesions measured by NIH semi quantitative scoring method after PAS and Masson staining. Ten fields were randomly selected from each slide to calculate the pathological scores. Results are based on the average scores from 4 to 8 animals per group. Data shown as mean ± sd. Y: young; O: old; Con: control; IP: isochronic parabiosis; HP: heterochronic parabiosis.
Figure 2 Youth blood environment reduced the expression of aging-associated markers in aged kidneys.

(A) Senescence-associated β-galactosidase (SA-β-gal) staining results of renal tissue 5 weeks after parabiosis surgery. Blue precipitates in aged mice exposed to a young circulation (old heterochronics) are less than old isochronic controls.

▲ Blue precipitates in the cytoplasm

(B) Expression of senescent biomarker p16 in the renal tissues. Western Blot analysis shows increased levels of p16 in the renal tissues of old mice compared to young mice and is restored to “youthful” levels in old mice after exposure to a young circulation (O-HP).

(C) Graph representing the percentage area of positive cells for each group mice. Positive cells were defined as the presence of blue precipitates in the cytoplasm. Ten fields were randomly selected from each slide and the percentage area of positive cells was counted. Data shown as mean ± sd.

(D) Graph representing quantitative analysis results of p16. Data shown as mean ± sd. Y: young; O: old; Con: control; IP: isochronic parabiosis; HP: heterochronic parabiosis.
Figure 3 Youth blood environment enhanced autophagy of the aged kidneys.

(A) Expression of autophagy makers Atg5, LC3 II and p62 in the renal tissues. Western Blot analysis shows autophagy-related protein Atg5 and LC3 II protein levels were significantly higher in old mice exposed to a young circulation when compared to old isochronic mice and autophagy degradation marker p62 significantly reduced in old mice exposed to a young circulation when compared to old isochronic mice.

(B) (C) (D) quantitative analysis results of Atg5, LC3 II and p62. Data shown as mean ± sd. Y: young; O: old; Con: control; IP: isochronic parabiosis; HP: heterochronic parabiosis.
Figure 4 Youth blood environment mitigated apoptosis of the aged kidneys.

(A) TUNEL staining results in the renal tissues, the percentage of positive cells (red arrow) in the O-HP group was significantly lower than the Ocon and O-IP groups, but still higher than those of Y-con, Y-IP and Y-HP groups.

(B) The apoptosis marker (total and cleaved caspase3) Western Blot analysis shows the ratio of total/cleaved caspase3 significantly increased in the O-HP group compared with the Ocon and O-IP groups, but still lower than those of Y-con, Y-IP and Y-HP groups.

(C) (D) quantitative analysis results of TUNEL staining and total/cleaved caspase3 ratio. Data shown as mean ± sd. Y: young; O: old; Con: control; IP: isochronic parabiosis; HP: heterochronic parabiosis.
Figure 5 Youth blood environment mitigated inflammation of the aged kidneys.

(A) Expression of inflammation-related indicators total/pNF-κB, IL-1β and IL-6 in the renal tissues. Western Blot analysis shows IL-1β and IL-6 significantly reduced in old mice exposed to a young circulation when compared to old isochronic mice, while the ratio of total/pNF-κB significantly increased in the O-HP group. 

(B) (C) (D) Quantitative analysis results of total/pNF-κB, IL-1β and IL-6. Data shown as mean ± sd. Y: young; O: old; Con: control; IP: isochronic parabiosis; HP: heterochronic parabiosis.
Figure 1
Figure 2
Figure 3
Figure 4

[Images showing microscopic images and Western blot analysis for caspase3 and cleaved caspase3. The images are labeled with various conditions (Y con, Y-IP, Y-HP, O con, O-IP, O-HP) and are accompanied by graphs showing quantification of TUNEL-positive cells and total/cleaved caspase3 levels.]
Figure 5

A) Western blot analysis of NF-kB, pNF-κB, IL-1β, IL-6, and β-actin in different groups.

B) Quantification of NF-κB expression in Y con, Y-IP, Y-HP, O-HP, O-IP, and O con groups.

C) Quantification of IL-1β expression in Y con, Y-IP, Y-HP, O-HP, O-IP, and O con groups.

D) Quantification of IL-6 expression in Y con, Y-IP, Y-HP, O-HP, O-IP, and O con groups.