Effects of Life-Long Exercise on Age-Related Inflammation, Apoptosis, Oxidative Stress, Ferroptosis Markers, and NRF2/KAEP 1/Klotho Pathway in Rat Kidneys

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Summary

To investigate the effects of life-long exercise (LLE) on age-related inflammatory cytokines, apoptosis, oxidative stress, ferroptosis markers, and the NRF2/KAEP 1/Klotho pathway in rats. Eight-month-old female Sprague-Dawley rats were divided into four groups: 1) LLE: 18-month LLE training starting at 8 months of age, 2) Old moderate-intensity continuous training (OMICT): 8 months of moderate-intensity continuous training starting at 18 months of age, 3) Adult sedentary (ASED): 8-month-old adult sedentary control group, and 4) Old sedentary (OSED): a 26-month-old sedentary control group. Hematoxylin eosin staining was performed to observe the pathological changes of kidney tissue injury in rats; Masson's staining to observe the deposition of collagen fibers in rat kidney tissues; and western blotting to detect the expression levels of IL-6, IL-1β, p53, p21, TNF-α, GPX4, KAEP 1, NRF2, SLC7A11, and other proteins in kidney tissues. *Results*: Compared with the ASED group, the OSED group showed significant morphological changes in renal tubules and glomeruli, which were swollen and deformed, with a small number of inflammatory cells infiltrated in the tubules. Compared with the OSED group, the expression levels of inflammation-related proteins such as IL-1β, IL-6, TNF-α, and MMP3 were significantly lower in the LLE group. Quantitative immunofluorescence analysis and western blotting revealed that compared with the ASED group, KAEP 1 protein fluorescence intensity and protein expression levels were significantly enhanced, while Klotho and NRF2 protein fluorescence intensity and protein expression levels were reduced in the OSED group. Compared with the OSED group, KAEP 1 protein fluorescence intensity and protein expression levels were reduced in the LLE and OMICT groups. Klotho and KAEP 1 protein expression levels and immunofluorescence intensity were higher in the LLE group than in the OSED group. The expression levels of GPX4 and SLC7A11, two negative marker proteins associated with ferroptosis, were significantly higher in the LLE group than in the OSED group, while the expression of p53 a cellular senescence-associated protein that negatively regulates SLC7A11, and the downstream protein p21 were significantly decreased. LLE may ameliorated aging-induced oxidative stress, inflammatory response, apoptosis, and ferroptosis by regulating Klotho and synergistically activating the NRF2/KAEP 1 pathway.

Keywords

Life-long exercise • Moderate intensity continuous training • Aging • Kidney tissue • Ferroptosis

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Introduction

The kidney is one of the most complex organs in terms of physiology, structure, and metabolism [1]. It plays a vital role in regulating water-salt balance,

excreting waste, and maintaining electrolyte balance in the system [2]. However, aging leads to changes in kidney structure and function, including renal fibrosis, progressive development of chronic kidney disease (CKD), oxidative stress, and inflammation, which account for the majority of renal aging mechanisms [2,3]. Oxidative stress causes cellular damage due to the excessive accumulation of endogenous reactive oxygen species (ROS) [4], which in turn triggers an inflammatory response, causing the secretion of pro-inflammatory cytokines, such as IL-1β, IL-6, and MMP-3. This results in a high inflammatory state in the renal tissue, promoting the development of CKD [5]. Therefore, chronic inflammation and accumulation of ROS contribute to kidney fibrosis. Senescence is also associated with defective autophagy and apoptosis [6,7]. Under normal physiological state, when cells receive apoptotic signals, such as oxidative stress or DNA damage, apoptotic vesicles are formed by protein hydrolysis. These vesicles promote phagocytosis and suppress excessive inflammatory responses, thereby protecting the kidney from injury and ensuing fibrosis [8]. However, cellular autophagy- or apoptosis-deficient kidney cells accumulate abnormal mitochondria and proteins and lack quality control over mitochondria, resulting in cellular dysfunction, a characteristic of the aging process [9]. This was fully characterized in an in vitro model of cisplatin-induced renal injury [10].

Ferroptosis is a recently discovered iron-dependent, non-apoptotic form of regulated cell death triggered by the reduced biological activity of GPX4 or accumulation of lipid peroxidation and ROS [11]. This process accelerates interstitial fibrosis and progressive decline in renal function during the aging process, thus exacerbating the development of CKD [12]. Regular physical activity is an effective measure for maintaining fitness, reducing oxidative stress, and preventing various chronic diseases [13]. LLE is associated with longer life expectancy and delayed onset of chronic diseases [14]. For example, aerobic exercise promotes the expression of ferroptosis inhibitors FPN1, FTH1, GPX4, and NRF2 in cardiomyocytes of obese rat, thereby reducing iron overload and ferroptosis in cardiomyocytes [15]. LLE promotes the metabolism and functional adaptation of T lymphocytes and monocytes, reduces oxidative stress and inflammatory responses, and delays aging-induced immune senescence [16]. In addition, the KAEP 1/NRF2 pathway serves as an essential oxidative stress defense mechanism in various tissues in organisms [17]; Activation of the NRF2 pathway inhibits ferroptosis, and the anti-aging protein Klotho synergistically activates the NRF2 pathway [18]. However, it remains unclear whether LLE promotes activation of the KAEP 1/NRF2 pathway in the aging kidney and whether Klotho exerts a synergistic activating effect in the kidney and ameliorates oxidative stress, inflammatory factors, and increased ferroptosis, ultimately alleviating CKD.

Based on the above observations, this study investigated the effects of LLE and 8 months of aerobic training on inflammatory cytokines, apoptosis, oxidative stress, ferroptosis markers, and the KAEP 1/NRF2/Klotho pathway in the kidney tissues of naturally aged rat.

Material and Methods

Animals

Animal experiments were designed in accordance with "Regulations on the Management of Laboratory Animals" and approved by the Ethics Committee of the Animal Experimentation Center of Nanjing Normal University (IACUU-1903006). Female Sprague-Dawley rats (8 months old) were purchased from the Guangdong Medical Laboratory Animal Center [animal license number: SCXK (Guangdong) 2018-0002].

All rats were fed ad libitum, and the Guangdong Medical Animal Experiment Center provided a national standard conventional rodent diet (56.8 % carbohydrates, 22.5 % proteins, 3.5 % lipids, and 17.2 % other nutrients). Feed supply was increased according to body mass growth of the rats, bedding was changed 2 to 3 times a week, and the temperature was maintained at 20 °C to 23 °C with a relative humidity of 50 % to 70 %. The rats were housed under natural light conditions, ensuring adequate ventilation and dry environment.

After 1 week of pre-adaptation, 8-month-old rats (n=12) were randomly selected as the ASED group and administered an intravenous injection of 10 % chloral hydrate at a dose of 4 mL/kg. The remaining animals were randomly divided into three groups: 1) 26-month LLE group with training starting at 8 months of age (LLE, n=12), 2) 8 months of moderate-intensity continuous training starting at 18 months of age (OMICT, n=12), and 3) a 26-month-old sedentary control group (OSED, n=12). Six rats were excluded from the study: four rats in the OMICT group (two died from causes unrelated to exercise and two from severe claw

infections) and two in the OSED group (severe eye infections).

Training protocol

A running table (model: FD000043; Guangzhou Feidi Biotechnology Co) was used for conducting animal experiments. Formal training was conducted after two weeks of acclimatization training. Further details on the calculation of maximum oxygen uptake (VO_{2max}) and training intensity have been described previously [19]. Before the formal training, both exercise programs were performed at a speed of 10 m/min for 1 min of warm-up on the running platform. Thereafter, both were performed at a constant speed of 17 m/min and finally at a speed of 10 m/min for 1 min of relaxation. The total exercise time for all the exercise groups was 45 min, and the slope was 0°. The OSED group was placed in the same environment to avoid interference. Exercise training was performed at the same time every morning [20]. A schematic of the experimental design is shown in Fig. 1.

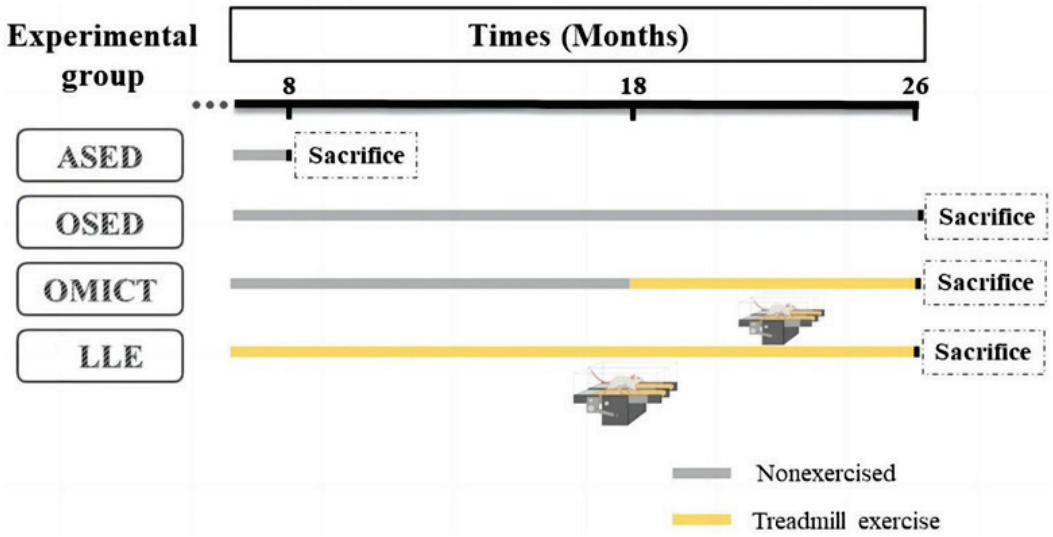

Fig. 1. Schematic diagram of experimental design. *Note:* ASED: Adult sedentary; OSED: Old sedentary; OMICT: Old moderate-intensity continuous training; LLE: Life-long exercise.

Sample collection

Renal tissue sampling was performed as follows: 48 h after the final training session, all rats were anesthetized with intravenous injection of 10 % chloral hydrate at a dose of 4 mL/kg, and rats were euthanized by severing the neck. Both kidneys were rapidly collected; one was sealed in a lyophilized tube, snap-frozen in liquid nitrogen, wrapped in tinfoil, and stored in a -80°C refrigerator for qRT-PCR and Western blotting experiments; the other was dehydrated and then fixed for immunofluorescence experiments.

Immunohistochemistry and immunofluorescence

Fresh kidney tissues were fixed in 10 % paraformaldehyde for 24 h, dehydrated by ethanol gradient, and embedded in paraffin. The tissue was sectioned using a semiautomatic rotary microtome at a thickness of 4 μm. Before dewaxing, the sections were baked in a 60 °C thermostat (Thermo, Scientific) for 60 min. For dewaxing, the tissue sections were soaked in xylene twice for 10 min each, followed by sequential soaking in anhydrous ethanol, 95 % ethanol, 85 % ethanol, and 70 % ethanol for 5 min each and then hydrated. Then, parts were subjected to hematoxylin-eosin (HE), Masson staining. Another portion was subjected to antigen repair and then 50-100 μL of ready-to-use goat serum (AR0009, Wuhan Dr. D. Biological Engineering Co., Ltd.) was added dropwise, and the mixture was incubated at room temperature for 20 min for blocking. Thereafter, the corresponding primary antibody (50-100 μL) was added dropwise on the slide and incubated overnight at 4 °C. The slide was washed with PBS for 20-30 min at room temperature, and secondary antibody was added dropwise on the slide. After incubation at 37 °C for 30 min, the slide was washed. The sections were observed under laser confocal microscopy (A1R/A1, Nikon, Japan) to study the expression of proteins, and 3-4 high expression areas were selected and photographed for further analysis.

RNA isolation and quantitative reverse transcription-polymerase chain reaction

Kidney tissues were added to TRIzol, total RNA was extracted after thorough homogenization, and total RNA concentration was determined using an ultra-micro spectrophotometer (OneDrop®OD-1000). The HiScript III 1st Strand cDNA Synthesis Kit was used for mRNA reverse transcription and the ChamQ SYBR qPCR Master Mix was used for mRNA quantification. All procedures were performed according to the manufacturer's instructions (ABI StepOnePlus, ABI Corporation, USA). GAPDH was used as the internal reference, and the relative quantification was performed using the 2-ΔΔCt method. Relevant primer sequences were designed according to the gene sequences in the NCBI database (Table 1), and the primers were synthesized by Shanghai Bioengineering Biotechnology Service Co.

Table 1. Related gene primer sequences

mRNAs	Forward (5′-3′)	Reverse (5′-3′)
Sestrin 1	CGGACCAAGCAGGTTCATCC	TGATGTTATCCAGACGACCCAAA
Sestrin 2	GAGTGCCATTCCGAGATCAAG	TAGTCCGGGTGTAGACCCATC
Sestrin 3	CGGAAGGACAAAAGAATCCGA	GTTCATCCGCCGTATTTGCT
SOD1	CCAGTTGTGGTGTCAGGACAGATTAC	GGACCGCCATGTTTCTTAGAGTGAG
SOD2	CAGACCTGCCTTACGACTATGG	CTCGGTGGCGTTGAGATTGTT
CAT1	CTATTGCCGTCCGATTCTCCACAG	CACAAGGTCCCAGTTACCATCTTCAG
GAPDH	TGCCGCCTGGAGAAACCTGC	TGAGAGCAATGCCAGCCCCA

Table 2. Primary antibodies used in this study

Primary antibodies	Manufacturer	Product	Primary
MMP-3	Bioss	bs-0413R	1:1000
TNF-α	Affinity	AF7014	1:500
IL-1β	Bioss	20448R	1:1000
IL-6	Bioss	4539R	1:1000
UCP 1	Santa Cruz	sc-293418	1:1000
Bcl-2	Bioss	bsm-33047M	1:1000
TNF-α	Calbiochem	#516557	1:1000
Caspase-3	Bioss	bs-0081R	1:1000
Bax	Santa Cruz	sc-7480	1:1000
P53	Bioss	bsm-33058M	1:1000
P21	Bioss	bsm-60698R	1:1000
SOD2	Bioss	bs-1080R	1:1000
GPX4	Abmart	T56959F	1:1000
SLC7a11	Abmart	T57046F	1:1000
KAEP 1	Bioss	bs-4900R	1:1000
Klotho	Bioss	bs-2925R	1:1000
NRF2	GB113808	Servicebio	1:1000
β-actin	Bioss	bs-0061R	1:10000

Western blotting

For protein isolation, 500 μL of RIPA lysis buffer and 10 μL of protease inhibitor were added to 50 μg of rat kidney tissue, homogenized in a high-speed tissue grinder for 2 min and then centrifuged. The middle layer was aspirated, sonicated for 30 s, and was

centrifuged. The supernatant was collected, mixed with the loading buffer, and boiled for 5 min. SDS-PAGE (10 %) was performed, and the resolved proteins were transferred to a PVDF membrane. The membrane was blocked with 5 % skimmed milk powder in 0.1 % Tween-20 in PBS, followed by incubation with the indicated primary antibody overnight at 4 °C. After incubation, the membranes were washed and incubated with a goat anti-rabbit peroxidase-conjugated secondary antibody (bs-0295G-HRP, Bioss) at a dilution of 1:10000 for 1 h at approximately 20 °C. Immunoreactive proteins were detected using Super Signal Chemiluminescent Substrate (Thermo Fisher Scientific) and measured using a ChemiDoc XRS+Gel Imaging System (721BR16568, Bio-Rad). ImageJ software was used to analyze the blots, and protein expression was normalized to β-actin levels. All antibodies used for western blotting are listed in Table 2.

Statistical analysis

The numerical variables were first tested for normality, and all groups were normally distributed and statistically described using mean ± standard deviation (SD). Differences between OSED, OMICT, and LLE groups were analyzed by one-way ANOVA followed by Tukey post hoc test; differences in age between ASED and OSED groups were assessed by independent samples t-test, with $P<0.05$ indicating significance, and $P<0.01$ indicating extreme significance. Analyses and graphing were performed using GraphPad Prism 9.0 Software (GraphPad Software, San Diego, California).

Results

Histopathological changes in the kidney

The results of HE staining, Masson's trichrome staining, and the degree of renal fibrosis are shown in Fig. 2 (a-c). In the ASED group, the glomerular morphology and tubular structure were intact. In the OSED group, the glomerular basement membrane was thickened, the glomerulus was atrophied, the tubular epithelium was edematous and necrotic, the tubular lumen was dilated, and the interstitium was thickened. In the OMICT and LLE groups, the extent of glomerular and tubular lesions was reduced (Fig. 2a). In the ASED group, the interstitial collagen was sparsely distributed and lightly colored, whereas in the OSED group, the glomerular and peritubular tissues were disorganized, the blue dark-stained collagen fibers increased, and the degree of renal fibrosis was significantly higher than that in the ASED group (Fig. 2b). In the OMICT and LLE groups, the degree of fibrosis was significantly reduced, and the degree of renal fibrosis was significantly lower than that in the OSED group. The reduction of fibrosis was more obvious in the LLE group than in the OMICT group (Fig. 2c).

Changes in the expression of renal inflammation-related proteins

We performed western blotting to examine the levels of inflammation-related protein markers in the kidneys of rats from each group (Fig. 3a-f). The OSED group showed a higher expression of IL-1β, IL-6, MMP-3, and TNF-α ($P<0.01$) than the ASED group, while UCP1 ($P<0.01$) protein content was significantly decreased. Compared with OSED, the LLE group showed significantly increased levels of UCP1 ($P<0.01$) proteins, and significantly reduced levels of IL-1β, IL-6, MMP-3, and TNF-α ($P<0.01$). Compared with the OSED group, the OMICT group showed a significant reduction in IL-1β and TNF-α ($P<0.05$) protein levels. In addition, compared with the OMICT group, the LLE group showed a significant reduction in IL-6 and MMP-3 ($P<0.05$) protein levels.

Changes in the expression of renal apoptosis-related proteins

As shown in Fig. 4a-f, western blotting was used to further analyze the expression of apoptosis-related proteins. Compared with the ASED group, the OSED group exhibited a significant increase in Caspase-3, Bax, p53, and p21 ($P<0.01$) protein levels, whereas Bcl-2 ($P<0.01$) protein level was significantly decreased. Compared with the OSED group, the LLE group showed significantly increased Bcl-2 ($P<0.01$) protein levels and significantly reduced levels of Caspase-3, Bax, p53, and p21 ($P<0.05$). Caspase-3, Bax, and p21 ($P<0.05$) protein levels were significantly lower in the OMICT group than in the OSED group, whereas Bcl-2 ($P<0.01$) protein expression was higher. In addition, compared with the OMICT group, the LLE group showed a significant reduction in Bax ($P<0.01$) and p53 ($P<0.05$) protein levels.

Changes in the expression of ferroptosis-related proteins and oxidative stress-related mRNAs and proteins in the kidney

As shown in Fig. 5a-j, compared with the ASED group, SOD2, SLC7a11, and GPX4 ($P<0.01$) protein expression decreased significantly in the OSED group.

SOD1 (P<0.01), SOD2 (P<0.01), and CAT (P<0.05) mRNA expression were lower in the OSED group than in the ASED group. Sestrin1, Sestrin2, and Sestrin3 mRNA expression were also lower in the OSED group than in the ASED group. The protein levels of SOD2 (P<0.01), SLC7a11 (P<0.01), and GPX4 (P<0.05) were significan-

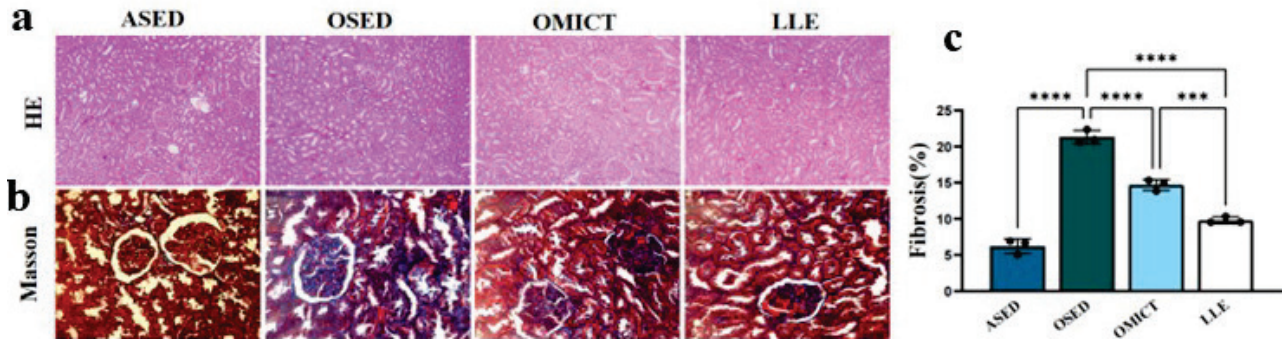

Fig. 2. Changes in the histopathological morphology of kidney. *Note:* H&E stain (×200) (**a**); Mosson stain (×400) (**b**); Comparison of the level of renal fibrosis between groups (**c**); Data are presented as means ± SEM. * indicates p<0.05, ***/**** indicates p<0.01. ASED: Adult sedentary; OSED: Old sedentary; OMICT: Old moderate-intensity continuous training; LLE: Life-long exercise.

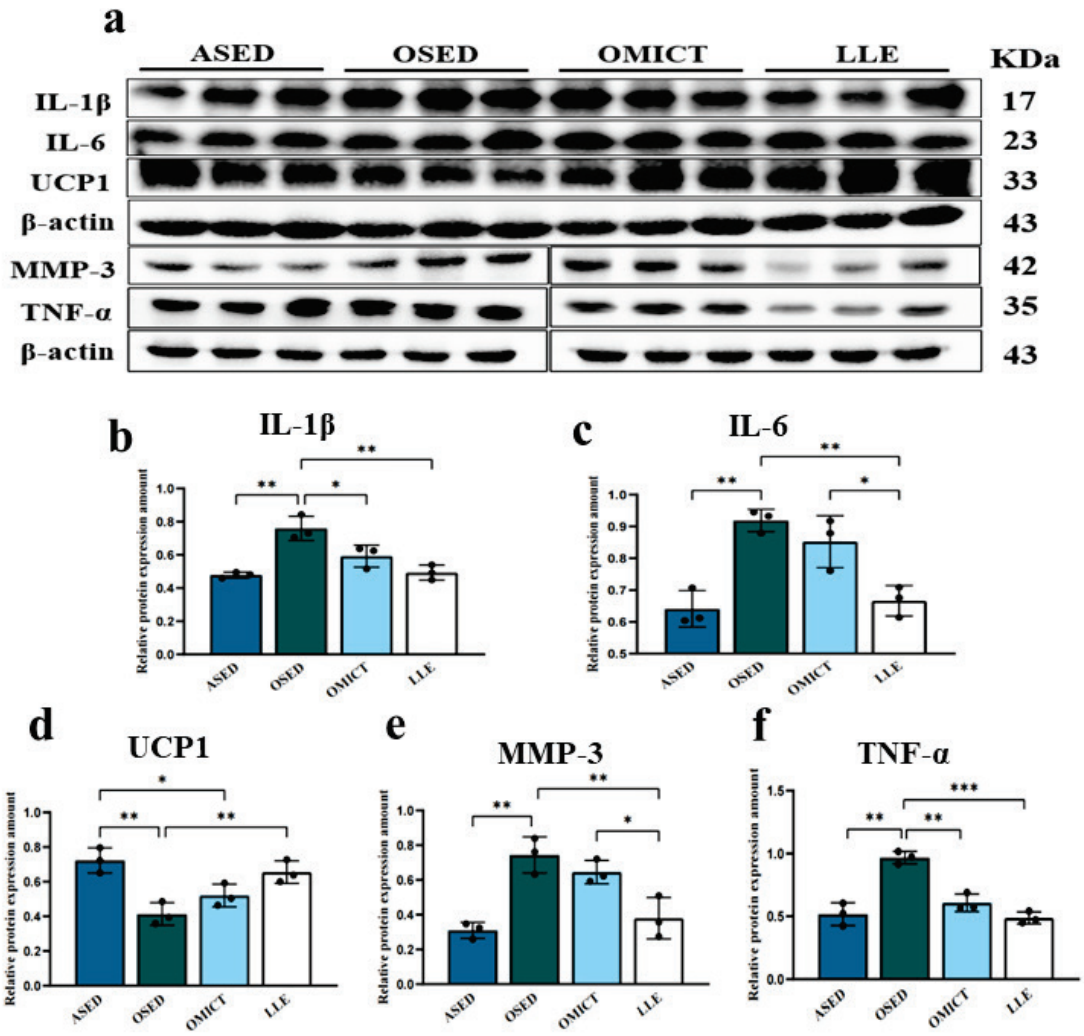

Fig. 3. Renal infammation-related proteins in each group of rats. *Note:* Western blotting bands of infammation-related proteins in renal tissue (**a**); Interleukin—1β, IL-1β in renal tissue (**b**); Interleukin-6, IL-6 in renal tissue (**c**); uncoupling protein 1, UCP1 in renal tissue (**d**); Matrix metalloproteinases-3, MMP-3 in renal tissue (**e**); Tumor Necrosis Factor-α, TNF-α in renal tissue (**f**). ASED: Adult sedentary; OSED: Old sedentary; OMICT: Old moderate-intensity continuous training; LLE: Life-long exercise. All data were analyzed by one-way ANOVA followed by Fisher's LSD post-hoc test. All data are presented as means±SEM. * indicates p<0.05; ** indicates p<0.01.

tly higher in the LLE group than in the OSED group. Moreover, compared with the OMICT group, SOD2 and SLC7a11 (P<0.05) protein levels were significantly increased in the LLE group. Compared with the OSED group, SOD1, and SOD2 (P<0.05) mRNA expression increased in the LLE group, whereas Sestrin1, Sestrin2, and Sestrin3 mRNA expression showed no significant differences.

Effects of life-long exercise on the NRF2/KAEP 1/Klotho pathway in kidneys

Immunofluorescence staining (Fig. 6a-d) showed that the OSED group exhibited weaker fluorescence for NRF2 (P<0.01)and Klotho (P<0.01) proteins and stronger fluorescence for KAEP 1 (P<0.01) proteins compared with the ASED group. Compared with the OSED group, the fluorescence for KAEP 1 (P<0.01) protein was weaker and that for NRF2 (P<0.01) and Klotho (P<0.05) proteins were stronger in the LLE and OMICT groups.

The altered NRF2/KAEP 1/Klotho pathway protein expression (Fig. 6e-h) in renal tissues suggests that, NRF2 (P<0.01) and Klotho (P<0.01) protein expression were significantly lower, while KAEP 1 (P<0.01) protein expression was higher in the OSED group than in the ASED group. KAEP 1 (P<0.05) protein levels were significantly lower, while NRF2 (P<0.05) and Klotho (P<0.05) protein expression was higher in the LLE groups than in the OSED group, and compared to the OMICT group, the LLE group had higher levels of Klotho protein (P<0.05).

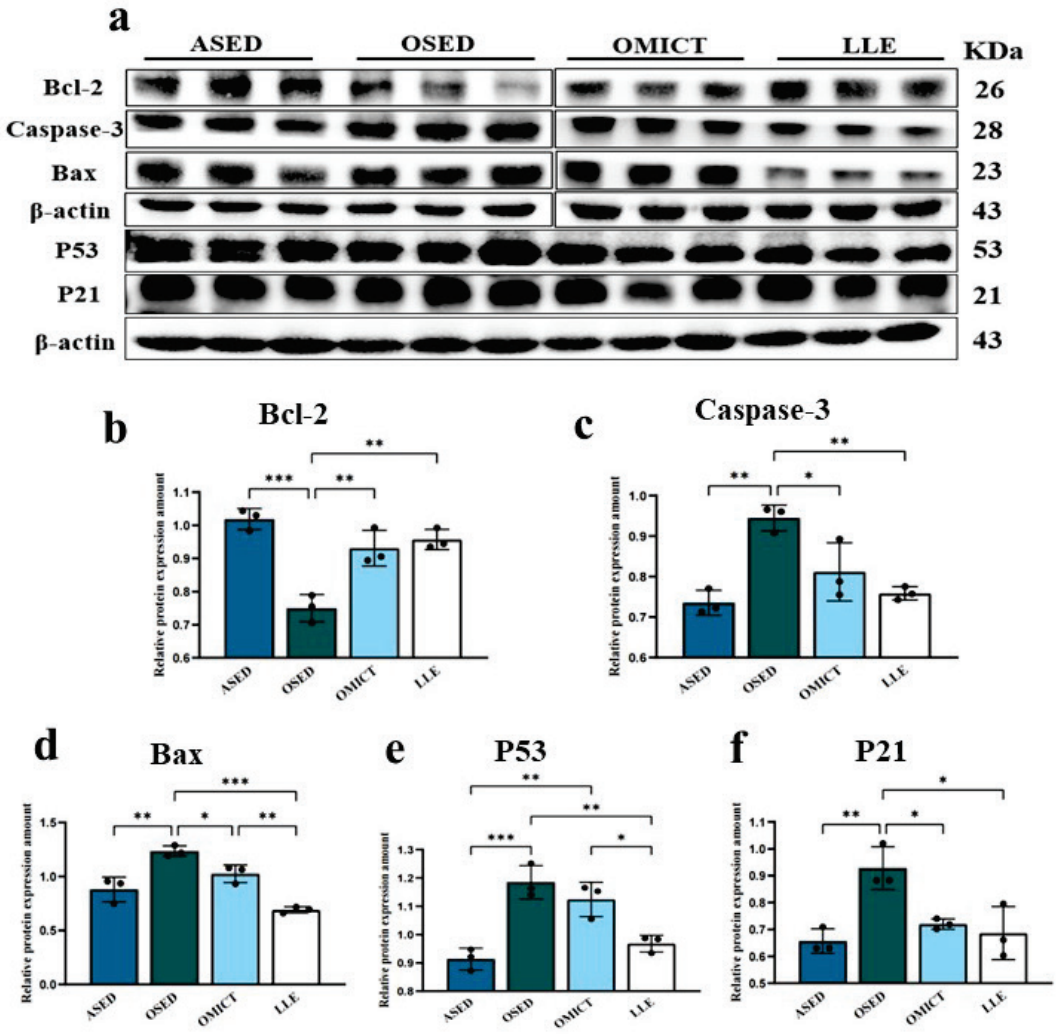

Fig. 4. Renal apoptosis-related proteins in each group of rats. *Note:* Western blotting bands of apoptosis-related proteins in renal tissue (**a**); B-cell lymphoma-2, Bcl-2 in renal tissue (**b**); Cysteinyl aspartate-specific proteinase-3, Caspase-3 in renal tissue (**c**); BCL2-Associated X, Bax in renal tissue (**d**); P53 in renal tissue (**e**); P21 in renal tissue (**f**). ASED: Adult sedentary; OSED: Old sedentary; OMICT: Old moderate-intensity continuous training; LLE: Life-long exercise. All data were analyzed by one-way ANOVA followed by Fisher's LSD post-hoc test. All data are presented as means±SEM. * indicates p<0.05; ** indicates p<0.01.

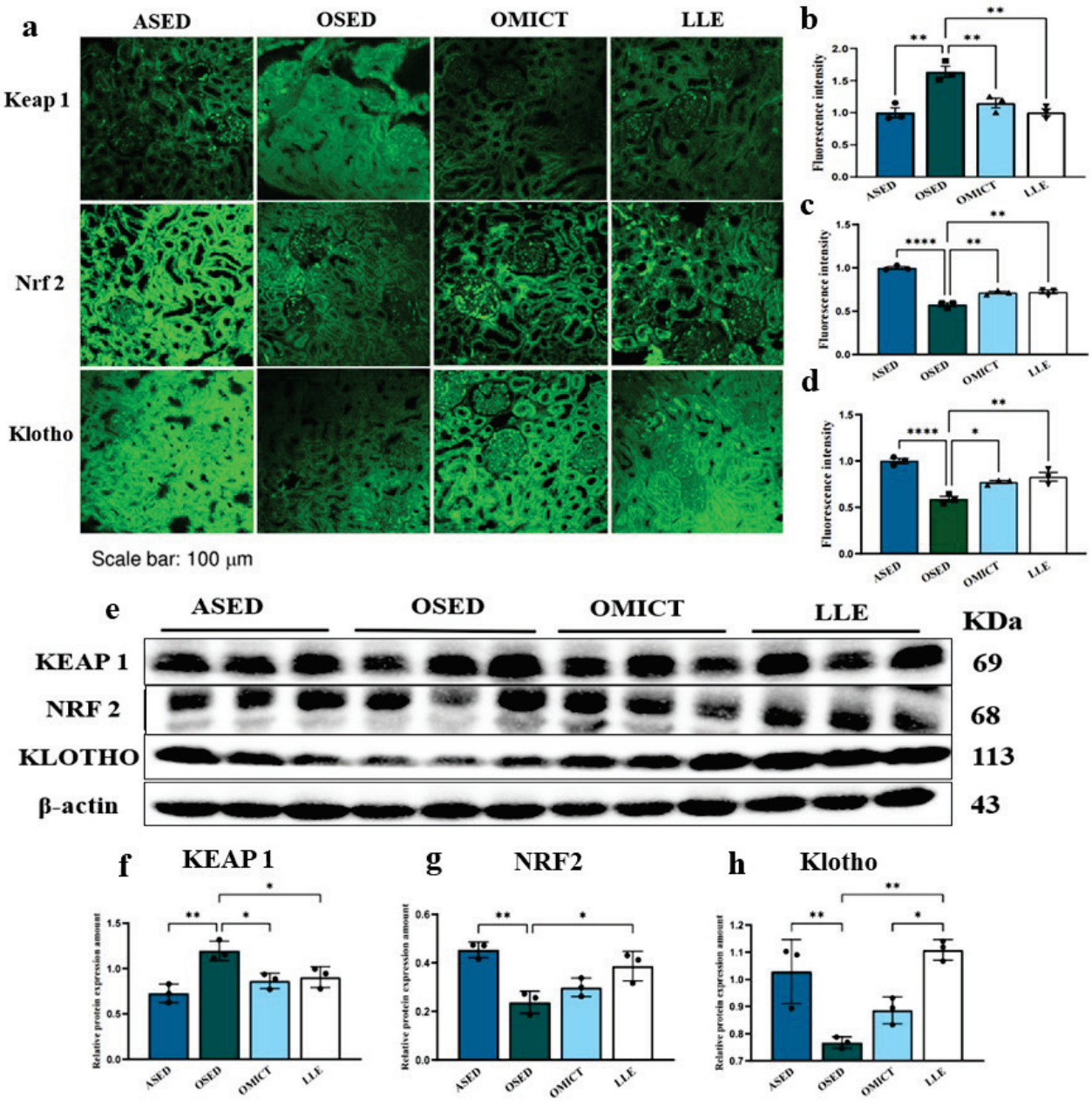

Fig. 5. Altered expression of renal NRF2/KAEP 1/Klotho pathway markers. Representative renal sections of rat in (**a**) stained by immunofluorescence for KAEP 1, NRF2, and Klotho. (**b**) Quantitation of renal fluorescence intensity of KAEP 1 in (a). (**c**) Quantitation of renal fluorescence intensity of NRF2 in (a). (**d**) Quantitation of renal fluorescence intensity of Klotho in (a). Western blot images of KAEP 1, NRF2, and Klotho (**e**); kelch-like ECH-associated protein-1, KAEP 1 in renal tissue (**f**); Nuclear factor erythroid2-related factor 2, NRF2 in renal tissue (**g**); Klotho in renal tissue (**h**). ASED: Adult sedentary; OSED: Old sedentary; OMICT: Old moderate-intensity continuous training; LLE: Life-long exercise. All data were analyzed by one-way ANOVA followed by Fisher's LSD post-hoc test. All data are presented as means±SEM. * indicates $p<0.05$; ** indicates $p<0.01$.

Discussion

Aging is an inevitable process that is associated with the onset of multiple age-related diseases [2]. As the main organ for removing metabolic waste and stabilizing the internal environment, the kidney is characterized by high metabolism and high sensitivity to aging. Structural changes and functional decline of the kidney during aging may significantly contribute to the onset of chronic kidney diseases, such as vascular sclerosis, interstitial fibrosis, and decreased glomerular filtration rate [5,21]. In particular, several key mediators, such as chronic inflammation, oxidative stress, and apoptosis, play vital roles [1]. In clinical practice, the treatment of renal diseases in the elderly is limited and the results are unsatisfactory. Therefore, it is necessary to identify non-

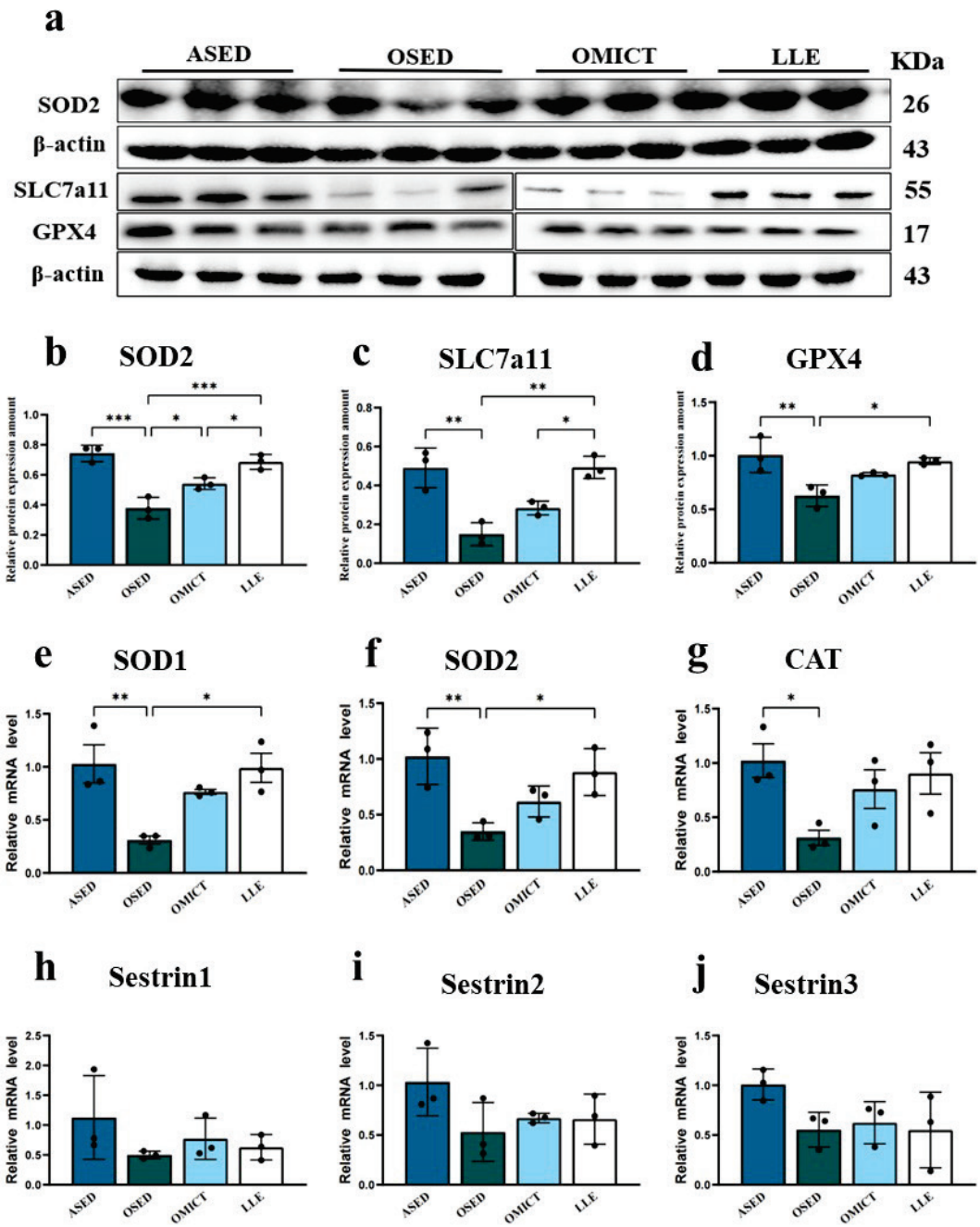

Fig. 6. Renal ferroptosis-related proteins and oxidative stress-related mRNAs and proteins in each group of rats. Altered expression of renal NRF2/KAEP 1/Klotho pathway markers. *Note:* Western blotting bands of oxidative stress and ferroptosis-related proteins in renal tissue (**a**); SuperoxideDismutase2, SOD2 in renal tissue (**b**); Recombinant Solute Carrier Family 7, Member 11, SLC7a11 in renal tissue (**c**); glutathione peroxidase 4, GPX4 in renal tissue (**d**); SOD1 mRNA expression (**e**); SOD2 mRNA expression (**f**); CAT mRNA expression (**g**); Sestrin1 mRNA expression (**h**); Sestrin2 mRNA expression (**i**); Sestrin3 mRNA expression (**j**). ASED: Adult sedentary; OSED: Old sedentary; OMICT: Old moderate-intensity continuous training; LLE: Life-long exercise. All data were analyzed by one-way ANOVA followed by Fisher's LSD post-hoc test. All data are presented as means ± SEM. * indicates $p<0.05$; ** indicates $p<0.01$.

pharmacological intervention modalities. In recent years, aerobic exercise has been extensively investigated as a non-pharmacological intervention in organ aging and may be an effective option to slow down renal aging [22]. Increased β-galactosidase activity increased fibrotic collagen deposition, upregulated expression of IL-1β, IL-18, α-SMA, and the aging-associated DNA double-strand breakmarker γH2AX protein were found to characterize renal cell aging [23]. In present research, as the exercise intervention cycle takes long time, to meet the demand of long-term experiments, we used Sprague Dawley rats as the experimental animal model, and the genetic background of rats is relatively stable, with small inter-individual differences, which are easy to be dome-

sticcated and to operate. Experimental results showed that rats in OSED group had obvious interstitial fibrosis of renal tissue, widening of the renal capsule cavity, obvious wrinkling, reduction in the number of glomeruli, and disorderly arrangement. However, repair of oxidative damage and narrowing of the renal capsule cavity were observed in all groups after the exercise intervention. The degree of collagen revealed by Masson's staining in the kidney cells of the LLE group was significantly lower than that of the OMICT group. However, it remains unclear whether LLE is superior to conventional regular moderate-intensity continuous training in delaying renal cellular senescence.

During aging, the body gradually develops a chronic low-grade systemic inflammatory response [24]. IL-1β and TNF-α induce secretion of downstream pro-inflammatory factors, whereas IL-10 counteracts immune cell activation and attenuates the inflammatory response [25]. High-intensity interval training (HIIT) was found to reduce serum inflammatory markers and IL-6 and upregulate serum IL-10 in aged rats [26]. We detected alterations in IL-6, IL-1β, TNF-α, MMP-3, and UCP1 protein expression in the renal tissue of aging rats after 18 months of LLE intervention. Although both exercise modalities tended to inhibit inflammatory factor protein expression in renal tissues, LLE had a higher inhibitory effect on the aforementioned inflammatory factor proteins than the OMICT group, especially IL-6, and MMP-3, two aging-associated secretory phenotype (SASP) pro-inflammatory factors. These results suggest that life-long exercise may be more effectively in reducing the secretion of inflammatory factors and subsequently slowing down the development of inflammatory infiltrates and aging kidney tissues compared to no exercise or short-term exercise.

Besides, oxidative stress levels are elevated, free radicals accumulate in the kidneys of aging organisms. SOD and CAT play essential roles in controlling oxidative stress and intercellular redox signaling [27]. In addition, stress activates the Sestrin protein, which reduces cellular damage caused by stressors. Studies have shown that the inhibition of the Sestrin protein in Drosophila leads to the accumulation of damage caused by oxidative stress [28]. The results of this experiment showed that the expression of SOD1, SOD2, CAT mRNA, and SOD2 protein was lower in the OSED group than in the ASED group, and the mRNA expression of Sestrin1, Sestrin2, and Sestrin3 showed a decreasing trend. This indicates that the balance between free radical generation and the antioxidant enzyme system is disrupted during aging, and that the kidney tends to age. After exercise intervention, renal SOD1 and SOD2 mRNA levels were significantly higher in the LLE group than in the OSED group, and SOD2 protein expression was significantly higher than that in the OMICT group. The expression of CAT followed a similar trend, but the differences were not significant. These results indicate that LLE to some extent improves the activity of antioxidant enzymes and the antioxidant capacity of aging kidneys more significantly compared to regular exercise, but the specific mechanisms require further investigations.

Related studies have shown that increased secretion of inflammatory factors and imbalance of oxidative stress lead to the accumulation of oxidants, which in turn causes cellular damage, initiating apoptosis, which further aggravates oxidative stress damage and inflammatory factor secretion, forming a vicious cycle [29]. Protein expression levels of Bax, Bcl-2, and Caspase-3 are commonly used to study the extent of apoptosis. Bcl-2 promotes cell survival by regulating the expression of cytochrome c and apoptosis-inducing factors, whereas upregulation of Bax induces apoptosis through the activation and release of caspases [30]. Caspase-3 is the most important apoptosis-inducing protease. Twelve weeks of aerobic exercise significantly reduces renal apoptosis and conferred protective effect on aging rat kidneys [31]. The results of this experiment suggest that the protein expression levels of Bax, Caspase-3, p21, and p53 were significantly higher, while that of Bcl-2 was significantly lower in the kidneys of OSED rats. The protein levels seem to indicate a greater resistance of senescent cells. After two different cycles of aerobic exercise, the expression levels of Bax and Caspase-3 proteins in the kidneys of rats in the OMICT and LLE groups were significantly decreased, whereas the expression level of Bcl-2 was increased. Indeed, Bax and p53 protein levels were more suppressed in the LLE group compared with the OMICT group, but there was no difference in p21, a downstream effector of p53. One idea to delay cellular senescence is to induce apoptosis by administering apoptosis to senescent cells so that they undergo apoptosis and are removed from the organism. These data suggest that LLE can indeed regulate the expression levels of apoptosis-related proteins such as Bax, Bcl-2 and Caspase-3 more significantly and have an effect on apoptosis, but whether apoptosis induction can be achieved still needs to be further explored.

Next, we analyzed the rat kidney KAEP 1/NRF2/Klotho pathway and ferroptosis. Ferroptosis is a newly identified mode of cell death caused by the accumulation of lipid peroxidation owing to iron overload. This process induces the proliferation and differentiation of renal interstitial fibroblasts, leading to renal fibrosis and promoting the development of CKD [12]. SLC7A11 promotes the synthesis of glutathione (GSH). GPX4 is the only enzyme of the GPX family identified so far that can reduce peroxides in lipid membranes and GSH is an essential cofactor in its activation. A growing number of studies have shown that NRF2 is a major regulator of antioxidant enzymes and cellular stress resistance, such as the regulation of redox homeostasis, energy metabolism, ferroptosis, proliferation, apoptosis, inflammatory response and mitochondrial physiology [32]. We found that GPX4, NRF2 and SLC7A11 protein expression was significantly downregulated in the OSED group, while KAEP 1 protein expression was upregulated. This suggests that aging leads to decreased GPX4 activity, increased cytoplasmic and lipid ROS levels, and increased inflammatory responses, ultimately leading to cellular ferritin deposition and ferroptosis. As an old partner of the classical activation mechanism of NRF2, KAEP 1, the pathway formed with NRF2, exists as an antioxidant defense mechanism in a wide range of tissues. Exposure to ROS and other environments modifies specific cysteine residues in KEAP1, interferes with the ubiquitylation of NRF2, and induces a series of cellular protective genes to be expressed, such as NQO1, GST, and GSH, etc., and the activation of this pathway also inhibits ferroptosis [33]. Furthermore, the NRF2 signaling pathway plays a key role in oxidative stress mediating the beneficial effects of exercise. Intermittent increases in oxidative stress induced through exercise episodes stimulate NRF2 activation and, when applied repeatedly, upregulate endogenous antioxidant defenses [32]. For example, exercise prevents cardiac and hepatic damage in rats by inhibiting apoptosis and activating the NRF2-Keap-1 pathway [34]. The present study demonstrated that whereas sustained training exercise at moderate intensity increased antioxidant capacity and inhibited ferritin deposition in aging kidneys through activation of the KAEP 1/NRF2 pathway, there was no difference between OMICT and LLE in this process.

The Klotho protein, which is highly expressed in the kidney, is involved in various biological activities, including calcium and phosphorus homeostasis, inhibition of inflammation, and inhibition of oxidative stress in addition to anti-apoptotic and anti-aging effects [35]. The klotho knockout mice also exhibit aging-related features such as reduced activity, gait disturbance, sarcopenia, and cognitive deficits; in contrast, genetic up-regulation of klotho extends the lifespan of wild-type mice and reverses the decline in physical function associated with aging [36]. Moreover, recent studies have shown that Klotho exerts cardioprotective effects by synergistically activating the NRF2 pathway and Brg1, thereby promoting HO-1 induction and protecting against AngII-mediated aortic smooth muscle cell apoptosis and senescence by activating NRF2 [37-38]. Activation of NRF2 by Klotho appears to be an important factor in the protection against kidney and aging-related diseases. Moreover, a growing number of studies have shown that exercise has beneficial effects on the alleviation of chronic inflammation associated with CKD, renal failure or aging, etc., and that its mediated activation of the classical wnt pathway may concomitantly cause an increase in the level of klotho, whereas exercise-mediated enhancement of the activity of the NRF2 pathway, etc., in turn, may potentially underlie an improvement in the biomarkers of inflammation [39-40]. Therefore, we speculate whether Klotho may improve renal senescence by synergistically activating the NRF2/KAEP 1 pathway mediated by exercise. In this regard, our results indicated that protein expression of Klotho showed a trend consistent with that of NRF2; Interestingly, the timing of exercise training initiation did not influence NRF2/KEAP1 expression in the kidney at old age. However, the level of Klotho protein expression after LLE was significantly higher than that after late moderate intensity exercise intervention. Therefore, we hypothesize that LLE may ameliorate renal aging by regulating Klotho and thus synergistically activating the NRF2/KAEP 1 pathway, but further studies are needed.

Here's another aspect worth paying attention to, the gender differences in aging. Traditional Chinese Medicine (TCM) considers kidney deficiency to be the root of aging [41]. Women are more likely to show it in haggard complexion, easy fatigue and memory loss, while men are more likely to show it in hair loss, movement disorders, memory loss, muscle atrophy and loss of libido [41]. Studies have shown that sarcopenia is more prevalent in elderly women, but progresses more severely in elderly men, which may be related to lower testosterone levels in women and a higher decline in testosterone levels in elderly men, which promotes

muscle mass and strength [42-45]. Studies also found that aging-induced severe reductions in the ability of knee muscle groups to perform maximal and rapid forceful contractions are more pronounced in males; and that bone metabolism in male rats enters a low-transition state earlier than in females [46-47]. In general, either female or male subjects are affected by hormones in basic research.

In conclusion, we demonstrated that both exercise regimens ameliorated senescence-induced oxidative stress, inflammatory response, apoptosis, and ferritin deposition in renal tissues to a certain extent, and that the improvement was more pronounced in LLE than in OMICT. This may be related to the fact that LLE synergistically activates the NRF2/KAEP 1 pathway through modulation of Klotho, which may be a key regulatory protein mediating the effects of exercise in ameliorating renal senescence. However, further studies are needed to confirm these effects and elucidate the underlying physiological mechanisms.

Limitations

First, only female rats were included in this study; exercise-induced protein adaptation may vary according to the sex. Second, no statements regarding body composition data were made in this study. Third, functional markers such as proteinuria/ albuminuria were not performed due to sample retention issues. Fourth, this study did not use fluorescent probes to detect intracellular Fe^{2+} ion distribution and concentration, and the relevant molecular mechanisms and control comparisons could not be directly obtained because the intervention was not performed directly at the molecular level using techniques such as gene knockout.

Author contribution

The study was conceived and designed by Fang-Hui Li and Xiao-Ming Yu. The literature retrieval, information collection, analysis, and writing of the first draft of this study were done by Xi-Kun Yuan and Pin-Shi Ni. The review and revision of the manuscript are completed by Zhi-Yu, Zhuang-Zhi Wang, Zhen-Hao Yan, and Chen-Kai Zhang. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

The rats selected for this experiment were trained and euthanized in compliance with the Animal Management Regulations of the Ministry of Health of China. This study was approved by the Ethics Committee of the Animal Experimentation Center of Nanjing Normal University (approval number: IACUU-1903006).

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