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Icariin, a natural flavonol glycoside, extends healthspan in mice

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Abstract

A major goal of aging research now is to find pharmacological manipulations in healthspan extension. Icariin is a flavonol isolated from medicinal herbal tonics. We have previously reported that icariin extended the healthspan of invertebrate models. Here, we showed that long-term treatment with icariin starting at 12 months of age extended healthspan and mean lifespan in C57BL/6 mice. In all our assays associated with heathspan, such as behavioral tests and bone density analysis, we found that icariin boosted healthy features in mice. We also presented data indicating that such beneficial effects of icariin were due to at least two mechanisms: reduced oxidative stress indicated by the induction of antioxidant proteins superoxide dismutase (SOD) activity and the decrease of oxidative marker malondialdehyde (MDA); maintained the genomic stability indicated by a reduction in DNA double-stranded breaks and down-regulation of DNA damage response genes. Our results indicated that icariin, a safe and widely used natural flavonol, extended healthspan and maintained genomic stability in a mammalian system.

Introduction

Over the last 2 decades, our understanding of the molecular processes of aging has improved greatly. Studies with model systems like *Caenorhabditis elegans* and *Drosophila melanogaster* have revealed the major signal pathways regulating lifespan [1]. Links between aging and physiological processes, such as oxidative damage, have also been well documented [2-4]. A major goal of aging research now is to translate our understanding of aging mechanisms into a wholesome improvement and disease free healthspan of individuals. An ideal anti-aging regent should provide the benefits of reduced age-related disease while maintaining and improving major functions of life [5, 6].

Icariin is a flavonol glycoside isolated from several species of plants in the genus *Epimedium*, which are commonly used as medicinal herbal tonics in treating aging-related diseases and boost male sexual function in Asian countries [7]. As the primary active component of *Epimedium* extracts and a natural flavonol, icariin's effects and related mechanisms have been studied extensively. It shows wide profile of health benefits including antioxidant, antidepressant, anti cancer, neuroprotective effects in animal studies, as well as stimulating osteoblast activity in bone tissue [7-10]. In consistence, our previous studies showed that the total *Epimedium* Flavonoids (EF), which contains icariin as the major component, delayed aging in *D. melanogaster* and *C. elegans* [11, 12]. We also reported that icariin and its metabolic derivative icariside II extended the healthspan in *C. elegans* [13]. Therefore, we tested whether icariin had similar effects in a mammalian system in the present study.

DNA molecules containing genetic information are critical in maintaining life. Recent studies suggested that genomic instability induced by DNA damage plays an important role in aging process[14-16]. DNA damage can be caused by spontaneous hydrolysis, different physical (UV, X-rays) or numerous chemical agents, including reactive oxygen species (ROS) produced by cellular metabolism. Under normal conditions, ROS are maintained at the physiological levels by several endogenous antioxidant systems. Physiological levels of ROS interact with redox state and play a role in mediating cell signaling, while pathological levels of ROS accumulated with aging can damage all components of the cell, including proteins, lipids, and DNA [17, 18]. Among the DNA lesions, double-stranded breaks (DSBs) in DNA are major threats to genome integrity [19]. So far, phosphorylated histone H2AX(γ –H2AX) has been demonstrated to be the most important DNA damage sensor molecule, and the presence of γ –H2AX foci in cells has been considered to reflect sites of DNA double-strand breaks[20, 21]. Flavonoids have received considerable attention for their anti-oxidative activity [22], and previous research has reported that icariin protects DNA against radical-induced oxidative damage [23]. These findings prompted us to further investigate whether icariin could maintain genome stability to extend the healthspan.

In our study, Cohorts of 12-month old C57BL/6 mice were provided with standard diet, standard diet plus 0.02% icariin or other intervention for the remainder of their lives. Our data showed chronic exposure of icariin extended healthspan and maintained genomic stability in mice. In sum, our study raised the possibility of a safe and natural flavonoid to promote healthy aging.

Experimental Procedures

Diet preparation

The diets employed in our study were as follows: the standard mouse diet, the standard mouse diet plus 0.06% EF and the standard mouse diet plus 0.02% icariin. The standard mouse diet (Pharmaceutical Biological Engineering Co., Ltd, Jiangsu China) for laboratory mice were made in accordance with the National Standard of the PRC (GB 14924.3-2001). EF and icariin were kind gifts from the laboratory of Weidong Zhang at the second Army Medicine University. The purity of icariin was 98% (HPLC), and EF contained 20% icariin, as determined by HPLC. EF or icariin was homogenized into the chow to create each diet, repectively.(Pharmaceutical Biological Engineering Co., Ltd, Jiangsu China).

Animals

All performance on mice was approved by Animal Care and Use Committee of Fudan University, permit number SCXK (Hu) 2010-0016 and in accordance with the guidelines for animal use of National Institutes of Health (NIH publication no. 86–23, revised 1985).

Eight to nine-week-old male C57BL/6 mice were purchased from Vital River Laboratories (Beijing, China). Throughout the study, all mice were housed in a suite of specific-pathogen free (SPF) room under identical environmental condition (12:12 hour light:dark cycle, 22～24℃,40～70% humidity) in the animal laboratory of

Shanghai Public Health Center. Sentinel mice were tested by the Shanghai Laboratory Animal Research Center quarterly to show the facility remained SPF throughout the period of the study. C57BL/6 mice have an easily irritable temperament and have a tendency to bite. Group-housed C57BL/6 mice display barbering behavior. So the mice in our study were housed individually in plastic laboratory mice cages with metal tops (L320*W202*H135 mm), using 1/4-inch corn-cob bedding (Pharmaceutical Biological Engineering Co., Ltd, Jiangsu China) after one week acclimation. Cages were arranged closely in lines. Mice were transferred to fresh cages every 14 days. All mice were given ad libitum access to water and the standard mouse diet until they were grouped when they were 12-month old. Water was sterile and acidified by addition of hydrochloric acid (pH 2.5-2.7).

Mice used in Life Span measurement

When the mice were 12-month old, 197 healthy mice were divided randomly into four groups: Control group (n=52), CR group (n=47), EF group(n=49), Icariin group(n=49). The mice in the control group received ad libitum access to the standard mice diet; the mice in the CR group received 60% of the control group's consumption; the mice in the EF group received ad libitum the standard mice diet plus 0.06% EF; and the mice in the icariin group received ad libitum the standard diet plus 0.02% icariin. The mice were free to the water access. Food intake and body weight were measured on a monthly basis for the duration the study. From the daily eaten lab chow per ad libitum fed control mouse, the CR fed mice received 60% once a day at 08:00[24]. Daily inspection was executed by experienced technicians at about 8:30 am and 16:30

pm every day during the study. The moments when the mice lost the vital signs (including temperature, heart rate and respiration) were recorded. The mice found dead were underwent gross necropsy immediately in the animal anatomy room. Neoplastic lesions at necropsy were fixed in 4% formalin and stained with hematoxylin and eosin (H&E) for histological examination. Survival curves were plotted using the Kaplan-Meier method, which included all available animals at each time point.

Mice used in Healthspan measurements

To investigate the effects of icariin on the life quality and to further explore its mechanisms, another 49 12-month-old healthy male C57BL/6 mice were divided into four groups as above: Control group (n=11), CR group (n=13), EF group (n=13), Icariin group (n=12). The treatments of the mice were the same as the above. Weight and intake of food and water were monitored monthly. The mice were housed in metabolic cages to determine output of faeces and urine monthly. When the mice were 24 months old, the number of each group was as follows: Control group, n=9; CR group, n=8; EF group, n=9; Icariin group, n=11. They were engaged into the behavioral tests including Morris water maze (MWM) and rotarod, and the 3-month-old healthy male C57/BL6 mice (n=10) were used as young control. The day after the above tests, all the mice were sacrificed and sampled for the following experiments including femur bone mineral density (BMD) measurement, histopathological observation, detection of γ–H2AX expression in tissues and the microarray analysis.

Morris water maze

The Morris Water Maze is a well accepted assay to assess spatial learning and memory capacities of rodents. The mice's cognitive function was assessed using the Morris water maze (MWM) as previously reported [25, 26]. The watermaze consisted of a large circular pool (1.5 m diameter), containing water at around 23±1℃. A platform located 2cm below the surface of the water in the southeast quadrant. A video camera was placed above the centre of the pool to capture images of the swimming mice, and this connected to a video, and a computer system running specialized tracing software. The training procedure included spatial training and probe trial. In spatial training the mice were released facing the wall from quasi-random locations along the edge of the pool (north,south,east or west). Mice were allowed 60 s to locate the hidden platform. After climbing onto the hidden platform, mice remained on the platform for 15 s. Unsuccessful attempts to locate the platform within 60 s resulted in the experimenter guiding the mice to the hidden platform and permitting 15 s of refuge on the platform. The tracking system measured the escape latency across trials, and parameters such as path-length, swim-speed, directionality in relation to platform location, and so on. To examine spatial reference memory, a probe test was administered 24 h after the 5-day spatial training. During the probe test, the platform was removed from the pool and the mouse was allowed to swim freely for 60 sec. Latency time (time to find platform, maximum 60 s) was record as measures of spatial learning. And platform cross, distance and time searching in the target quadrant where platform was absent were measures of reference memory in the probe trial. .

Rotarod experiment

Rotarod test is a performance test that evaluates balance, grip strength and motor coordination of the subjects usually a rodent. The parameter of a rotarod test is the duration time that the animal stays on the rotating rod at the accelerating rotarod. In brief, the animals were placed on the roller lane of the rotarod and the time was started. When the animal drop safely into its own lane, the time latency to fall and rotation speed were automatically recorded. The mice were given a habituation trial on day 1 where they were placed on the rotarod at a constant speed (4 rpm) for 60 s. The following day each mouse was given three trials during which the rotarod started at 4 rpm and accelerated to 40 rpm over a period of five min. The maximum trial length was 5 min and there was a 30 min rest period between each trial.

Measurement of femur BMD

Right femurs were dissected, and adherent soft tissues were removed. Subsequently, the BMD of the femur bone specimens was measured by a trained and certified radiologic technologist using dual-energy X-ray absouptiometry (Hologic Discovery densitometer, Hologic, Inc, Bedford, MA). The results were expressed in grams per square centimeter.

Histopathological observations

The heart, liver, spleen, lung, kidney, and brain from the mice in the healthspan subgroup were fixed and embedded. Tissue microarrays were made accordance with the experimental design. Two tissue cores (1 mm in diameter each) were obtained from each tissue sample. The tissue cores were precisely arrayed into a new paraffin block using a tissue microarray workstation (Beecher Instruments, Sliver Spring, MD) as previously described [27]. Briefly, the instrument consisted of thin-walled stainless steel needles with an inner diameter of approximately 600 μm and a stylet used to transfer and empty the needle contents. The tissue microarray was stained with hematoxylin and eosin (H&E) for histological examination.

Detection of γ-H2AX expression in tissues

The tissue microarray containing heart, liver, spleen, lung, kidney, and brain tissues was stained by immunohistochemical method for detection of γ-H2AX. Briefly, tissue microarray slides were mounted on charged polylysine-coated slides. Sections were deparaffinzed in xylene and rehydrated through a graded alcohol series to distilled water. The slides were subjected to antigen retrieval by microwave irradiation in 10 mx citrate buffer, pH 6.0, in a 750-W oven for 30 min, at an estimated temperature of 95–97° C. The slides were then cooled to room temperature and washed in PBS. Endogenous peroxidase activity was blocked by incubation of the slides in hydrogen peroxide and methanol. Anti-γ-H2AX antibodies (rabbit polyclonal to gamma H2A.X, phospho-Serine 139; ab2893, Abcam) were applied overnight at

4°C at a 1:200 dilution in an immunostainer. Negative control sections were treated identically, but with the primary antibodies omitted. The antibody was detected by standard indirect immunoperoxidase procedures (LSAB, DAKO). Diaminobenzidine was used as a chromogen, and light hematoxylin was used as a counterstain. Lesions that demonstrated the presence of nuclear staining were considered to be positive for γ-H2AX expression. Immunoreactivity was quantified by counting the percentage of immunopositive cells based on nuclear staining. Cytoplasmic staining in the absence of nuclear staining was not considered immunopositive. The expression of γ-H2AX was determined by 2 investigators.

SOD and MDA detection

Liver tissues were weighed, and homogenized in Tris-HCl (5 mmol/L containing 2 mmol/L EDTA, pH 7.4). Homogenates were centrifuged (1000 r/min, 10 min), and the supernatants were used immediately for measurement of SOD and MDA according to the manufacturer's instructions (Nanking Jiancheng Bio-engineering Research Institute, Nanking, China).

Microarray analysis and real time PCR

The Oligo GEArray microarray experiment was carried out by the Shanghai Kangchen Biotechnology Company. In brief, total RNA was isolated from liver tissues of 24-month-old mice (n=4 in each group) with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cRNA was synthesized, labeled, and purified using a TrueLabeling-ATMTM Linear RNA

amplification kit and a SuperArray ArrayGrade cRNA purification kit (SuperArray Inc., USA). A SuperArray (Oligo GEArray mouse DNA Damage Signaling Pathway Microarray) was used following the manufacturer's protocol to compare the gene expression profiles of the groups. The SuperArray contained 114 functionally well-characterized genes associated with the ATR/ATM signaling network, including transcriptional targets of the DNA damage response (cell cycle arrest, apoptosis, and DNA repair). Controls consisted of 4 normalizable genes (glyceraldehyde-3-phosphate dehydrogenase, beta-2-microglobulin, heat-shock protein 90-a, and b-actin) and a blank. Chemiluminescence detection was carried out using a chemiluminescence detection kit (SuperArray Inc.). Chemiluminescence microarray images were obtained from X-ray film using a desktop scanner. Integrated microarray data were analyzed using the GEArray Expression Analysis Suite (SuperArray Inc.). Changes in gene expression detected in the microarray were validated by Real-time PCR. Real-time PCR was performed accordance with previous report [28].

Statistics

Kaplan-Meier survival curves were compared. The survival curves of compounds treatments groups and the control were crossed at the last points which don't meet the requirement for a consistent hazard ratio of Long-rank test. Thus we employed the Gehan-Breslow-Wilcoxon test between the curves. Comparisons between control and treatments groups were performed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test; comparison between old and

young group was performed using student's t test. Analyses were performed using GraphPad Prism 5.04 for Windows (GraphPad Software, San Diego, CA). A two-tailed value of $P<0.05$ were considered significant.

Results

1. Icarrin extended the mean lifespan of C57BL/6 mice.

The survival curves separated shortly after the onset of the compounds treatments (Fig. 1b). As shown in the Fig.2 and Table.1, icariin treatment extended 8% of mean lifespan(vs control: Chi square=4.3, p=0.03 in Gehen-breslow survival test). And EF treatment resulted in a 4% extension of mean lifespan (vs control: Chi square=2.7, p=0.09 in Gehen-breslow survival test). Although 4 mice in CR group were dead in the initiation, CR had the most remarkable lifespan extension compared to the others (Fig. 2c) and it extended 25% of mean lifespan.

To determine the statistic significance of the increases in maximal lifespan between control and treatments groups, we compared the proportion of living mice in each group after 90% had died of the control in the contingency table [29]. As shown in Table.1, 10% of the control mice were alive at these ages, compared with 43% of the CR mice (CR vs control: p=0.0002). Although the icariin and EF treatment groups showed a trend towards increased maximum lifespan (20% of the EF-treated mice, and 18% of the icariin-treated mice), they were not significantly different from the control group (EF vs control: p=0.1651; icariin vs control: p=0.2551).

When the animal was found dead during the daily inspection, a gross necropsy was executed immediately. Based on gross evaluation, histopathologic examination of neoplastic tissues was tested by an experienced veterinary pathologist. The details of the mice which presented tumors were shown in Table S1.

2. Icariin treatment improved physical performance of aging mice.

To investigate whether icariin affected the physical responses of mice, we monitored body weight, intake, and output of mice each month. Body weight and intake of food were monitored in all mice, and the intake of water, output of faeces and urine with metabolic cages were monitored in mice of healthspan subgroup. Change of body weight in each group was shown in Fig3a. Prior to the start of treatment, there was no significant weight difference between the groups (means ± SEM, control 38.4 ± 0.85, CR 38.4 ± 0.87, EF 37.4 ± 0.81, icariin 38.9 ± 0.89; P=0.63). During the initial months, body weights of EF and icariin-treated animals were no significant difference from the control mice. But from 18 to 24 months, mice on the EF and icariin diet were lighter than the control mice (means ± SEMs: control 39.3 ±0.35, EF 36.2 ±0.28, icariin 37.1 ± 0.67 g; $P < 0.01$ *vs* the control, pooled data). Mice in CR group lose weight significantly during the initial two months (from 38.4 ± 0.87 to 23.9 ±0.35 grams, mean ± SEM). Then mice in CR group maintained the body weight at 24.5±0.34 (mean ± SEM) grams from 15 to 32 months of age. Throughout the study, the body weights of mice in CR group were much lower than those of control or compounds-treated mice.

Although mice on icariin or EF diet were slightly lighter than control mice, they consumed more calories compared to the control (Fig. 3b). There were no differences in water consumption or excretory between icarrin-treatment group and control group (Fig. 3b). Water consumption and the excretory of CR group were significantly higher than their counterparts of control.

To determine whether icariin treatment improved the behavioral characteristics in later life, we examined the Morris water maze (MWM), rotarod and bone mineral density (BMD) in the mice of 24 months old.

MWM is a well-accepted and frequently used cognitive test in terms of both learning and memory. Improvements in spatial acquisition can be indicated by the escape latencies in hidden platform tests. The reference memory can be measured by the number of crossing platform, the time and the distance in the target quadrant in the probe test. During the 5-day acquisition training, young mice spent less latency time after two days training while the mice in interventions groups did not show significant differences compared to the old control (Fig.4a). In the probe test, mice in the icariin group and CR group both exhibited increased number of crossing platform, longer searching distances, and more time spent in the target quadrant compared to the control (Fig.4b-d).

To investigate the effects of icarrin on balance and motor coordination, we tested the time required for our experimental animals to fall from an accelerating rotarod (4-–40 rpm over 5 min). As shown in Fig.4e, the latency to fall from the rotarod in the 24-month-old control mice was significantly less than 3-month-old mice. This decline was attenuated in mice of all intervention groups, which exhibited much longer latency times compared with control mice of the same age.

Bone density loss from osteoporosis is a major cause of disability and death in the elderly [30]. As shown in Fig.4f, we found that the BMDs of old mice were significantly lower than those of the young mice. Treatment with icariin significantly increased BMD compared to the control (old). Notably, icariin treatment led to the same high level of BMD of 24-month-old mice as their 3-month-old young counterparts. The comparison of BMD between CR group and the control (old) group was not significantly different.

2. Icariin significantly decreased the expression of γ-H2AX.

To detect the expression of γ-H2AX in the major tissues of mice, we employed immunohistochemistry analysis of tissue microarrays. Representative images were shown in Fig. S2a-c. Labeling indices for γ-H2AX (LI) were determined as the number of positive nuclei in 100 cells in 3 randomly selected fields. We found that the mean LI values for γ-H2AX in the heart, liver, spleen, lung, kidney, and brain increased significantly in 24-month-old (control) mice compared with 3-month-old (young) mice (Fig.5a–f). The LI values in the liver, kidney, lung, and heart tissues of

icariin-treated mice were significantly reduced compared with those of the control mice (Fig.5a–d). There was no difference between the LI values of the spleen and brain tissues in icariin-treated mice and control mice (Fig.5e–f). Mice of CR group only lowered the LI values of liver and kidney (Fig.5a–b).

In order to investigate whether long-term administration of the compounds caused side effects, we did HE stain of the tissue microarray. There were no significant histopathological abnormalities of the heart, spleen, lung, and brain tissues between control and treated mice. Instead, icariin or EF treatment attenuated the aging-associated degeneration of the liver.

4. Icariin downregulated the expression of genes related to the DNA damage response.

Next, we employed a DNA damage signaling pathway microarray to determine whether icariin treatment maintained genome stability in transcriptional level. Stringent criteria for array analysis (≥ 2-fold change in expression) was used, and the differential expression of genes related to the DNA damage signaling pathway in each group was as shown in Fig.6a. Compared to 24-month-old mice (Old), 48 genes were differentially expressed in 3-month-old mice (Young); of these 48 genes, 5 were up-regulated, and 43 were down-regulated. Compared to the control group (Old), the expression levels of 29 genes in the DNA damage signaling pathway were altered upon icariin treatment; Of these 29 genes, 6 were up-regulated, and 23 were down-regulated. Similar patterns of gene expression were also observed in the

EF-treated group and CR-treated group.

The majority down regulated genes encodes mismatch repair, damage DNA binding, and base-excision repair proteins. Among the few genes that were up-regulated, we noticed that Ataxia telangiectasia mutated (ATM) and breast cancer type 1 susceptibility protein (BRCA1) were up-regulated in young group as well as the intervention groups. Results were confirmed by qPCR (Fig.6b, Fig.6c).

5. Icariin increased SOD activity and decreased MDA in the liver.

SOD and MDA levels were determined in the supernatants of liver tissue lysates from mice in each group. As shown in Fig.7a, SOD activity in the control group (Old) was significantly lower than that in the young group (Young: 3-month old mice). With icariin or EF treatment, SOD levels increased significantly compared to the control group (Old). Surprisingly, the level of SOD of 24-month-old mice exposed in icariin treatment was as high as the 3-month-old mice. SOD levels of mice in CR group did not show difference from the control (Old). In agreement with these data, MDA levels were significantly lower in icariin or EF group compared with the control group (Old) (Fig. 7b). Levels of MDA in CR group were also lower than the control group.

Discussion

In present study, we used male C57/BL6 mice as experimental model to test the effect of icariin on lifespan. We found that mice had improved survival after icariin feeding. icariin treatment extended 8% of mean lifespan(vs control: Chi square=4.3, p=0.03 in Gehen-breslow survival test). As far as the maximum lifespan is concerned, icariin prolonged the maximum life span of mice by 6%. However, when we compared the proportion of living mice in each group after 90 had died, it was not significantly different from the control group in statistic. Our previous studies on EF, which contains icariin as a major constituent, showed that EF delayed aging in *D. melanogaster* and *C. elegans* [11, 12]. In our present study, we found that EF extended 4% of mean lifespan in mice which were treated with the diet plus 0.06% EF. In our study, EF contained about 20% icariin, so the diet plus 0.06% EF contained approximately 0.012% icariin which was nearly half dose of the icariin group. It suggested icariin might be responsible for the effects of EF and the effects of icariin on lifespan might be dose-dependent. It was consistent with the previous studies that CR had positive effects on both mean and maximum lifespan. The extension size of the mean and maximum lifespan of the CR group were in the range as reported [31]. In our study, we also found that the major impact of CR on lifespan was in the late life (900-1200 days) compared to the control. However the major impact of icariin group on lifespan was in the middle life (500 to 700 days). The curves of control and icariin group met at the end point indicating that icariin might improve the health condition rather than slow the rate of aging.

Although life expectancy is an important indicator to measure whether interventions can slow aging, the goal of aging research is not just a simple extension of life, but to extend the healthy life. So we tested a set of parameters to assess whether quality of life was maintained by icariin treatment.

Cognitive impairment was one of major hazards of late life quality. The Morris water maze is one of the most widely used tasks in behavioral neuroscience for studying the psychological processes and neural mechanisms of spatial learning and memory. The neuroprotective effect of icariin has been extensively recognized[32]. Oral administration of icariin potently improved the ability of spatial learning and memory in Alzheimer's disease model rats[33], and attenuated D-galactose-induced rats' behavioral dysfunction and neurodegeneration[34]. In our study, we found that spatial learning and memory impairment with age was attenuated by icariin treatment.

Neuromuscular coordination decreases with age, which seriously affects the quality of life in old age[35] . The rotarod provides an easy way to test the effects of drugs, brain damage, or diseases on motor coordination or fatigue resistance in rodents. In our study, it showed that the time latency of 24-month old mice was much shorter than that of 3-month old mice, suggesting that the neuromuscular coordination decreased significantly with aging. However, icariin-fed mice improved their motor skills as they aged.

Bone density loss from osteoporosis is a major cause of disability and death in the elderly, mostly due to subsequent fractures, which seriously affect the health of the elderly population. The effects of icariin on the skeletal system have been extensively investigated. The potential of icariin to increase bone formation and reduce bone absorption made it a promising drug candidate which could enhance BMD value, improve biomechanical properties and bone microstructure and suppress increased bone turnover[32]. In our present study, it was found that the femur bone mineral density of 24-month-old mice decreased significantly as compared with that of 3-month-old mice; while icariin could significantly improve the femur bone mineral density of old mice, which suggested that icariin could attenuate the bone density loss with age.

. Altogether, these data indicated that icariin could improve age-related changes in mice such as impaired cognition, decreased neuromuscular coordination, and reduced bone mineral density, leading to prolonging the healthspan.

The free radical theory of aging is one of the most prominent theories to explain aging. Prolonged persistence of oxidative stress and inefficient antioxidant defense are considered as major factors influencing cellular aging. Although the free radical theory of aging is questioned recently[36], there is solid evidence supporting that oxidative damage may play an important role in health span [37]. Findings in laboratory mice clearly show that the progression and severity of many age-related diseases can be exacerbated or blunted by altering oxidative stress through modulation of the expression of antioxidant genes [37]. Antioxidant is a proven effect of icariin[38, 39], thus we measured the SOD and MDA. As expected, icariin treatment increased SOD

activity and decreased MDA, suggesting that reduced oxidative stress might be an important mechanism of icariin's effect in healthspan extension.

Although DNA is fairly stable, many sources of DNA alterations have an effect on its structure and integrity. DNA lesions can be caused by either endogenous (reactive oxygen species (ROS) resulting from metabolic processes) or exogenous (ionizing radiation (IR), UV) agents. Recent evidence suggests that genomic instability caused by DNA damage plays an important role in the aging process [40]. The accumulation of DNA damage is thought to contribute to the physiological decay associated with the aging process. Double-stranded breaks in DNA are major threats to genome integrity because they can result in chromosomal aberrations that can affect, simultaneously, many genes, and lead to cell malfunctioning and cell death. When a double strand break is introduced in the DNA the cell is signaling this by phosphorylating the histone protein H2AX [41]. Using immunohistochemistry, it is possible to visualize these breaks as large foci with antibodies specific for the phosphorylated from γ-H2AX [42]. In the present study we detected the γ–H2AX expression in tissues such as heart, liver, spleen, lung, kidney and brain of mice. And the results showed that γ–H2AX expressions in the heart, liver, spleen, lung, kidney, and brain increased significantly in old control group, compared with the young control group. However, icariin could significantly reduced the γ–H2AX expression in the liver、kidney、lung and heart tissues of old mice. These results suggested that DNA damage accumulation in old mice increased compared with young mice, but icariin could reduce the DNA damage accumulation with aging. The results were also

consistent with the microarray test which showed that the genes related to DNA damage response were up-regulated in the old control group mice, while icariin treatment could down-regulated the expression of DNA damage response related genes.

In response to genomic insults cells trigger a signal transduction pathway, known as DNA damage checkpoint, whose role is to help the cell to cope with the damage by coordinating cell cycle progression, DNA replication and DNA repair mechanisms[43]. The mammalian protein kinase ataxia telangiectasia mutated（ATM） is a key regulator of the DNA double-strand-break response. ATM deficiency cause ataxia telangiectasia (AT), a genetic disorder that is characterized by premature aging[44].The recent study show that ATM plays a role in mediating survival in the face of both spontaneous and induced DNA damage [45]. Our study found that the expression of ATM was lower in old control mice which was consistent with the previous study, which showed that aged, 22-month-old mice showed limited activation of ATM targets, though high numbers of cells with DNA damage foci were found [46]. Icariin, EF and CR intervention all could up-regulated the expression of ATM in old mice, which indicated that icariin, EF and CR treatment might enhance the sensitivity of the DNA repair system and reduced the DNA damage.

In the present study, we also found an interesting phenomenon that mice in the EF and icariin groups weighed less than the controls, but more than those in CR group. However they consumed more calories without increasing outputs compared to control group. These results suggested that the substance were perhaps not blocking uptake or absorption of nutrients, which could result in a CR-like response. Although DNA damage and oxidative stress all contribute to aging, metabolic dysfunction is a common hallmark of aging at least in invertebrates[47]. A common feature of aging-related disease is the involvement of metabolic systems in general, and the mitochondria in particular[48, 49]. Recently the beneficial effects of icariin on metabolic syndrome are recognized. Icariin could reduce the levels of serum total cholesterol and low-density lipoprotein cholesterol of rabbits fed with high-cholesterol diet[50], and ameliorate streptozocin-increased activities of lactate dehydrogenase, acid phosphatase, gamma-glutamytranspeptidase and alpha-glucosidase in the epididymis and reduce serum lipid and fructose levels[51]. Icariin also ameliorated streptozocin-induced rat diabetic retinopathy [52] and nephropathy [53]. The recent study conducted by Lu et al also indicated that icariin was a novel PPARα agonist, activated lipid metabolism gene expression in liver, which could be a basis for its lipid-lowing effects and its beneficial effects against diabetes [54]. It was reported that old mice were significantly heavier than young mice, mostly because of an increase in fat mass and had a tendency to decreased energy expenditure[47]. So the beneficial effects of icariin on lipid metabolism likely in part explained our result that the EF and icariin groups had lower body weights than the controls. Furthermore, the result that the

icariin-treated mice were lighter yet eat more than controls suggested that icariin might improve the energy metabolism of mice. In particular, mitochondria are crucial in energy metabolism and mitochondrial function has been linked to the aging process in a number of ways[55]. So a deeper and comprehensive investigation into the effects of icariin on energy metabolism and mitochondrial function are needed in future.

Taken together, we presented that icariin extended mean lifespan as well as boosting several health features in late life. The healthspan extension of icariin treatment might due to the reduced oxidative stress and the maintenance of genome stability. However specific mechanisms by which icariin improved healthspan of mice need further investigation. In this study, we also showed that long-term administration of icariin did not result in serious adverse effects as there were no significant histophathological changes in the heart, spleen, lung and brain tissue between control and icariin groups. Instead, icariin or EF treatment attenuated the aging-associated degeneration of the liver.

Conclusion

Several studies have reported lifespan or healthspan extension using chemical treatments in simple model organisms; in contrast, only a few of these treatments have been found to be efficacious in mammalian systems [56-60]. Our study provided precious data of a safe and natural flavonoid to extend healthspan in mice. The results suggest that investigation into human effects might lead to promising new health interventions, but I wouldn't go further than that.

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Author Contributions

The authors have made the following declarations concerning their contributions. Conceived and designed the experiments: SQZ, CWJ, JHH, XMZ, and ZYS. Performed the experiments: SQZ, WJC, and JHH. Analyzed the data: SQZ, WJC XLC, XMZ, and ZYS. Contributed reagents/material/analysis tools: BW, XSJ. Wrote the paper: SQZ and WJC.

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Figure Legends

Fig.1 Experiment procedure. (a) Mice used in Life Span measurements. (b) Mice used in Healthspan measurements

Fig.2 Icariin increased survival. (a) Chemical structure of icariin. (b, c, d, e) Kaplan-Meier survival curves for mice treated with control (Control, the standard mice diet, n=52), calories restriction (CR, 60% of the standard mice diet, n=47), EF (the standard mice diet plus 0.06% EF, n=49) or icariin (the standard mice diet plus 0.02% icariin, n=49). Interventions started from the 12 months old till death.

Table.1 Detailed parameters of survival curves of the groups.

Fig. 3 Icariin-treated mice were lighter yet eat more than controls. (a) Body weight. Body weights of all the mice were monitored throughout the study. **(b)** Intake and output. Food intake was monitored in all the mice of the study; the intake of water, output of faeces and urine with metabolic cages were monitored in mice of healthspan subgroup. Repeated-measures ANOVAS followed by Dunnett's post hoc test were used for comparisons. *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus control group. .

Fig.4 Icariin improved physical performance. (a-d) Morris water maze performance. a) Escape latencies during the 5-day acquisition training period; comparisons between control (old) and intervention groups, All $F(3,33)<2.88$, all $P>0.05$; old control versus young control group by student's *t* test, $p<0.05$ b) Platform crosses; comparisons between control (old) and intervention groups, $F(3,33)=3.775$, $p=0.0196$; old control versus young control group by student's *t* test, $p<0.05$. c) Searching distance in the target quadrant in the probe test; comparisons

between control (old) and intervention groups, F (3,33)=4.063, p=0.0146; old control versus young control group by student's *t* test, p<0.05. d) Searching time in the target quadrant in the probe test, comparisons between control (old) and intervention groups,F(3,33)=3.103, p=0.0398. **(e)** Time to fall from an accelerating rotarod; comparisons between control (old) and intervention groups, F (3,33)=4.604, p=0.0085; old control versus young control group by student's *t* test, p<0.05. **(f)** Bone mineral density (BMD) of the right femur bone; comparisons between control (old) and intervention groups; F (3,33)=3.258, p=0.0338; old control versus young control group by student's *t* test, p<0.05. The measures were performed when the mice were 24 months old, and the 3-month-old (3-M) healthy male C57/BL6 mice were used as young control; n=9(Old), n=8(CR), n=9(EF), n=11(icariin), n=10(Young); comparisons among old control, CR, EF and icariin groups were performed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test, *p<0.05, **p<0.01, ***p<0.001 versus old control group; comparison between old and young was performed using student's *t* test. #p<0.05 versus young control group by student's *t* test; Error bar indicate s.e.m.

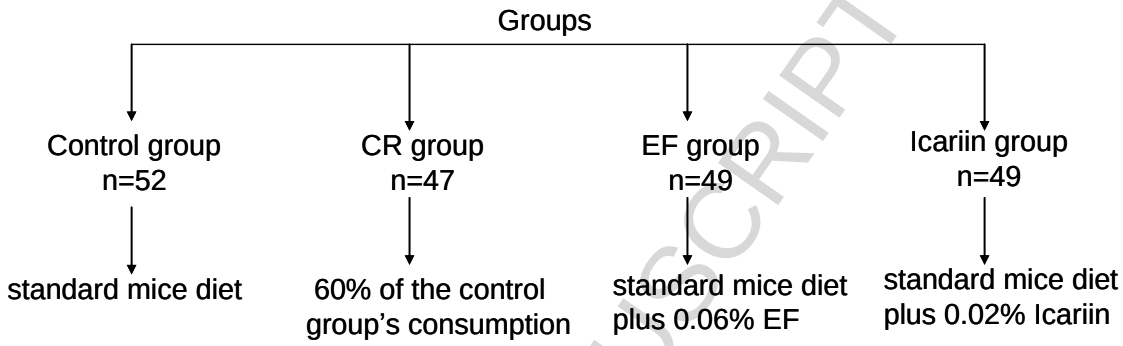
Fig. 5 Icariin significantly decreased γ-H2AX in the liver, kidney, heart, and lung. Percent of γ-H2AX positive cells in the **(a)**liver, F(3,28)=3.929, p=0.0185, (b) kidney, F(3,28)=5.337, p=0.0049, **(c)** heart, F(3,28)=5.374, p=0.0047, **(d)** lung, F(3,28)=3.026, P=0.0461, **(e)** spleen, F(3,28)=0.2187, p=0.8824 and **(f)**brain, F(3,28)=0.2694, P=0.8468; n=8 in each group; comparisons among old control, CR, EF and icariin groups were performed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test, *p<0.05, ***P* < 0.01 versus old control group; comparison between old and young was performed using student's *t* test. #p<0.05 versus young control group by student's *t* test; Error bar indicate s.e.m.

Fig. 6 Icariin downregulated the expression of genes related to the DNA damage response. (a) Microarray analysis (Total RNA was extracted from the liver, n=4 in each group): Heat map of genes involved in DNA damage; Green represented down-regulation, and red represented up-regulation. **(b,c)** Real-time PCR: Relatively mRNA levels of ATM and BRCA1 in liver tissues. n=9(Old), n=8(CR), n=9(EF), n=11(icariin), n=10(Young); comparisons among old control, CR, EF and icariin groups were performed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test, *p<0.05, **p < 0.01 versus old control group; comparison between old and young was performed using student's *t* test. #p<0.05 versus young control group by student's *t* test; Error bar indicate s.e.m.

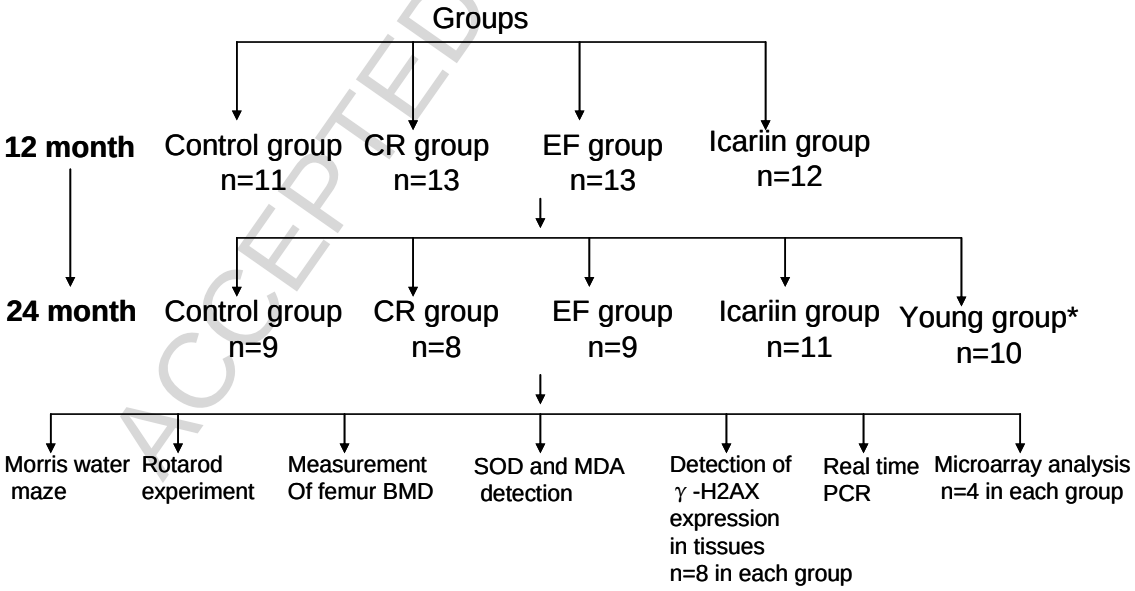
Fig.7 Icariin modulated SOD and MDA levels in the liver. (a) SOD levels in supernatants of liver tissue lysates. **(b)** MDA levels in supernatants of liver tissue lysates. n=9(Old), n=8(CR), n=9(EF), n=11(icariin), n=10(Young); comparisons among old control, CR, EF and icariin groups were performed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test, *p<0.05, **$P < 0.01$ versus old control group; comparison between old and young was performed using student's *t* test. #p<0.05 versus young control group by student's *t* test; Error bar indicate s.e.m.

Fig.1

a. Mice used in Life Span measurements



b. Mice used in Healthspan measurements



* The 3-month-old healthy male C57/BL6 mice (n=10) were used as young control.

Fig.2

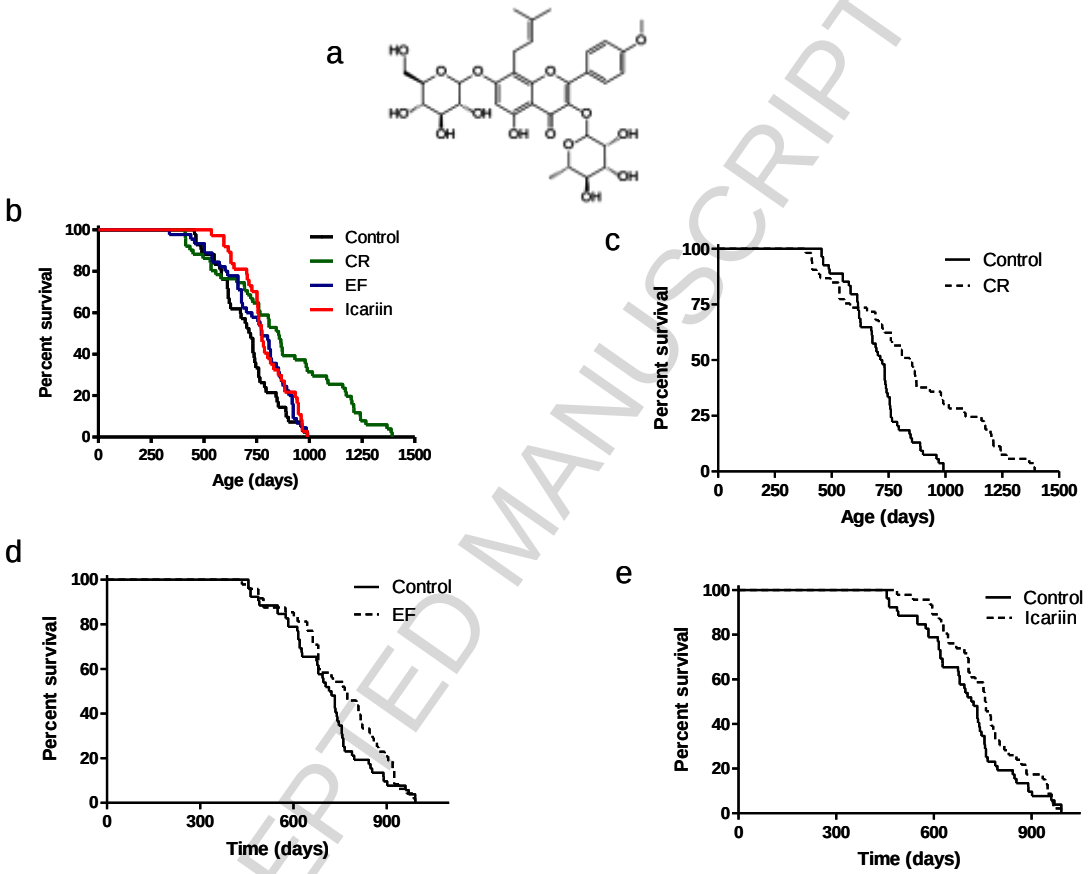


Fig.3

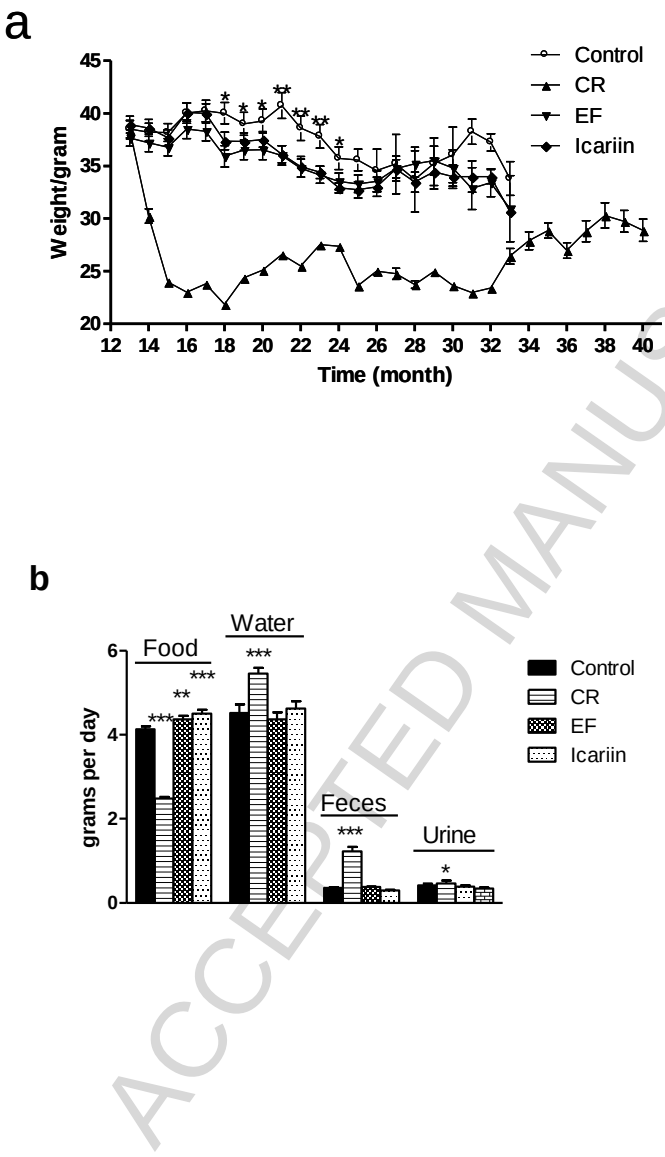


Fig.4

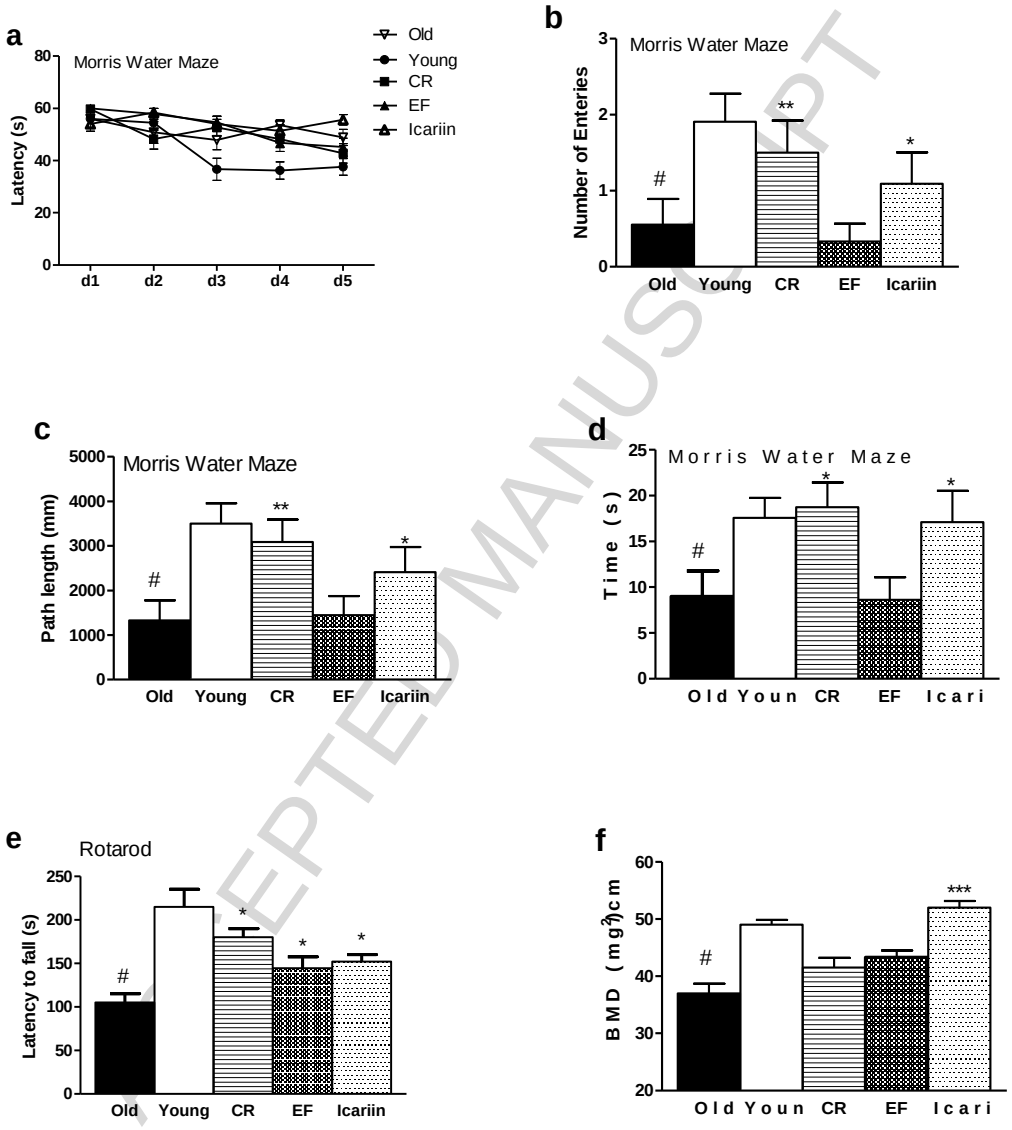


Fig.5

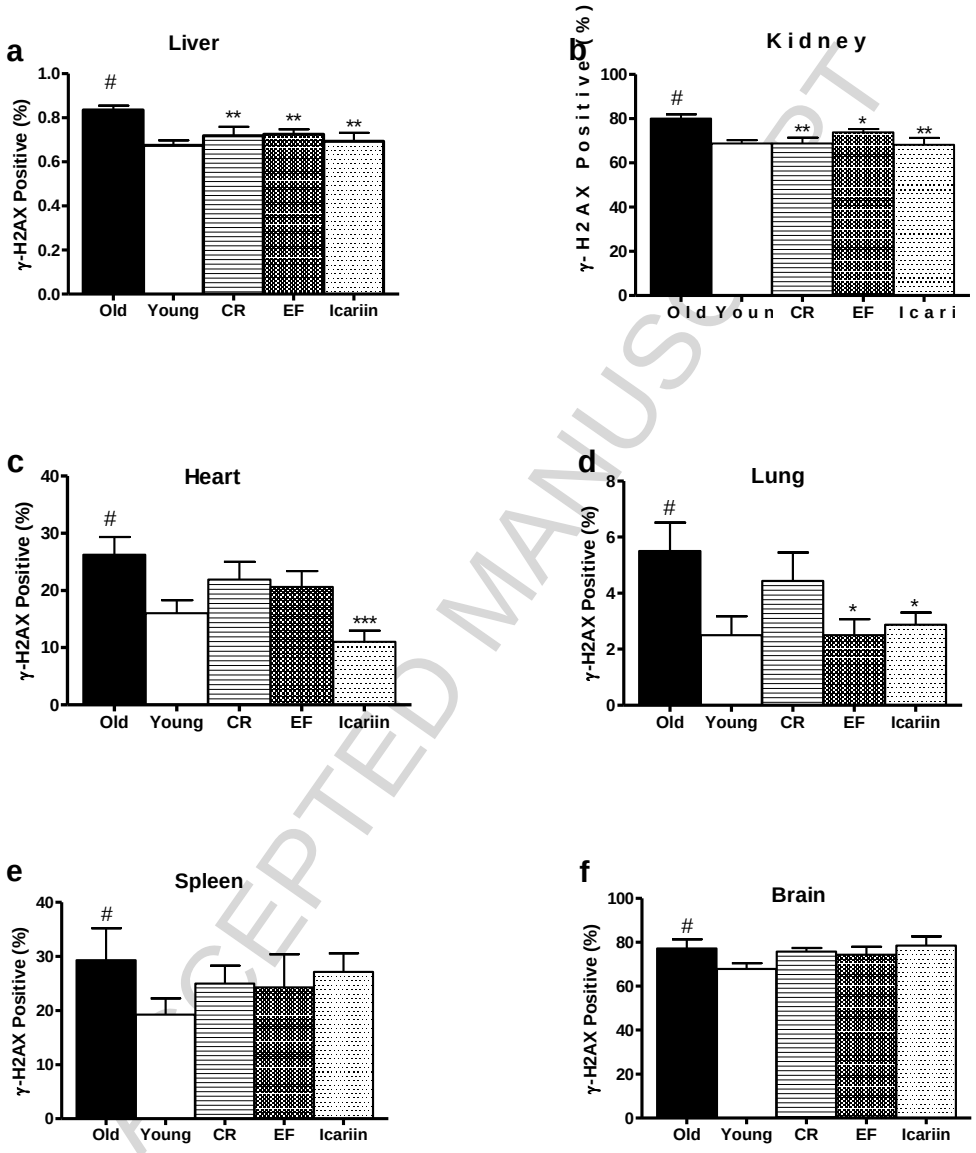


Fig.6

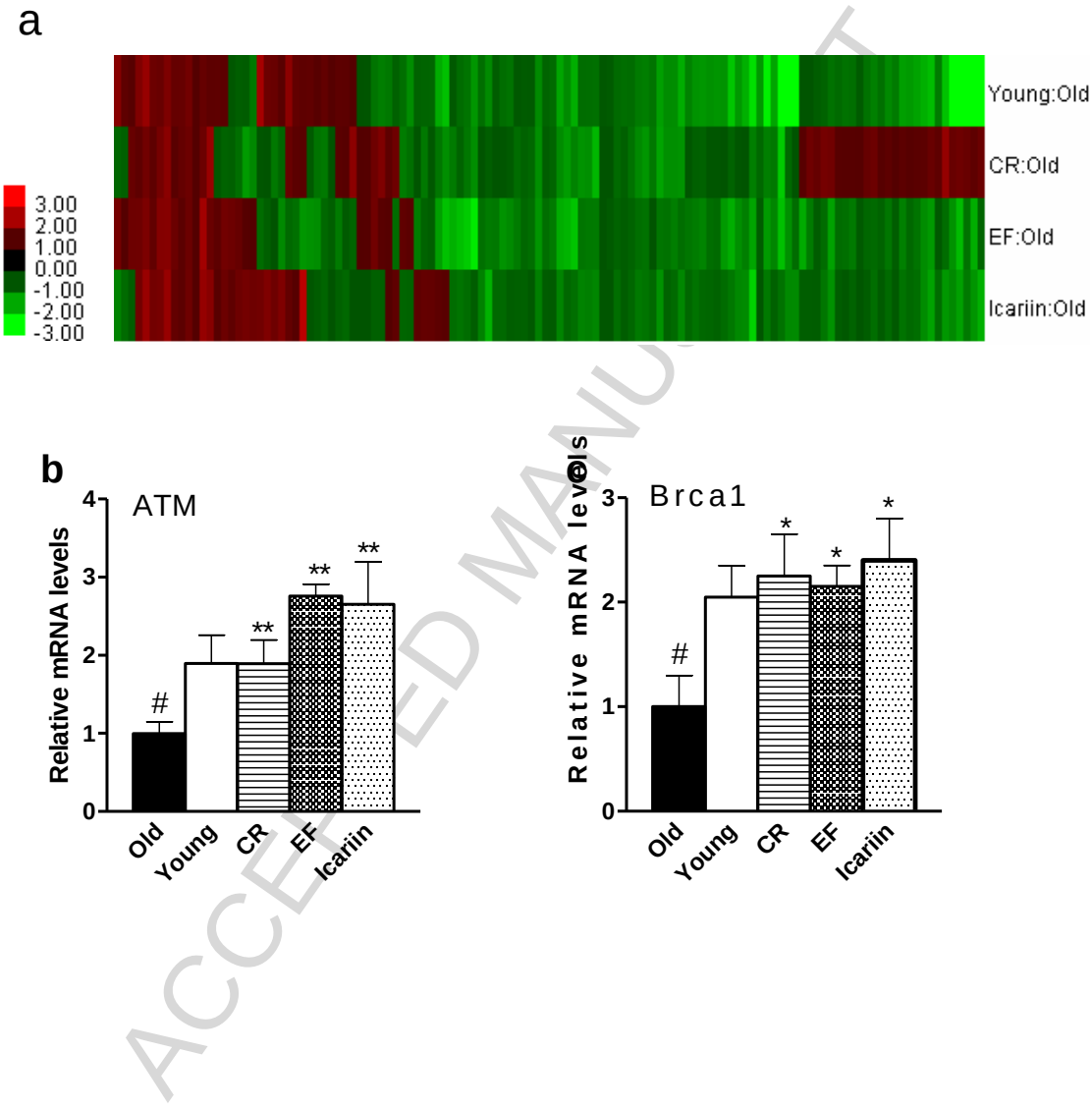


Fig.7

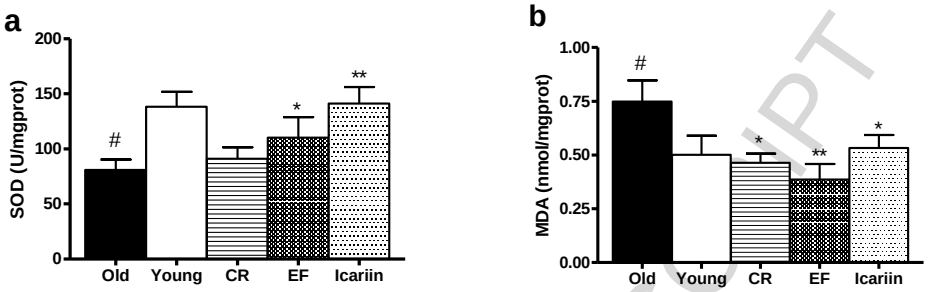


Table.1

Group (n)	Mean lifespan	(Extension)	Maximum lifespan[1]	(Extension)	90th%[2]	p-value[3]
Control (52)	702		898		10%	
CR(47)	879	(25%)	1246	(39%)	43%	0.0002
EF(49)	727	(4%)	926	(3%)	20%	0.1274
Icariin(49)	758	(8%)	953	(6%)	18%	0.2033

1. Ages of 90th percentile 2.Proportion of living mice at 90th

3.Comparision of surviving proportion of mice at the 90th percentile age.

Highlights

1. Icariin, a flavonol isolated from herbal epimedii, extended lifespan in C57BL/6 mice.
2. Icariin boosted healthy features in mice such as cognitive function, neuromuscular coordination and bone density.
3. Icariin exhibited protective effects on the maintenance of genomic stability.
4. Icariin was a safe natural flavonoid to extend healthspan in mice.