World Cancer Research Journal WCRJ 2021; 8: e1919

URSOLIC ACID PROTECTS THE KIDNEYS FROM THE CONSEQUENCES OF AGING THROUGH INCREASING THE EXPRESSION OF SIRT1, SIRT6 AND α-KLOTHO IN THE MICE MODEL

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Abstract – *Objective:* *In our previous studies on the anti-aging effects of ursolic acid (UA) on various tissues including skeletal muscle, heart, hypothalamus and pancreas, the protective and anti-aging roles of UA on renal tissue remained elusive. In this study, we aim to address this issue.*

Materials and Methods: *In this research we used 20 aged male mice C57/BL6. After optimizing the conditions for treatment, UA was dissolved in corn oil and administrated with 200 mg/kg doses twice a day for 7 days in the form of intra-peritoneal injection. The animals were anaesthetized and perfused in order to analyze the tissues through Immunofluorescence (IF) and histochemical tests.*

Results: *The findings showed that UA significantly increased SIRT1 (~4 folds),* $p<0.001$. *Furthermore, UA enhanced SIRT6 up-regulation (~5 folds),* $p<0.001$. *With regard to the prominent role of α-Klotho in the control of CKD progression, our findings obviously showed that UA enhanced α-Klotho expression (~3.51 folds),* $p<0.001$.

Conclusions: *By enhancing SIRT1 expression, it seems that UA may be a new therapeutic agent to increase resistance to many causal factors in the development of renal diseases, including diabetic nephropathy. It led to a decrease in glomerular cell apoptosis, inflammation, sodium reabsorption, blood pressure, and interstitial fibrosis, and an increase in autophagy and renal lipid metabolism. Also, enhancing of SIRT6 in the kidney of aged mice using UA promotes metabolism regulation, DNA repair and longevity. Finally, through up-regulation of α-Klotho, UA prohibits the kidney vulnerability, renal fibrosis and induces kidney regeneration.*

KEYWORDS: *Ursolic Acid, SIRT1, SIRT6, α-Klotho.*

LIST OF ABBREVIATIONS: *Ursolic acid (UA), SIRT1 and SIRT6 (sirtuin, silent mating type information regulation 2 homolog)1 and 6, Chronic kidney disease (CKD), Renin-Angiotensin-Aldosterone System (RAAS), calorie restriction (CR), Phosphate buffer saline (PBS), Insulin like growth factor 1 (IGF-1), Transient receptor potential cation channel subfamily V member 5 (TRPV5), lipopolysaccharide (LPS).*

Aging is an undeniable process resulting from gradual changes over time. Cell growth and cell division, as well as the precision of macromolecules in the cell, reduces with aging, resulting in impaired physiological function of various organs that leads to death[1]. As previous research has shown, aging increases the risk of developing diseases such as cancer, diabetes, cardiovascular disorders, neurological and renal diseases[2]. According to previous studies, the main role of

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kidneys is to maintain hemostasis, i.e. they control the fluid level, balance of electrolytes and other factors to keep the internal environment of body stable. Some of the main functions of kidney include antioxidation, recovery of dietary nutrients, pH regulation, regulation of blood osmolality, regulation of blood pressure and secretion of active compounds such as calcitriol, renin, and erythropoietin[3]. Based on our studies, UA is a lipophilic compound that has medicinal properties and can be found abundantly in apple peels as well as in plants such as rosemary, oregano, *etc.*[4,5]. UA has been identified as an anti-diabetic[6], anti-cancer, anti-inflammatory[7], anti-HIV[8], anti-bacterial[9], liver protective[10] and anti-oxidant agent[11].

It has been shown that UA prevents skeletal muscle breakdown and increases its volume and strength in laboratory animals[12,13]. In addition, it could be considered as a suitable candidate to treat liver malignancy[10]. Moreover, UA significantly reduced serum index enzymes (creatine kinase, lactate dehydrogenase) caused by heart attack, indicating its therapeutic effect in reducing complications after a heart attack[14]. Finally, UA lowers blood glucose, plasma fats, albumin excretion, urea, etc. in diabetic mice, making it a good treatment to reduce kidney damage and protect the kidneys[15]. Numerous changes occur during the aging process that affect kidneys' nutrient uptake, absorption, digestion, and metabolism. One of the main functions of the kidneys is to expel waste products from the blood. When they are damaged, the blood cleaning and excretion of body waste is not done properly, a condition called chronic kidney failure. This could lead to anemia, high blood pressure, and bone problems[2]. Indeed, kidney aging is a multifactorial process in which sex, race, genetic background and several mediators, including chronic inflammation, oxidative stress, Renin-Angiotensin-Aldosterone System (RAAS), the inability to repair the kidneys and cardiovascular diseases play an important role[16]. Biomarkers such as SIRT1, SIRT6, and α-Klotho can play a role in delaying aging. As recent studies indicate, SIRT1 plays an important role in inflammation and fibrosis, inhibition of renal cell apoptosis, and regulation of lipid metabolism, stress resistance, blood pressure, sodium balance, differentiation and aging[17,18]. In addition, SIRT6 has a main role in regulating metabolism, DNA repair and longevity. SIRT6 knockouting in mice shows a significant phenotype of premature aging similar to kidneys aging[19]. It was also reported that the levels of SIRT1 and SIRT6 increased in the brain and kidneys of rats fed with a calorie restriction (CR) diet, while only the level of SIRT6 increased in the heart[19]. Finally, α-Klotho, a gene that suppresses aging and increases longevity, was discovered in 1997[20]. Alpha-Klotho deficiency contributes to reduced life expectancy, infertility, atherosclerosis, skin atrophy, osteoporosis, hypogonadism, premature thymus vulnerability, misplaced calcification, bone mineral dysfunction, pulmonary emphysema, and hearing impairment, while α-Klotho gene expression prolongs longevity[21]. In contrast, excessive expression of α-Klotho reverses the aging process[22]. Studies conducted on UA have shown that it increases the level of anti-aging biomarkers of SIRT1, SIRT6 and α-Klotho[10,12]. Moreover, the kidneys are one of the organs that are most susceptible to severe structural and functional damage during old age compared to other organs[23]. In this study, we evaluated the effect of UA on the expression of these biomarkers in kidney tissue of C57BL/6 mice with Immunofluorescence microscope, which is a strong and practical method generally used by researchers to show that UA increases SIRT1, SIRT6 and α-Klotho in the kidney and thus improves its function both in *in vitro* and *in vivo* conditions.

MATERIALS AND METHODS

Materials

2.1. UA was purchased from Sigma-Aldrich (St. Louis, MO, USA, U6753) with high purity (90%). Antibodies specific for SIRT1 were provided by (Cambridge, United Kingdom, Biotech, Life Sciences, Ab110304), SIRT6 (Sigma-Aldrich, St. Louis, MO, USA, S4322) and α-Klotho (Cambridge, United Kingdom, Biotech, Life Sciences, MAB1819). Goat anti-rabbit FITC (Ab6717), donkey anti-rabbit (SC-2095), Goat anti-mouse FITC (Ab97022) and Goat anti-mouse (ab6787) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Abcam (Cambridge, United Kingdom). Paraformaldehyde, Triton X-100, DAPI, Tris-HCl, and NaCl were purchased from Sigma-Aldrich Company (St. Louis, MO, USA) and all other chemicals were purchased from Merck Company (Kenilworth, NJ, USA).

Animal study

In this research, we used 20 inbred aged C5BL/6 male mice (20 months old) prepared from Pasteur Institute of Iran. Within 3 weeks of onset treatment, the mice were housed in colony cages with

12 h light/12 h dark cycles to adopt the new condition and kept on standard chow (Harlan Teklad formula 7013). UA was dissolved in corn oil (20 mg/ml) and administrated in 200 mg/kg doses through intra-peritoneal injection (i.p)[13]. The mice were classified into 3 groups, one group received UA, another received placebo (corn oil) and the control group received distilled water. UA was administrated twice a day for 7 days[13]. Finally, for Immunofluorescence (IF) and histochemical tests, all animals were weighted and anaesthetized by an i.p injection of ketamine/xylazine and then fixed with 4% paraformaldehyde and 2.5% glutaraldehyde.

Animal Ethical statement

To evaluate UA effects on some proteins which modified in kidneys of aged mice, we tried to follow from standard regulation which applied for animals. Hence, all animal procedures were approved by the Institutional Animal Care and Committee of the Endocrinology and Metabolism Research Center, Tehran University of Medical Sciences (Tehran, Iran) with ethical code IR.TUMS.REC.1396.282.

Tissue preparing for immunofluorescence test

After anaesthetizing and perfusing of mice, the kidneys were properly isolated and immersed in 4% paraformaldehyde and 2.5% glutaraldehyde overnight in order to fix them[24]. Then, the samples were impregnated with 30% sucrose and stored in 4°C for IF tests. Serial cross-sections (8 mm thicknesses) were made using a cryostat microtome at -25°C, mounted onto the glass slides, and then stained for cytoplasm and nucleus detection. In this experiment, slides were blocked in room temperature for 10 min prior to staining. Next, the cytoplasm staining was performed by Hematoxylin for 5 min, washed with PBS and water, and the nuclei staining was done by Eosin for 5 min, washed with PBS and water, dehydrated by descending alcohol, mounted onto glass slides and then visualized using a bright-field microscope (Nikon, Tokyo, Japan, TE2000-S) and captured by camera (TCH-1.4CICE).

Immunofluorescence microscopy

To identify the mentioned anti-aging proteins in the hypothalamus tissue, cryosection slides were first dehydrated in Room Temperature (RT) for 10 min. Then, the slides were embedded in PBS for 10 min (rehydration) and exposed to 0.1 normal HCl for 20 min. Afterwards, it was replaced by Borate Buffer for 5 min. Next, they were washed by PBS (2-5 min). To evaluate nucleus antigen, tissue was made permeable by Triton X-100 (3% in PBS) for 30 min and then washed by PBS (2 -5 min). In the next step, the semi-prepared tissues were blocked by goat-serum (500 ml goat-serum in 4.5 cc PBS) for 45 min in RT. The first antibodies were added based on optimized protocol in 4°C overnight. Likewise, SIRT1 (1–1000 diluted in blocking buffer), SIRT6 (2–4 mg/ml diluted in blocking buffer), α-Klotho (8-25 mg/ml diluted in blocking buffer). Then, the slides were washed in PBS (2-5 min). Afterwards, the secondary antibodies were included for 2 h in 37°C: FITC-conjugated goat anti-mouse IgG1 (1–500 diluted in blocking buffer) and FITC-conjugated goat anti-rabbit IgG1 (1–1000 diluted in blocking buffer). Next, the slides were washed in PBS (2-5 min). In the last step, to identify tissue nucleus, 50 ml of DAPI (40, 6-diamidino-2-phenylindole) diluted in PBS was used on each slide for 2 min in dark, and then removed from tissue surface and washed by PBS (2-5 min)[25]. Finally, the slides were embedded in PBS, visualized by bright-field microscope (Nikon, Tokyo, Japan, TE2000-S), captured by camera (TCH-1.4CICE) and the images were analyzed with the LSM 510 image browser software.

Statistical analysis

The results of this study were analyzed by one-way ANOVA. Each experiment was done at least three times, and the data were presented as the mean ± SEM, where applicable.

RESULTS

Ursolic acid increases SIRT1 in the kidney of aged mice

Based on our previous studies on the rejuvenating effects of UA on hypothalamus, liver and skeletal muscle in the mice[10,12,26], we further confirmed this phenomenon in the kidneys of aged mice. Therefore, we decided to use a new strategy to check the expression of SIRT1 protein level. As shown in Figure 1, SIRT1 protein significantly increased in the mice receiving UA (94.22 ± 10.35) compared to mice receiving corn oil (69.26 ± 3.5) and distilled water (24.22 ± 3.6) (p<0.001) (Figure 1).

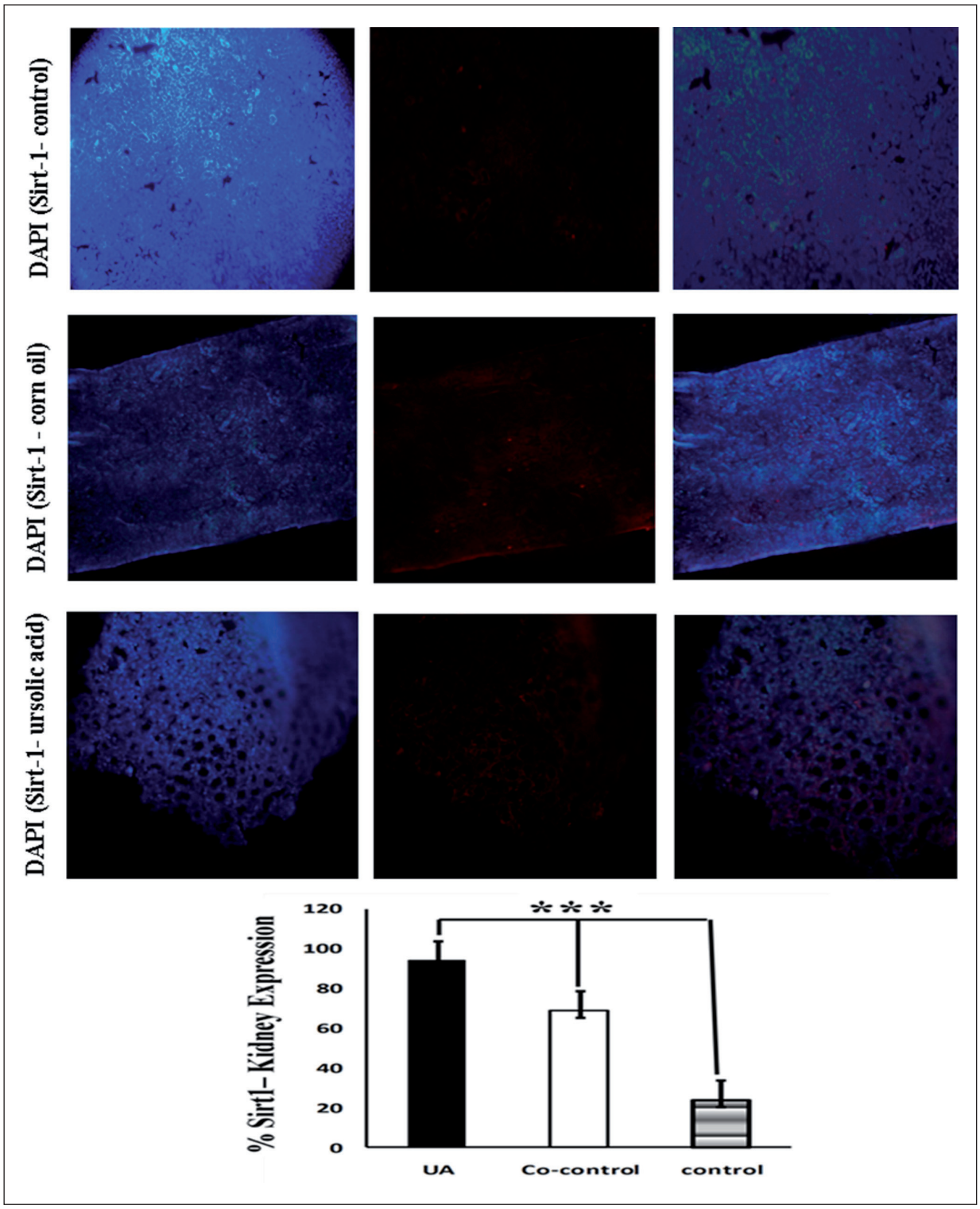

Fig. 1. UA increases SIRT1 protein level in aged mice's kidney. Expression of nuclear SIRT1 on the kidney tissue. The mice (C57BL/620 aged-month, n = 20) were treated with 200 mg/kg of UA which was dissolved in corn oil (20 mg/ml). UA was administrated twice a day for 7 days through i.p injection, and the tissue was cryosectioned (8 mm) and stained with FITC-anti-SIRT1 monoclonal antibody. *A*, UA + Corn Oil treated mice (UA); *B*, The control mice which received vehicle alone (Corn Oil, CO, USA); *C*, The control mice which only received distilled water. p value was determined by one away ANOVA test. Data are presented as mean ±SEM (*p<0.05, **p<0.01, ***p<0.001). Scale bar 50 mm. DAPI staining shows entire cell populations.

Ursolic acid up-regulates SIRT6 in the kidneys of aged mice

As relevant studies indicate the key role of kidneys and functional changes that occur as a result of kidney aging, we examined the UA effect on the SIRT6 protein level in old mice. Our findings illustrated that UA significantly increased SIRT6 overexpression (84 ± 11.91) in comparison to mice that only received corn oil as placebo (8.3 ± 0.53) and distilled water (17.63 ± 0.17) (p<0.001) (Figure 2).

α-Klotho protein was up-regulated in the kidneys of aged mice which received UA

As shown by previous studies, a gradual decrease in the level of secretory α-Klotho protein in the urine occurs during CKD progression in patients[27]. In our recent study on the anti-aging effects of UA[28], UA was administrated to evaluate the level of α-Klotho protein in the kidney of aged mice. The findings showed that UA significantly increased α-Klotho level (22.65 ± 1.43), in comparison with cases which only received corn oil (9.88 ± 0.84) and distilled water (6.37 ± 0.08) (p<0.001) (Figure 3).

DISCUSSION

Aging reduces the function of biological systems in all body organs. In fact, whether aging is a disease or not is still an important physiological and scientific issue. Kidney aging is a complex and multifactorial process that causes chronic kidney disease in older people during their lifetime[16]. As known, reduced calorie intake delays age-related changes in the kidneys. These changes include glomerulosclerosis, ischemic injuries, vessel wall thickening, and intracranial tissue hardening. There have been observations of the effect of low-calorie diets on delaying kidney aging[29]. Hence, appropriate diets and various pharmacological approaches have been tested to delay the aging process or reduce its function in the elderly[16].

Despite studies on different functions of UA[28,30], we studied its effect on the biological biomarkers such as α-Klotho, SIRT1 and SIRT6 in the renal tissue of old mice. The results of all experiments on renal tissue showed that the level of α-Klotho protein in UA-treated mice increased significantly (Figure 3). It is worth mentioning that α-Klotho plays a pivotal role in the regulation of aging and the improvement of age-related diseases in mammals. Thus, high expression of α-Klotho will increase life expectancy about 20 to 30%. One interesting point is that α-Klotho expression is present in a limited number of bonds, but deficiency in α-Klotho gene expression leads to several pseudo-aging phenotypes in almost all systemic organs in mice[22]. According to α-Klotho functions in the kidney, it acts as a hormone that enters the bloodstream in the following three ways: (a) RNA intermittent monitoring, (b) Proteolytic divisions and (c) Sodium-potassium ATPase transfer[31,32]. Besides, α-Klotho acts as a regulator of fibroblast growth factor, so that when too much phosphate is produced, factor-23 fibroblast growth factor (FGF-23) begins to be secreted from the bone and work in the kidneys, leading to the excretion of phosphate from the urine[33]. There is a direct relationship between α-Klotho, fibroblast growth factor and age[34]. It regulates signals (IGF1) and insulin[35]. However, it could resist oxidative stress at the cellular and organic levels in mammals[36]. It also acts as a glucuronidase and activates TRPV5 ion channels[37,38]. Moreover, it protects endothelial dysfunction and controls nitric oxide production[31,39]. In fact, α-Klotho affects the intracellular signaling pathways as follows[31]:

1. p53/p21: the expression of genes p53 and p21, which causes cell death, increases with age. Therefore, α-Klotho prevents cell death by regulating p53/p21 signal pathway.
2. it affects Wnt signal pathways.

α-Klotho deficiency increases the vulnerability of the kidney to acute insults, weakens kidney regeneration an induces renal fibrosis. In addition to direct renal effects, α-Klotho deficiency also causes and intensifies disturbed mineral metabolism, secondary hyperparathyroidism, vascular calcification, cardiac hypertrophy and fibrosis. Recent studies suggested that nuclear α-Klotho and cytoplasm α-Klotho are also bioactive molecules to protect cells from senescence and apoptosis[40,41]. Although, SIRT6 has been implicated as a potential regulator of longevity and has important roles in cytoprotective functions, its molecular targets, biological functions and possible roles in renoprotection are largely unknown. When discussing mitochondrial biogenesis and kidney diseases, it should be noted that mitochondrial biogenesis and its associated processes improve metabolic pathways such as fatty acid oxidation and increase antioxidant defense mechanisms that mitigate injury from aging, tissue hypoxia and glucose or fatty acid overload, all of which contribute to the pathogenesis of acute and chronic kidney disease[42]. Moreover, the results have shown that UA increases SIRT1 in kidney tissue. As shown, SIRT1 plays an important role in inflammation, apoptosis of renal and fibrous cells, and has protective effects on organs in oxidative stress, including kidney; it also controls lipids, autophagy, blood pressure and sodium balance[17].

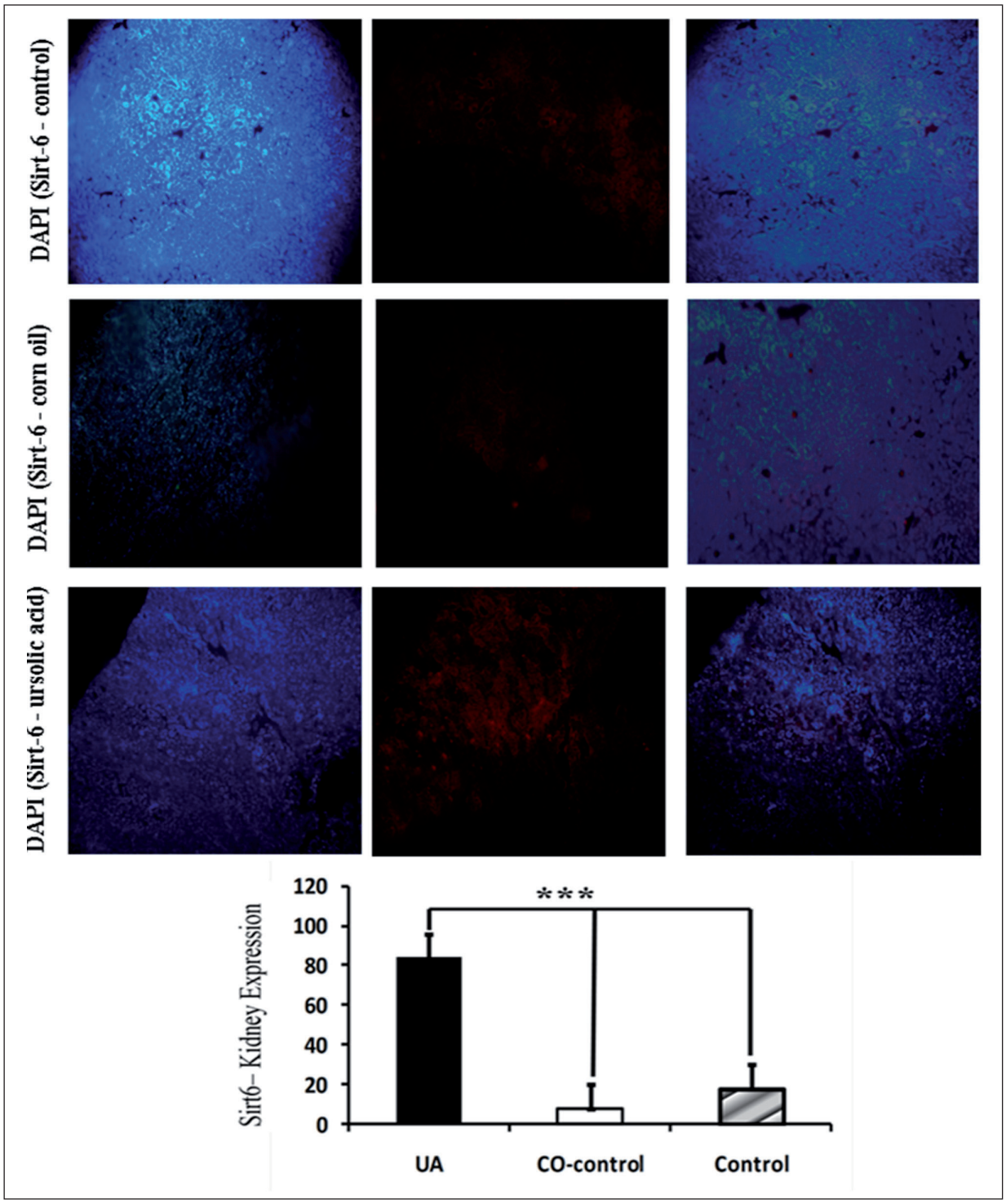

Fig. 2. SIRT6 protein was overexpressed in UA-treated mice. Nuclear SIRT6 expression was detected in aged mice's kidney. The mice (C57BL/6, 20 aged-month, n = 20) which received, *(A)* UA + Corn Oil (200 mg/kg), UA, *(B)* Corn Oil (CO) and *(C)* distilled water *(C)*. The tissue was processed by cryostat microtome (8 mm) and stained with FITC-anti- SIRT6 monoclonal antibody. p value was determined by one away ANOVA test. Data are presented as mean ± SEM (*p< 0.05, **p<0.01, ***p<0.001). Scale bar 50 mm. DAPI staining shows entire cell.

Studies indicate that the removal of SIRT1 in mice causes severe kidney changes, including diabetes, acute kidney damage and LPS caused by kidney inflammation. In addition, loss of SIRT1 in epithelial cells of renal tubules exacerbates the damage caused by renal fibrosis[18,43]. It was recently reported that a novel type of communication between two different kidney compartments mediated by SIRT1 prevents the above-mentioned disorders[44-46].

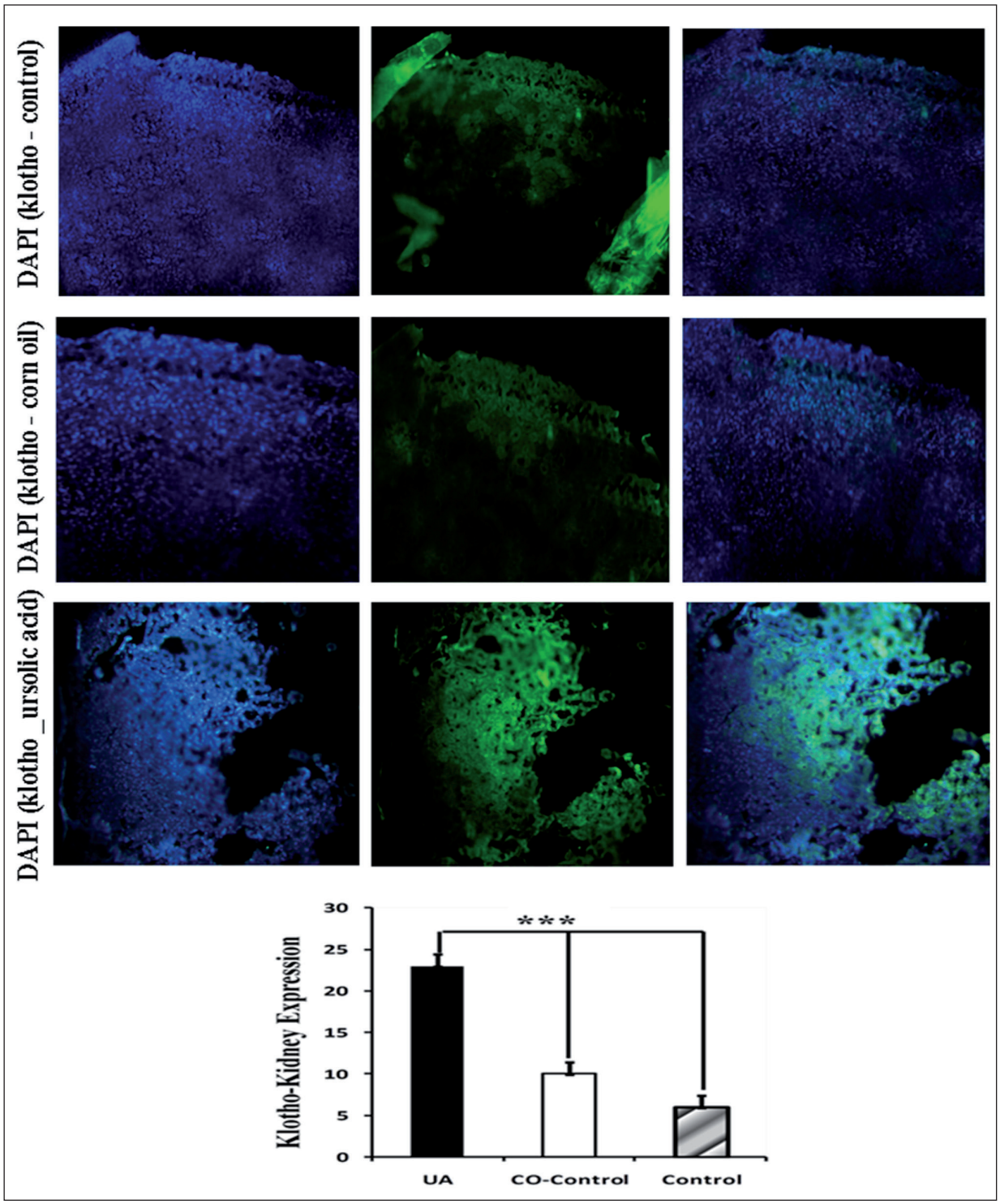

Fig. 3. UA enhances anti-aging α-Klotho level in aged mice's kidney. The mice were treated with, *(A)* UA + Corn Oil (UA) (ip) containing 200 mg/kg UA twice a day for 7 days, *(B)* Corn Oil (CO) and *(C)* distilled water *(C)*. The cryosection of kidney tissue was prepared (8 mm) and stained with FITC-anti- α-Klotho monoclonal antibody. p value was determined by one away ANOVA test. Data are presented as mean ± SEM (*p<0.05, **p<0.01, ***p<0.001). Scale bar 50 mm. DAPI staining shows entire cell

CONCLUSIONS

The kidney has a key role in governing of aging. In order to validate the findings of our previous study on rejuvenation effects of UA in the hepatic, skeletal muscle and hypothalamus, our results in this study support the anti-aging effect of UA through enhancing metabolic sensor proteins level (SIRT6 and SIRT1). The activation of SIRT1 in the kidney may be a new therapeutic target to

increase resistance to many causal factors in the development of renal diseases, including diabetic nephropathy. Also, SIRT6 is of great importance in regulating metabolism, DNA repair and longevity. As mentioned earlier, α-Klotho is originally known as an aging suppressor gene. In fact, α-Klotho plays an important role in intracellular signaling pathways such as p53/p21 and Wnt [31]. In this study, we found that UA increased α-Klotho in mice under UA treatment; therefore, it seems that UA delays aging or ameliorates it through elevation of anti-aging biomarkers in kidney. Taken together, further studies are required to evaluate the contribution of UA to age-sensitive traits, in addition to its effect on hypothalamus, kidney and skeletal muscle as prominent organs in controlling of aging.

Acknowledgements:
The authors of this manuscript are obligated to thank Young Scientists Festival, Jamilli Charitable Foundation, Tehran, Iran that provided financial support for this project.

Conflict of interest:
We declare that there is no conflict of interest.

REFERENCES

1. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. Cell 2013; 153: 1194-1217.
2. Wei SY, Pan S, Li B, Chen YM, Lin SL. Rejuvenation: turning back the clock of aging kidney. J Formos Med Assoc 2019; 119: 898-906.
3. Sands JM, Verlander JW. Functional anatomy of the kidney. Comprehensive Toxicol 2018; 1-26.
4. Liu J. Oleanolic acid and ursolic acid: research perspectives. J Ethnopharmacol 2005; 100: 92-94.
5. Mallavadhani UV, Mahapatra A, Pattnaik B, Vanga N, Suri N, Saxena AK. Synthesis and anti-cancer activity of some novel C-17 analogs of ursolic and oleanolic acids. Med Chem Res 2013; 22: 1263-1269.
6. Aly HF, Ebrahim ME, Metawaa HM, Hosni EA-MA, Ebrahim FM. In vitro and in vivo evaluation of the antidiabetic effect of different extracts of Nepeta cataria in streptozotocin induced diabetic rats. J Am Sci 2010; 6: 364-386.
7. Pádua TA, de Abreu BS, Costa TE, Nakamura MJ, Valente LM, das Graças Henriques M, Siani AC, Rosas EC. Anti-inflammatory effects of methyl ursolate obtained from a chemically derived crude extract of apple peels: potential use in rheumatoid arthritis. Arch Pharm Res 2014; 37: 1487-1495.
8. Han B, Peng Z. Anti-HIV triterpenoid components. J Chem Pharm Res 2014; 6: 438-443.
9. Do Nascimento PG, Lemos TL, Bizerra AM, Arriaga ÂM, Ferreira DA, Santiago GM, Braz-Filho R, Costa JGM. Antibacterial and antioxidant activities of ursolic acid and derivatives. Molecules 2014; 19: 1317-1327.
10. Gharibi S, Bakhtiari N, Jalalvand EM. Ursolic acid mediates hepatic protection through enhancing of anti-aging biomarkers. Curr Aging Sci 2018; 11: 16-23.
11. Tsai SJ, Yin MC. Antioxidative and anti-inflammatory protection of oleanolic acid and ursolic acid in PC12 cells. J Food Sci 2008; 73: H174-H178.
12. Bahrami SA, Bakhtiari N. Ursolic acid regulates aging process through enhancing of metabolic sensor proteins level. Biomed Pharmacother 2016; 82: 8-14.
13. Kunkel SD, Suneja M, Ebert SM, Bongers KS, Fox DK, Malmberg SE, Alipour F, Shields RK, Adams CM. mRNA expression signatures of human skeletal muscle atrophy identify a natural compound that increases muscle mass. Cell Metab 2011; 13: 627-638.
14. Radhiga T, Rajamanickam C, Senthil S, Pugalendi KV. Effect of ursolic acid on cardiac marker enzymes, lipid profile and macroscopic enzyme mapping assay in isoproterenol-induced myocardial ischemic rats. Food Chem Toxicol 2012; 50: 3971-3977.
15. Ling C, Jinping L, Xia L, Renyong Y. Ursolic acid provides kidney protection in diabetic rats. Curr Ther Res 2013; 75: 59-63.
16. Bolignano D, Mattace-Raso F, Sijbrands EJ, Zoccali C. The aging kidney revisited: a systematic review. Ageing Res Rev 2014; 14: 65-80.
17. Kitada M, Kume S, Takeda-Watanabe A, Kanasaki K, Koya D. Sirtuins and renal diseases: relationship with aging and diabetic nephropathy. Clin Sci 2013; 124: 153-164.
18. Gao R, Chen J, Hu Y, Li Z, Wang Sh, Shetty S, Fu J. Sirt1 deletion leads to enhanced inflammation and aggravates endotoxin-induced acute kidney injury. PLoS One 2014; 9: e98909.
19. Kanfi Y, Shalman R, Peshti V, Pilosof SN, Gozlan YM, Pearson KJ, Lerrer B, Moazed D, Marine JC, Cabo RD, Cohen HY. Regulation of SIRT6 protein levels by nutrient availability. FEBS Lett 2008; 582: 543-548.
20. Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, Ohyama Y, Kurabayashi M, Kaname T, Kume E, Iwasaki H, Iida A, Shiraki-Iida T, Nishikawa S, Nagai R, Nabeshima Y-I. Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature 1997; 390: 45-51.
21. Manolis AS. Klotho, spinning the thread of life: an anti-ageing gene. Hosp Chronicles 2012; 7: 129-132.
22. Kurosu H, Yamamoto M, Clark JD, Pastor JV, Nandi A, Gurnani P, Mcguinness OP, Chikuda H, Yamaguchi M, Kawaguchi H, Shimomura I, Takayama Y, Herz J, Kahn CR, Rosenblatt KP, Kuro-o M. Suppression of aging in mice by the hormone Klotho. Science 2005; 309: 1829-1833.
23. John GB, Cheng C-Y, Kuro-o M. Role of Klotho in aging, phosphate metabolism, and CKD. Am J Kidney Dis 2011; 58: 127-134.
24. Hernández A, Goodwin ML, Lai N, Cabrera ME, McDonald JR, Gladden LB. Contraction-by-contraction Vo2 and computer-controlled pump perfusion as novel techniques to study skeletal muscle metabolism in situ. J Appl Physiol 2010; 108: 705-712.
25. Kobelt P, Tebbe JJ, Tjandra I, Bae H-G, Rüter J, Klapp BF, Wiedenmann B, Monnikes H. Two immunocytochemical protocols for immunofluorescent detection of c-Fos positive neurons in the rat brain. Brain Res Protoc 2004; 13: 45-52.
26. Ahmadzadeh S, Noori AR, Bakhtiari N, Hemmati R. Elucidating the anti-aging ursolic acid through evaluation of SIRT1 and SIRT6 in the mice skeletal muscle tissue. Biomacromol J 2019; 5: 47-57.

27. Rotondi S, Pasquali M, Tartaglione L, Muci ML, Mandanici G, Leonangeli C, Sales S, Farcomeni A, Mazzaferro S. Soluble α-Klotho serum levels in chronic kidney disease. Int J Endocrinol 2015; 2015: 872193.
28. Bakhtiari N, Hosseinkhani S, Soleimani M, Hemmati R, Noori-Zadeh A, Javan M, Tashakor A. Short-term ursolic acid promotes skeletal muscle rejuvenation through enhancing of SIRT1 expression and satellite cells proliferation. Biomed Pharmacother 2016; 78: 185-196.
29. McKiernan SH, Tuen VC, Baldwin K, Wanagat J, Djamali A, Aiken JM. Adult-onset calorie restriction delays the accumulation of mitochondrial enzyme abnormalities in aging rat kidney tubular epithelial cells. Am J Physiol Renal Physiol 2007; 292: F1751-F1760.
30. Bakhtiari N, Hosseinkhani S, Tashakor A, Hemmati R. Ursolic acid ameliorates aging-metabolic phenotype through promoting of skeletal muscle rejuvenation. Med Hypotheses 2015; 85: 1-6.
31. Wang Y, Sun Z. Current understanding of klotho. Ageing Res Rev 2009; 8: 43-51.
32. Saito Y, Nakamura T, Ohyama Y, Suzuki T, Iida A, Shiraki-Iida T, Kuro-o M, NabeshimaY-I, Kurabayashi M, Nagai R. In vivo klotho gene delivery protects against endothelial dysfunction in multiple risk factor syndrome. Biochem Biophys Res Commun 2000; 276: 767-772.
33. Kuro-o M. A potential link between phosphate and aging—lessons from Klotho-deficient mice. Mech Ageing Dev 2010; 131: 270-275.
34. Gkentzi D, Efthymiadou A, Kritikou D, Chrysis D. Fibroblast growth factor 23 and Klotho serum levels in healthy children. Bone 2014; 66: 8-14.
35. Utsugi T, Ohno T, Ohyama Y, Uchiyama T, Saito Y, Matsumura Y, Aizawa H, Itoh H, Kurabayashi M, Kawazu S, Tomono S, Oka Y, Suga T, Kuro-o M, Nabeshima Y, Nagai R. Decreased insulin production and increased insulin sensitivity in the klotho mutant mouse, a novel animal model for human aging. Metabolism 2000; 49: 1118-1123.
36. Mitani H, Ishizaka N, Aizawa T, Ohno M, Usui S-i, Suzuki T, Amaki T, Mori I, Nakamura Y, Sato M, Nangaku M, Hirata Y, Nagai R. In vivo klotho gene transfer ameliorates angiotensin II-induced renal damage. Hypertension 2002; 39: 838-843.
37. Tohyama O, Imura A, Iwano A, Freund J-N, Henrissat B, Fujimori T, Nabeshima Y-I. Klotho is a novel β-glucuronidase capable of hydrolyzing steroid β-glucuronides. J Biol Chem 2004; 279: 9777-9784.
38. Chang Q, Hoefs S, Van Der Kemp A, Topala C, Bindels R, Hoenderop J. The β-Glucuronidase Klotho Hydrolyzes and Activates the TRPV5 Channel. Science 2005; 310: 490-493.
39. Palmer RM, Ferrige A, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 1987; 327: 524-526.
40. Liu F, Wu S, Ren H, Gu J. Klotho suppresses RIG-I-mediated senescence-associated inflammation. Nat Cell Biol 2011; 13: 254-262.
41. German DC, Khobahy I, Pastor J, Kuro-o M, Liu X. Nuclear localization of Klotho in brain: an anti-aging protein. Neurobiol Aging 2012; 33: 1483.e25-1483.e30.
42. Weinberg JM. Mitochondrial biogenesis in kidney disease. J Am Soc Nephrol 2011; 22: 431-436.
43. Simic P, Williams EO, Bell EL, Gong JJ, Bonkowski M, Guarente L. SIRT1 suppresses the epithelial-to-mesenchymal transition in cancer metastasis and organ fibrosis. Cell Rep 2013; 3: 1175-1186.
44. Hasegawa K, Wakino S, Yoshioka K, Tatematsu S, Hara Y, Minakuchi H, Washida N, Tokuyama H, Hayashi K, Itoh H. Sirt1 protects against oxidative stress-induced renal tubular cell apoptosis by the bidirectional regulation of catalase expression. Biochem Biophys Res Commun 2008; 372: 51-56.
45. Hasegawa K, Wakino S, Simic P, Sakamaki Y, Minakuchi H, Fujimura K, Hosoya K, Komatsu M, Kaneko Y, Kanda T, Kubota E, Tokuyama H, Hayashi K, Guarente L, Itoh H . Renal tubular Sirt1 attenuates diabetic albuminuria by epigenetically suppressing Claudin-1 overexpression in podocytes. Nat Med 2013; 19: 1496-1504.
46. Hasegawa K, Wakino S, Yoshioka K, Tatematsu S, Hara Y, Minakuchi H, Sueyasu K, Washida N, Tokuyama H, Tzukerman M, Skorecki K, Hayashi K, Itoh H. Kidney-specific overexpression of Sirt1 protects against acute kidney injury by retaining peroxisome function. J Biol Chem 2010; 285: 13045-13056.