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Protective Effects of Myricitrin and Vitamin E on Nephropathy of Aging Mice Model Induced By D-Galactose

D-Galaktoz ile İndüklenen Farelerde Yaşlanma Modelinin Nefropatisi Üzerine Mirisitrin ve Vitamin E'nin Koruyucu Etkileri

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ABSTRACT

Aim: Aging occurs in cells and tissues due to oxidative stress in physiological conditions. D-galactose (DG) is widely used to cause aging in animal studies. In this study, the renal protective effects of myricitrin and vitamin E in the aging mice model induced by DG was evaluated.

Material and Methods: Subcutaneous DG injection was used for induction of the aging model. 72 female mice were randomly divided into six groups: All groups were received DG at 500 mg/kg/d for six weeks. In the last 28 days, the groups treated with myricitrin subcutaneously received 5, 10, and 20 mg/kg/d, and the vitamin E group received 100 mg/kg/d by gavage. Urine and plasma albumin, BUN, creatinine levels, MDA, TAC, and kidney histological changes were evaluated.

Results: Plasma albumin was significantly decreased ($p=0.001$), but a significant increase in urine albumin ($p=0.001$), BUN ($p<0.001$), and creatinine ($p=0.010$) levels was observed in the DG group when compared with the control. Also, a significant increase in MDA levels ($p=0.002$) along with a significant decrease in TAC ($p=0.012$) was observed. Histopathological changes such as congestion of erythrocytes ($p<0.001$), infiltration of inflammatory cells ($p<0.001$), and proximal tubule cell damage ($p=0.004$) significantly increased, while glomerulus diameter significantly decreased ($p=0.038$) in comparison to the control. Administration of myricitrin and vitamin E showed a significant ameliorative effect on all studied variables.

Conclusion: The improvement effects of myricitrin on DG-induced kidney damage was approximately equivalent to vitamin E. Myricitrin and vitamin E could have beneficial effects on the nephropathy of aging model.

Keywords: Aging; D-galactose; nephropathy; mice; myricitrin.

ÖZ

Amaç: Fizyolojik koşullarda oksidatif strese bağlı olarak hücre ve dokularda yaşlanma meydana gelir. D-galaktoz (DG), hayvan çalışmalarında yaşlanmaya neden olmak için yaygın olarak kullanılmaktadır. Bu çalışmada, DG ile indüklenen farelerde yaşlanma modelinde mirisitrin ve vitamin E'nin renal koruyucu etkileri değerlendirildi.

Gereç ve Yöntemler: Yaşlanma modelinin uyarılması için subkutan DG enjeksiyonu uygulandı. 72 dişi fare rastgele şekilde altı gruba ayrıldı: Tüm gruplara altı hafta boyunca 500 mg/kg/gün DG verildi. Uygulamanın son 28 günü mirisitrin ile tedavi edilen gruplara subkutan olarak 5, 10 ve 20 mg/kg/gün, E vitamini grubuna ise gavaj yoluyla 100 mg/kg/gün uygulandı. İdrar ve plazma albümini, BUN, kreatinin seviyeleri, MDA, TAC ile böbrek histolojik değişiklikleri değerlendirildi.

Bulgular: Kontrol grubu ile karşılaştırıldığında, DG grubunda plazma albumin düzeyi önemli ölçüde azalırken ($p=0.001$), idrar albümini ($p=0.001$), BUN ($p<0.001$) ve kreatinin ($p=0.010$) düzeylerinde anlamlı bir artış gözlendi. Ayrıca MDA düzeylerinde anlamlı artış ($p=0.002$) ile birlikte TAC'de de anlamlı düşüş ($p=0.012$) gözlendi. Kontrol ile karşılaştırıldığında, eritrosit konjesyonu ($p<0.001$), inflamatuar hücrelerin infiltrasyonu ($p<0.001$) ve proksimal tübül hücre hasarı ($p=0.004$) gibi histopatolojik değişiklikler önemli ölçüde artarken, glomerül çapı önemli ölçüde azaldı ($p=0.038$). Mirisitrin ve E vitamininin uygulanması, çalışılan tüm değişkenler üzerinde önemli bir iyileştirici etki göstermiştir.

Sonuç: Mirisitrinin DG'nin neden olduğu böbrek hasarı üzerindeki iyileştirme etkileri yaklaşık olarak E vitaminine eşdeğer idi. Mirisitrin ve E vitamini, yaşlanma modelinin nefropatisi üzerinde faydalı etkilere sahip olabilir.

Anahtar kelimeler: Yaşlanma; D-galaktoz; nefropati; fare; mirisitrin.

INTRODUCTION

Aging is a process that the mechanism of body organs changes with time gradually. This process can enhance the risk of damage to various parts of the body, including the kidneys (1).

Body weight loss, occurs in some experimental diabetic models such as streptozotocin administration (2) while, in the diabetic models in which, a carbohydrate such as sucrose is used to induce diabetes, body weight increases (3). Also, the body weight of mice increases in the high-fat diet (4). In contrast to these studies, one study showed that the body weight of diabetic rats did not change significantly in the high-fat diet (5). D-galactose (DG) can cause the progression of aging in the brain, kidney, and liver (6), and this aging effect is contributed to nephropathy (7). In addition, DG induces oxidative stress by increasing lipid peroxidation that causes similar symptoms to normal aging (8). Many morphological changes are observed in the kidney with aging such as glomerular and tubular destruction. In addition, the number and volume of glomeruli and kidney tubules decrease in the aging process (9). Antioxidant agents are capable to protect cells and tissues against oxidative damage. Flavonoids have strong medicinal properties such as antioxidant, anti-inflammatory, and anti-diabetic effects (10). Myricitrin is a main component of *Myricacerifera* that has antioxidant and anti-inflammatory properties (11). Myricitrin could decrease oxidative damage by decreasing MDA levels and ameliorated antioxidant enzyme activity during cell damage caused by reactive oxygen species (ROS). In addition, this antioxidant agent has protective properties against cellular apoptosis caused by oxidative stress (12). In a previous study, myricitrin improved diabetic nephropathy by enhancement of antioxidant enzyme activity and reduction of oxidative stress (13). Vitamin E (Vit E) has antioxidant and anti-inflammatory effects. Protective effects of Vit E on kidney damages have been cleared previously (14). The purpose of this study was to investigate the protective effects of myricitrin and Vit E on DG-induced kidney damages in mice.

MATERIAL AND METHODS

Animals

In our study, 72 adult female NMRI mice, 4 months old (25-30 g) were taken from Ahvaz Jundishapur University of Medical Sciences (AJUMS) animal facility. The study was performed according to the principles and guidelines of AJUMS laboratory animal care with the code of the ethics committee (IR. AJUMS.REC.1398.010). Mice were kept in a 12 hour light/12 hour dark cycle at 20±4 °C and with free access to water and rodent chow.

Sample Size

The number of animals was determined according to our previous studies with considering the values of α=0.05 and β=0.2, and with the help of Minitab software. Assuming a 35% drop, 12 mice in each group were placed (15,16).

Chemicals

Myricitrin was purchased from Ava Chem San Antonio, U.S.A (purity 98%), and DG was bought from Merck, Germany. Xylazine2% and Ketamine10% from Alfasan Co. (Netherlands), malondialdehyde (MDA) and total antioxidant capacity (TAC) kits were purchased.

Experimental Design

The design of this experiment is schematically presented in Figure 1. In the current study, after one week of acclimatization, the aging model was induced by subcutaneous (SC) injection of DG 500 mg/kg/d for six weeks (17). The animals were divided randomly into six groups, as well as, 12 animals were placed in each group:

1) Control: mice administered SC vehicle; normal saline for six weeks (0.1 ml) and drinking water by gavage from the beginning of the third week to the end of the experiment.
2) DG: mice injected SC with DG for six weeks and concomitant gavage of normal saline from the beginning of the third week to the end of the experiment.
3-5) Myricitrin+DG: DG mice that simultaneously treated by gavage with myricitrin 5, 10, and 20 mg/kg/d from the beginning of the third week to the end of the experiment.
6) DG+Vit E: DG mice that received Vit E 100 mg/kg/d by gavage from the beginning of the third week to the end of the experiment (18).

Finally, in order to urine collection, animals were placed in metabolic cages for 24 hours. The animals were anesthesiaed by the combination of ketamine and xzylazin (90/10 mg/kg, respectively) 24 hours after the last drug treatment, sacrificed, and plasma samples were obtained after cardiac puncture, blood collection, and centrifuging at 3000 rpm for 15 min. Plasma and urine samples were stored at -20 °C and used for the evaluating of BUN, albumin, and Cr levels. After removing the kidneys and washing them with normal saline, the left kidney

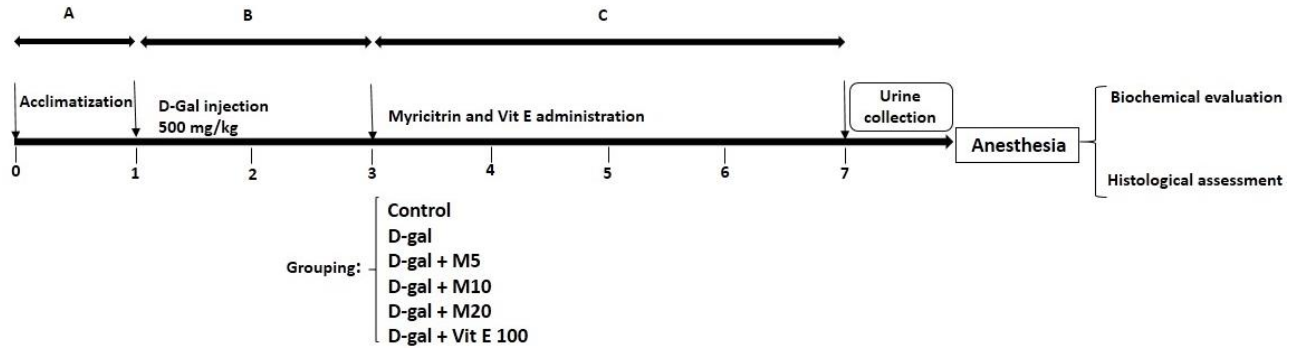

Figure 1. Diagrammatic representation of experimental protocol (time: week)

A: Acclimatization lasted a week, B: induction of aging by D-galactose injection (500 mg/kg), C: Treatment with myricitrin (5, 10 and 20 mg/kg) and Vitamin E (100 mg/kg), at the end of third week, mice divided into following groups: Control, DG: D-galactose 500 mg/kg/d, DG+M5: D-galactose 500 mg/kg/d + myricitrin 5 mg/kg, DG+M10: D-galactose 500 mg/kg/d + myricitrin 10 mg/kg, DG+M20: D-galactose 500 mg/kg/d + myricitrin 20 mg/kg, DG+Vit E 100: D-galactose 500 mg/kg/d + Vitamin E 100 mg/kg. After anesthesia, the biochemical and histological assays were evaluated

snap-frozened in liquid nitrogen, and reserved at -80 °C to evaluate MDA and CAT activity. Another one used for histological study.

Experimental Measurement

The left kidney was defrized and homogenized in ice-cold Tris-HCl buffer (0.1 M, pH 7.4, ratio 1:4 w/v), centrifuged for 15 min at 10,000 g, and supernatants were maintained at -20 °C and used for measurement of kidney MDA and CAT activity by commercial assay kits (ZellBio GmbH, Germany). Also, the concentration of albumin, BUN, and Cr were measured by auto analyzer (BT3000, Italy) devices and biochemical assay kits (Pars Azmoon, Iran).

Histological Assessment

The kidney washed and fixed in 10% neutral formalin solution, dehydrated through a series of graded alcohol, placed in paraffin, cut into 5 μm sections using a microtome (Leica RM 2125, Leica Microsystems Nussloch GmbH, Germany) and stained with hematoxylin and eosin (H&E) to evaluate tissue changes such as red blood cells (RBC) congestion, inflammation, and proximal tubular cell damage under a digital research microscope (BMZ-04-DZ, Behin Pajouhesh ENG. CO., Iran).

Statistical Analysis

The data were presented as mean±standard deviation and analyzed by GraphPad Prism 9 for windows (GraphPad Software, San Diego, CA). The Shapiro-Wilk test was used to examine normality and the Levene test for homogeneity of variance. One-way analysis of variance (ANOVA) was conducted for differences among the groups followed by post hoc Tukey's HSD test. Significant difference was set at $p<0.05$.

RESULTS

Effect of Myricitrin and Vitamin E on Body Weight

As shown in Figure 2, body weight was significantly higher in DG compare to myricitrin treated groups ($p<0.001$) and Vit E received group ($p<0.001$). Kidney weight in DG ($p<0.001$) and M5 ($p=0.020$) groups was significantly lower than control. Kidney weight in M5 ($p=0.002$), M10, M20, and Vit E groups were significantly higher ($p<0.001$) than DG. The percentage of the kidney to body weight ratio was markedly reduced in the DG compared to the control ($p<0.001$). Myrycitrin receiving mice and Vit E group significantly improved it ($p<0.001$).

Effect of Myricitrin and Vitamin E on Kidney Function

Urine albumin had a significant difference between DG and control groups ($p=0.001$). Administration of M5 ($p=0.050$), M10 ($p=0.010$), M20 ($p=0.009$), and vitamin E ($p=0.006$), showed a significant decrease in urine albumin levels in comparison to the DG group. Also, the plasma albumin level of DG was markedly lower than control ($p=0.001$) and the ameliorative effect of M5, M10 groups ($p=0.002$), M20 ($p=0.005$), and Vit E ($p=0.003$) was observed. There was a significant increase of BUN in the DG ($p<0.001$) compared to the control, and administration of M10 ($p=0.010$) and Vit E ($p=0.020$) significantly decreased it. Decreased levels of BUN in M5 and M20 groups were not significant compared to the DG. Plasma levels of Cr increased in the DG group compared to control ($p=0.010$), and it was decreased in M5, M10, Vit E groups ($p=0.040$), and M20 ($p=0.010$, Table 1).

Effect of Myricitrin and Vitamin E on Antioxidant Activity in the Kidney

A dramatic increase effect of DG on lipid peroxidation was observed through increasing of MDA levels in DG mice ($p=0.002$). Also, DG had a decreasing effect on TAC

Table 1. Effect of different doses of myricitrin and Vit E on urine albumin and plasma level of albumin, BUN and creatinine

	Control	DG	DG+M5	DG+M10	DG+M20	DG+Vit E	p
Urine Alb (mg/24h)	155.3±18.78	232.7±21.74**	180.0±19.48$^{\#}$	169.9±17.3$^{\#}$	168.6±24.82$^{\#\#}$	165.5±26.96$^{\#\#}$	**0.001**
Plasma Alb (g/dl)	2.92±0.08	2.65±0.1**	2.90±0.08$^{\#\#}$	2.90±0.06$^{\#\#}$	2.88±0.05$^{\#\#}$	2.90±0.04$^{\#\#}$	**<0.001**
BUN (mg/dl)	52.5±3.07	70.6±2.96***	59.6±5.03	56.7±2.6$^{\#}$	59.8±2.69	57.5±9.52$^{\#}$	**0.002**
Creatinine (mg/dl)	0.22±0.04	0.37±0.06*	0.24±0.04$^{\#}$	0.25±0.05$^{\#}$	0.23±0.05$^{\#}$	0.25±0.04$^{\#}$	**0.008**

DG: D-galactose 500 mg/kg/d, DG+M5: D-galactose 500 mg/kg/d + myricitrin 5 mg/kg, DG+M10: D-galactose 500 mg/kg/d + myricitrin 10 mg/kg, DG+M20: D-galactose 500 mg/kg/d + myricitrin 20 mg/kg, DG+Vit E: D-galactose 500 mg/kg/d + Vitamin E 100 mg/kg, Alb: albümin, *: different from Control, $^{\#}$: different from DG, 1 symbol $p<0.050$, 2 symbols $p<0.010$, 3 symbols $p<0.001$

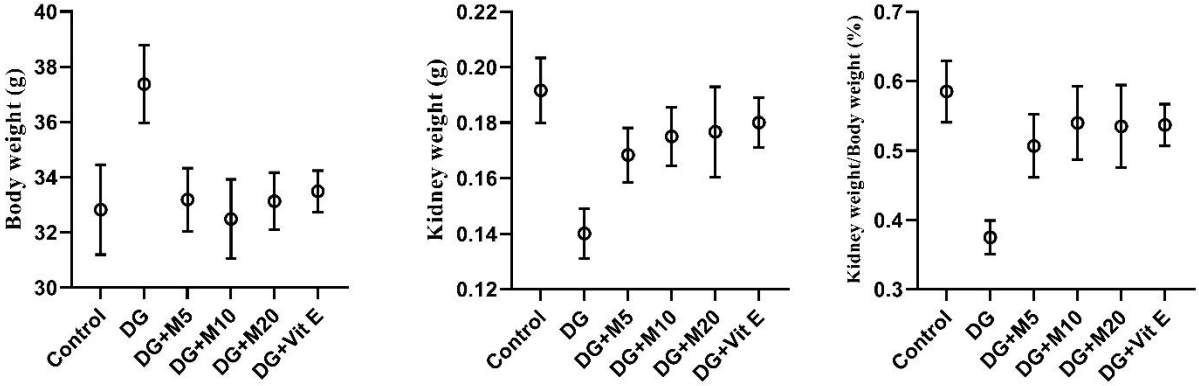

Figure 2. The effect of myricitrin and vitamin E on the body weight, kidney weight, and kidney to body weight ratio

DG: D-galactose 500 mg/kg/d, DG+M5: D-galactose 500 mg/kg/d + myricitrin 5 mg/kg, DG+M10: D-galactose 500 mg/kg/d + myricitrin 10 mg/kg, DG+M20: D-galactose 500 mg/kg/d + myricitrin 20 mg/kg, DG+Vit E: D-galactose 500 mg/kg/d + Vitamin E 100 mg/kg

level, as observed in DG animals (p=0.012). Myricitrin and Vit E remarkably decreased lipid peroxidation of kidney tissue through decreasing MDA and increasing TAC levels. The effect of M5 on reducing MDA (p=0.008) and increasing TAC (p=0.035) was more pronounced than M20 group (Table 2).

Effect of Myricitrin and Vitamin E on Renal Histopathology

As shown in Table 3 and Figure 3, the natural appearance of the kidney decreased in DG mice, and treatment with myricitrin and Vit E improved it. Glomerulus diameter markedly decreased in DG (p=0.038), M5 (p=0.044), and M10 (p=0.047) in comparison with the control group, but M20 and Vit E improved these changes (p=0.049). Furthermore, proximal tubule damage (brush border loss, tubular dilation, and vasodilatation) was increased in the DG, M5, and M10 groups (p=0.009), and it was effectively improved by the M10 (p=0.046), M20 and Vit E (p=0.008). Inflammatory cell infiltration has occurred in groups that received DG and administration of myricitrin and Vit E had a preventive effect. Furthermore, it was revealed that accumulation of RBCs had a remarkable increase in the DG group (p<0.001), and treatment with M10 (p=0.048), M 20, and Vit E (p<0.001) effectively improved this parameter.

DISCUSSION

The present study indicated that the renal dysfunction induced by DG improved by the administration of myricitrin and Vit E. In agreement with this result, it was shown that DG promotes aging alterations (15). Also, we found that DG can induce nephropathy through increases in urine albumin, BUN, plasma Cr, and a decrease of plasma albumin level. Increased renal biomarkers may be due to acute nephropathy that is related to tubular dysfunction (19). Endogenous plasma creatinine levels indicate changes in kidney function caused by diet and diabetes in mice (20). It is widely known that BUN and Cr are two important factors in the evaluation of renal function (21). Also, an increase in BUN level is a predictable factor during renal damage (22). Plasma Cr is a more specific factor than urea level to evaluate kidney function; because Cr has all the features needed for a perfect filtration indicator. It was revealed that elevation of urea and plasma Cr along with a reduction of plasma albumin level occurs in nephropathy (23). The increase of BUN and Cr levels are related to glomerular filtration dysfunction caused by DG. The present study indicated that myricitrin could ameliorate albumin, BUN, and Cr levels by improvement of the filtration function.

It was considered that DG promotes oxidative damage in the kidney of rodents and causes the excessive generation of ROS and diminishes endogenous antioxidant activity (24). Thereby, inhibition of oxidative stress provides a therapeutic strategy against renal injury. Previously, the protective effects of Vit E on kidney function was investigated. It was shown that vit E improves the antioxidant defense system by suppression of free radicals (25). Myricitrin eliminates the overproduction of ROS during kidney injury in rats (26). Also, a previous study revealed that myricitrin and Vit E had a preventive effect against DG induced lipid

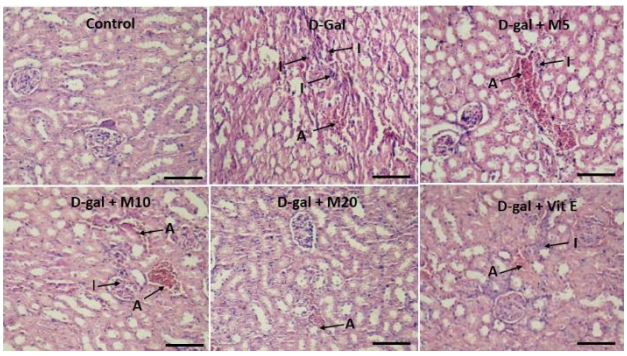

Figure 3. Effect of myricitrin and vitamin E on renal histology (scale bar: 50 µm)

DG: D-galactose 500 mg/kg/d, DG+M5: D-galactose 500 mg/kg/d + myricitrin 5 mg/kg, DG+M10: D-galactose 500 mg/kg/d + myricitrin 10 mg/kg, DG+M20: D-galactose 500 mg/kg/d + myricitrin 20 mg/kg, DG+Vit E: D-galactose 500 mg/kg/d + Vitamin E 100 mg/kg, I: inflammation; A: accumulation of red blood cells

Table 2. Effect of different doses of myricitrin and Vit E on MDA and TAC level in the kidney

	Control	DG	DG+M5	DG+M10	DG+M20	DG+Vit E	p
MDA (µM/g tissue)	6.08±0.7	8.85±0.6**	5.32±0.5$^{\#\#\#}$	7.12±0.5$^{\#}$	7.60±0.4$^{\$\$}$	6.83±0.4$^{\#}$	**0.004**
TAC (mM/g tissue)	0.77±0.02	0.5±0.04*	0.83±0.08$^{\#\#}$	0.71±0.03$^{\#}$	0.63±0.07$^{\$}$	0.76±0.10$^{\#}$	**0.034**

DG: D-galactose 500 mg/kg/d, DG+M5: D-galactose 500 mg/kg/d + myricitrin 5 mg/kg, DG+M10: D-galactose 500 mg/kg/d + myricitrin 10 mg/kg, DG+M20: D-galactose 500 mg/kg/d + myricitrin 20 mg/kg, DG+Vit E: D-galactose 500 mg/kg/d + Vitamin E 100 mg/kg, MDA: malondialdehyde, TAC: total antioxidant capacity, *: different from Control, $^{\#}$: different from DG, $^{\$}$: different from M5, 1 symbol p<0.050, 2 symbols p<0.010, 3 symbols p<0.001

Table 3. Effect of different doses of myricitrin and Vit E on kidney histology

	Control	DG	DG+M5	DG+M10	DG+M20	DG+Vit E	p
Glomerulus diameter (µm)	239.3±16.3	190.2±12.1*	195.4±9.3*	210.5±13.8*	225.7±12.9$^{\#}$	223.8±13.4$^{\#}$	**0.025**
Proximal tubule damage (%)	0.1±0.04	0.6±0.06**	0.53±0.11**	0.33±0.07$^{**\#}$	0.16±0.05$^{\#\#}$	0.17±0.04$^{\#\#}$	**0.004**
Inflammatory cell infiltration	0.09±0.01	2.1±0.31***	0.83±0.23$^{***\#}$	0.52±0.18$^{***\#\#}$	0.12±0.04$^{*\#\#\#}$	0.11±0.03$^{*\#\#\#}$	**<0.001**
Accumulation of RBCs	0.08±0.02	2.3±0.19***	1.82±0.25***	1.12±0.29$^{***\#}$	0.52±0.09$^{**\#\#\#}$	0.58±0.1$^{**\#\#\#}$	**<0.001**

DG: D-galactose 500 mg/kg/d, DG+M5: D-galactose 500 mg/kg/d + myricitrin 5 mg/kg, DG+M10: D-galactose 500 mg/kg/d + myricitrin 10 mg/kg, DG+M20: D-galactose 500 mg/kg/d + myricitrin 20 mg/kg, DG+Vit E: D-galactose 500 mg/kg/d + Vitamin E 100 mg/kg, RBCs: red blood cells, *: different from Control, $^{\#}$: different from DG, 1 symbol p<0.050, 2 symbols p<0.010, 3 symbols p<0.001

peroxidation in the reproductive system (15). In addition, a report verified that DG increased lipid peroxidation (MDA) and reduced SOD in the testis of DG-induced aging mice model (27). In the present study, myricitrin and Vit E restored the antioxidant defense system through enhancement of the antioxidant enzyme activity and reduction of MDA in the kidney of DG treated mice. It seems that myricitrin at a low dose has a better function than Vit E.

Hypoalbuminemia routinely occurs in nephropathy (28). Albuminuria is one of the main symptoms of kidney damage (29). Structural dysfunction of the glomerular filtration barrier causes passes of serum protein into the urine and eventually causes albuminuria. It seems that, reduction of plasma albumin in DG mice is associated with renal dysfunction in our study. In agreement with our study, it has been reported that proximal damage is also involved in the reduction of serum albumin due to oxidative stress induced by DG (30). In another study, it was shown that increased proximal damage of the glomerulus in the DG receiving mice decreased the functional capacity of the nephrons (31). Decreased plasma albumin levels can also be related to decreased reabsorption of albumin by damaged tubules. This may be the cause of albuminuria in the DG group. Myricitrin and Vit E improved tubular damages and glomerular degradation that might be the cause of albuminuria due to DG.

In carbohydrate-induced diabetic models such as sucrose, body weight enhances due to the accumulation of fat mass in the abdominal area (3,32). Therefore, it is concluded that the ratio of organ weight to body weight decreases in these models. In the current study, due to the carbohydrate nature of DG, body weight increased and the percentage of the kidney weight to body weight ratio markedly decreased in the DG group. Administration of myricitrin and Vit E could improve this ratio near to the normal range. Furthermore, the diameter of the glomerulus decreased in the DG group, whereas this reduced effect is markedly lower in mice that also received myricitrin or vitamin E. It has been reported that the total number of glomeruli in the kidney reduces with age (33). Thus, a reduction in the percentage of the kidney weight to body weight in the DG group may be related to a decrease in the diameter of the glomerulus and loss of glomeruli.

DG can cause infiltration of inflammatory cells in the kidney tissue. Flavonoids have anti-inflammatory effects (34). An increase in inflammatory cells in kidney tissue shows that DG disrupts the function of enzymes and proteins in the interstitial tissue of the kidney, imbalances the antioxidant defense system, generates ROS, and eventually causes an inflammatory response (35). Therefore, a decrease in inflammatory cell number in mice that received myricitrin and Vit E is associated with their anti-inflammatory activities. It was reported that Vit E diminishes the peroxidation of unsaturated membrane lipids through the scavenging of oxygen (36). In this study, Vit E has improved histological alterations and plasma levels of albumin, BUN, and creatinine in comparison to the DG group. So, we found that Vit E is a protective component for kidney tissue against peroxidative damage. Myricitrin at low doses exerted antioxidant properties by reducing MDA level.

CONCLUSION

In brief, our study showed that myricitrin, given its high antioxidant properties, may be a candidate for the prevention or treatment of aging-relative disorders approximately equivalent to Vit E.

Ethics Committee Approval: The study was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Science (07.05.2019, IR.AJUMS.REC.1398.010).

Conflict of Interest: None declared by the authors.

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Author Contributions: Idea/Concept: AA; Design: VR, MO; Data Collection/Processing: VR, LK, MO; Analysis/Interpretation: AA; Literature Review: VR, AA; Drafting/Writing: AA; Critical Review: VR, MO.

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