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ORIGINAL ARTICLE

Hyperglycemia-induced oxidative stress in isolated proximal tubules of mouse: the in vitro effects of myricitrin and its solid lipid nanoparticle

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ABSTRACT

Context: The hyperglycemia (Hyper) induces oxidative stress in kidney tubular cells. Myricitrin (Myr) has an antioxidant effect along with low bioavailability.

Objective: The present research investigated the effects of Myr and its solid lipid nanoparticles (SLN) on isolated proximal tubules exposed to the hyperglycemic condition.

Materials and Methods: In this experimental study, the proximal tubules of mice were dissected by the microdissection method and the tubules were prepared for experimental or Real Time-PCR measurement.

Results: The malondialdehyde level, transforming growth factor-β, nuclear factor kappa B and Bax genes expression increased in Hyper and decreased in Hyper + Myr and its SLN-treated groups compared to Hyper. Superoxide dismutase, total antioxidant capacity, the viability of proximal tubules and Bcl-2 gene expression decreased in untreated Hyper and increased in all treatment groups compared to Hyper.

Conclusion: The hyperglycemia-induced oxidative disorder, inflammation and apoptosis in proximal tubules and that administrating Myr and its SLN improved them.

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KEYWORDS
Hyperglycemia; myricitrin; solid lipid nanoparticle; proximal tubule; mouse

Introduction

The hyperglycemic condition induces oxidative stress, inflammation and attenuates natural antioxidant defenses. Oxidative stress can impair cellular function via oxidized membrane lipids or inactive enzymes (Shokrzadeh *et al.* 2017). Kidney tubular function changes during hyperglycemia (Hyper). Recently, it has been observed that the hyperglycemic medium initiates oxidative stress in both mesangial and kidney tubular epithelial cells leading to their dysfunction and apoptosis via the formation of reactive oxygen species (ROS) (Manda *et al.* 2015). Hyper can cause an up-regulation of the transforming growth factor-β (TGF-β) and nuclear factor kappa B (NF-κB) expression by increasing oxidative stress. TGF-β and NF-κB have deleterious effects on the pathogenesis of various diabetic complications by the decreased capacity of the cellular antioxidant defense. Flavonoids and related phenolic compounds protect various cell types from oxidative stress through different mechanisms such as direct and indirect antioxidant activities (Han *et al.* 2005). Myricitrin (Myr) is a natural flavonoid glycoside belonging to the flavonol subgroup. It is abundantly distributed in the fruits, branches, bark and leaves of *Myrica rubra*. Myr has antioxidant, antinociceptive, anti-inflammatory, anti-apoptotic and antifibrotic activities (Wang *et al.* 2017). Our previous research revealed that this plant-derived antioxidant improves hyperglycemia-induced oxidative stress and decreases the cellular apoptosis of myotubes (Ahangarpour *et al.* 2018a).

Flavonoids have shown low bioavailability under experimental conditions (Thilakarathna and Rupasinghe 2013). Flavonoid glycoside is large and highly polar, thus, it cannot cross the membranes easily and may be metabolized by glycosidases enzymes inside the cell. Therefore, these events can lead to reducing the biological activity of flavonoid glycoside (Fernandez *et al.* 2009). Solid lipid nanoparticles (SLNs) have been employed to increase the storage stability, absorption and bioavailability and reduce side effects of some compounds such as flavonoids (Hu *et al.* 2004). Accordingly, based on the effect of hyperglycemia on inducing oxidative stress in proximal tubules and the antioxidant effects and poor bioavailability of Myr, the present research

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investigated the antioxidant, anti-inflammatory and antiapoptotic effects of this flavonoid glycoside and its SLN on isolated proximal tubules exposed to the hyperglycemic condition.

Materials and methods

Reagents

Myr (Purity 98%; AvaChem Scientific, USA), D-glucose, Saline 0.9%, Tween80 (Sinopharm chemical reagent co Ltd, China), Ketamine & Xylazine (Alfasan, Netherland), Phosphate buffered saline (PBS) (Pharmaceutical Technology Development Center of AJUMS, Ahvaz, Iran; pH:7.4), Dulbecco's Modified Eagle's Medium (DMEM) (Solar bio, South Korea), Penicillin-streptomycin (Sigma-Aldrich, Canada), malondialdehyde (MDA), total antioxidant capacity (TAC), catalase (CAT) (Zellbio Co, Germany), and superoxide dismutase (SOD) (Randox. United Kingdom) assay kits, RNeasy Mini Kit (Qiagen, Valencia, CA), cDNA Synthesis Kit, Sybergreen (Takara, Japan), TGF-β, NF-κB, the primers of Bax and Bcl-2 (Microsynth, Switzerland), Fetal bovine serum (FBS), Thiazolyl blue tetrazolium bromide (MTT), Dimethyl sulphoxide (DMSO) (Bio Idea, Iran).

Preparation and characterization of SLNs of Myr

SLNs of Myr was prepared according to the cold homogenization method explained in the previous study (Ahangarpour *et al.* 2018b).

Preparation of kidney for microdissection of the proximal tubule

The three months old male NMRI mice in this experimental study (25–30 g) were obtained from the Ahvaz Jundishapur University of Medical Sciences (AJUMS) animal facility and, were treated in accordance with the principles and guidelines on animal care of AJUMS as reviewed by an ethics committee (IR.AJUMS.REC.1395.136), and kept at a 20 °C ± 4 °C temperature with a 12 h light/12 h dark cycle. They had access to tap water and commercial chow *ad libitum*. The microdissection of the proximal tubules was performed as described by Schafer *et al.* (1997) and Levillain and Hus-Citharel (1998) with a little modification. In brief, the mice were anesthetized by Ketamine/Xylazine (70/10 mg/kg). The left kidney was exposed, removed and washed in the PBS (10 mL) containing penicillin-streptomycin (100 U/mL–100 µg/mL, respectively) 5x, 3x, 2x and 1x successively. Following that, the kidney decapsulated was cut in 1.0-mm-thick sagittal slices and was immersed in the DMEM medium containing 0.5 mg/mL of collagenase P under 5% CO_2 at 37 °C condition for 10 min with mild shaking. After centrifugation at 3000 rpm for 2 min, the enzyme-containing solution was removed and the cold DMEM medium was replaced. Finally, the proximal tubules were detected under a light microscope (Figure 1).

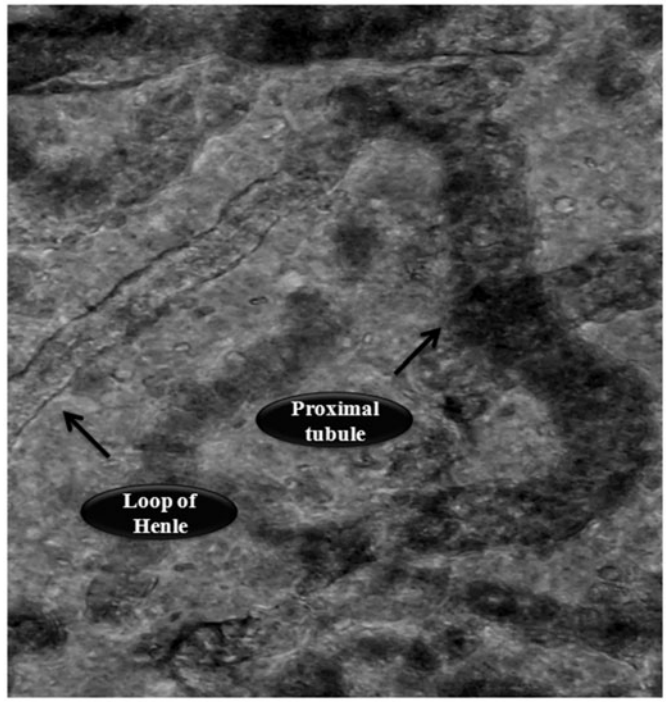

Figure 1. Isolated proximal tubule. (×40 magnification).

Experimental design

Proximal tubules separated in 25cm^2 cell culture flasks and divided into 8 groups ($n = 3$; 8 proximal tubules in each culture flask): control (maintained in the medium containing 5 mM D-glucose), Hyper (maintained in the medium containing 25 mM D-glucose), Hyper + Myr 1, 3 and 10 µM, Hyper + SLN containing Myr 1, 3 and 10 µM (maintained in the medium containing 25 mM D-glucose and Myr or its SLN for 48 h) (Huang *et al.* 2014, Senesi *et al.* 2016). After the treating period, all proximal tubules were separated using trypsin-EDTA 0.05% and centrifuged at 1300 rpm for 15 min. Then re-suspended in 0.5 mL of PBS (pH: 7.4), and lysed by the administration of a Teflon homogenizer. Next, the samples were kept at −80 °C after centrifuging at 2000 rpm for 10 min and until the experimental and real-time PCR measurements were made.

MDA, TAC, CAT and SOD measurement

The homogenate of the proximal tubule's supernatant was used to measure the levels of MDA, TAC, CAT and SOD by specific commercial kits.

MTT assay experiment

The isolated proximal tubules were kept in the medium containing 0.5 mg/mL of 3–(4, 5-Dimethylthiazol-2-yl) and 2, 5-Diphenyltetrazolium Bromide for 4 h (at 5% of CO2 and 37 °C). Subsequently, 100 µL DMSO was added to the achieved crystals of purple formazan and was shaken for 15 min. Then, the MTT concentration absorbance was read at 540 nm using an ELISA reader (Absorbance Microplate Reader, USA) (Son *et al.* 2015).

Assessment of pro-inflammatory and apoptotic gene expression

Total RNA was purified from the proximal tubule by RNeasy mini kit according to the commercial instruction. cDNA was synthesized using Reverse Transcriptase kit according to the manufacturer's instructions. The Real-time PCR protocol was performed using SYBR Green Master Mix in ABI Step One Plus instrument (Thermofisher, USA), and was prepared in duplicate. This protocol was conducted according to our previous study. The ratio of the relative TGF-β, NF-κB, Bax and Bcl-2 gene expression level was calculated using a comparative CT method ($2^{-\Delta\Delta CT}$) related to the expression level of GAPDH as the endogenous reference gene. The efficiency of the primers was investigated before employing this method. The forward and reverse primer sequences regarding TGF-β, NF-κB, Bax, Bcl-2 and GAPDH genes were (Son *et al.* 2015):

TGF-β forward primer: 5′-GCCTGGACACACAGTACAGC-3′
TGF-β reverse primer: 5′-TTGCAGGAGCGCACAATCAT-3′
NF-κB forward primer: 5′-GAGCTGCTGCATTTCCAGGT-3′
NF-κB reverse primer: 5′-AGGCCTGTTCCCTCTGACTC-3′
Bax forward primer: 5′-GCTGGACATTGGACTTCCTC-3′
Bax reverse primer: 5′-ACCACTGTGACCTGCTCCA-3′
Bcl-2 forward primer: 5′-GCTGGACATTGGACTTCCTC-3′
Bcl-2 reverse primer: 5′-GCTGGACATTGGACTTCCTC-3′
GAPDH forward primer: 5′-ACCCAGAAGACTGTGGATGG-3′
GAPDH reverse primer: 5′-TTCTAGACGGCAGGTCAGGT-3′

Statistical assessment

The data obtained were analyzed by SPSS software (version 16) with one-way analysis of variance (ANOVA) and *post hoc* least significant difference (LSD) tests. Furthermore, the data were represented as the mean ± standard error of the mean (SEM), and the statistical differences were considered significant at $p < .05$.

Results

Solid lipid nanoparticle characterizations

As demonstrated in our previous study, the particle appearance was spherical (Figure 2(A)), the mean particle size was 76.1 nm (50–150 nm) (Figure 2(B)).

Lipid peroxidation and antioxidant enzymes

The results of the MDA assessment indicated a significant increase in Hyper compared to the control group. This lipid peroxidation factor decreased in Hyper + Myr 1 μM, Hyper + SLN containing Myr 10 μM ($p < .01$) and other groups ($p < .05$) compared to Hyper (Figure 3(A)). SOD and TAC levels decreased in the untreated Hyper group compared to the control group ($p < .01$). Moreover, the tubular level of SOD increased in Hyper + Myr 1 μM ($p < .01$), Hyper + SLN containing Myr 10 μM ($p < .001$) and other groups ($p < .05$) compared to Hyper (Figure 3(B)). TAC measurement showed a significant increase in Hyper + Myr 1 ($p < .05$), 3 and 10 μM ($p < .01$) and Hyper + SLN containing Myr 1 ($p < .05$), 3 and 10 μM ($p < .01$) groups compared to Hyper (Figure 3(C)). CAT level revealed no significant differences between all groups in proximal tubules (Figure 3(D)).

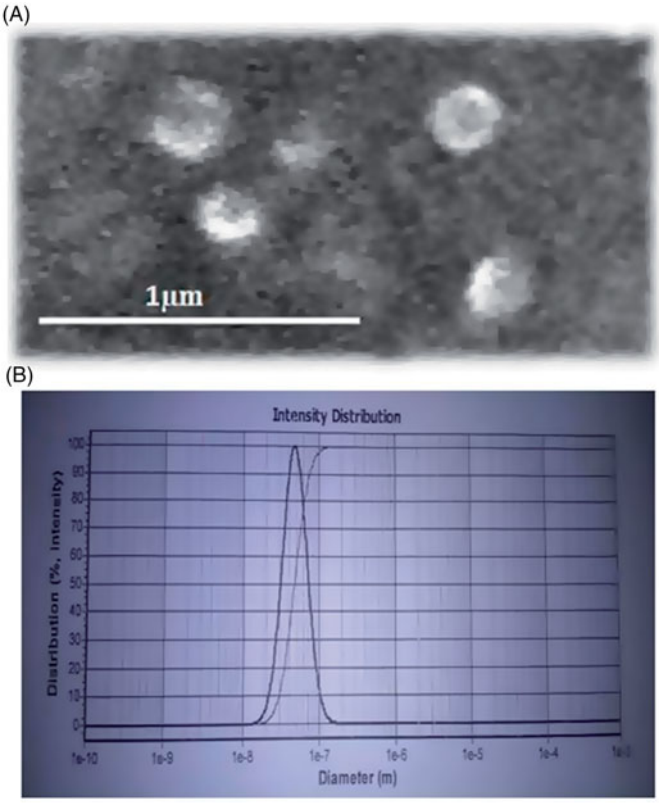

Figure 2. (A) The nanoparticles appearance under scanning electron microscope (SEM), (B) the nanoparticle size diagram.

The MTT level of proximal tubules

The viability of proximal tubules decreased in Hyper ($p < .001$) and Hyper + Myr 1 μM ($p < .05$) compared to the control group. This variable increased in Hyper + Myr 1 μM ($p < .01$) and other groups ($p < .001$) compared to Hyper (Figure 4).

Effect of Myr and its SLN on TGF-β and NF-κB gene expression

TGF-β showed a significant increase in untreated hyperglycemic proximal tubule ($p < .01$) and a significant decrease in hyperglycemic Myr 3 ($p < .01$) and 10 μM ($p < .001$) and SLN containing Myr-treated 10 μM ($p < .001$) proximal tubule compared to the control group. This gene expression decreased in Hyper + Myr 1 ($p < .05$), 3 ($p < .01$) and 10 μM ($p < .001$) and Hyper + SLN containing Myr 1, 3 ($p < .05$) and 10 μM ($p < .001$) compared to Hyper (Figure 5(A)). NF-κB assessment revealed a remarkable increase in Hyper ($p < .001$), Hyper + Myr 1, 3 and 10 μM ($p < .01$) and Hyper + SLN containing Myr 1 and 3 μM ($p < .05$) compared to the control group. This variable decreased in all doses of treated Myr ($p < .05$) and Hyper + SLN containing Myr 1, 3 ($p < .01$) and 10 μM ($p < .001$) groups compared to Hyper (Figure 5(B)).

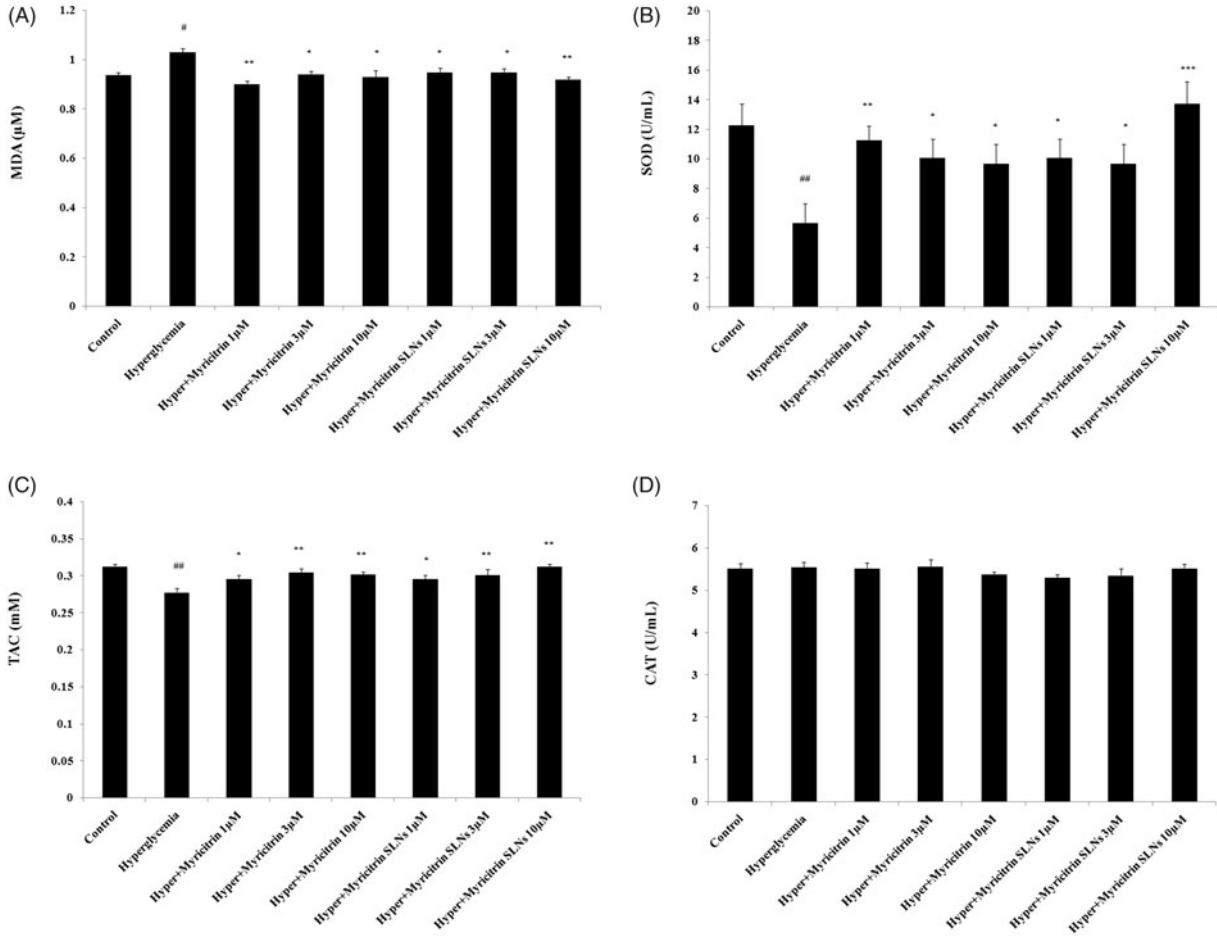

Figure 3. The effects of myricitrin and SLN containing myricitrin on lipid peroxidation and antioxidant enzyme level of the proximal tubules. Data are presented as mean ± SEM; $n = 3$; eight proximal tubules in each cell culture flask; $^{\#}p < .05$ and $^{\#\#}p < .01$ compared with the control, $^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$ compared with the Hyper (one-way analysis of variance (ANOVA)), followed by *post hoc* least significant difference (LSD) tests). (A) Malondialdehyde (MDA), (B) Superoxide dismutase (SOD), (C) Total antioxidant capacity (TAC), (D) Catalase (CAT).

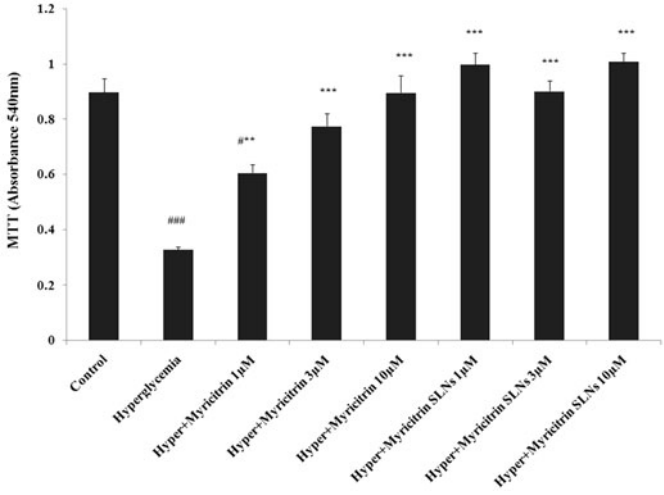

Figure 4. The MTT level in proximal tubules. Data are presented as mean ± SEM; $n = 3$; eight proximal tubules in each well plate; $^{\#}p < .05$, $^{\#\#}p < .01$ and $^{\#\#\#}p < .001$ compared with the control, $^{*}p < .05$ and $^{***}p < .001$ compared with the Hyper (one-way analysis of variance (ANOVA)), followed by *post hoc* least significant difference (LSD) tests).

Apoptotic and antiapoptotic gene expression

Bax gene expression increased in Hyper ($p < .001$), Hyper + Myr 1 ($p < .001$) and 3 μM ($p < .01$) and Hyper + SLN containing Myr 1 and 3 μM ($p < .05$) compared to the control group. This gene assessment revealed a significant decrease in Hyper + Myr 1 μM ($p < .05$) and other groups ($p < .001$) when compared with Hyper (Figure 6(A)). The proximal tubule level of Bcl-2 decreased in Hyper ($p < .05$) and increased in other groups ($p < .001$) compared to the control group. This antiapoptotic gene expression increased in Hyper + Myr 1 μM ($p < .05$) and other groups ($p < .001$) compared to Hyper (Figure 6(B)).

Discussion

The results of the present study indicated that Myr and its SLN decreased the lipid peroxidation and increased the level of antioxidant enzymes in proximal tubules which were altered by hyperglycemia. The hyperglycemic condition induced diabetic nephropathy through the production of free radicals and oxidant stress. The results of in vivo and in vitro studies indicated that the overproduction of superoxide anion and ROS in proximal tubules occurs in response to high glucose concentrations (Rosca *et al.* 2012). Antioxidant agents such as taurine have a beneficial effect on hyperglycemia-induced tubular damage by inhibition of oxidative stress and act as an endogenous antioxidant in tubule cells (Verzola *et al.* 2002). Therefore, consistent with previous studies, Myr and SLN containing Myr improved lipid peroxidation

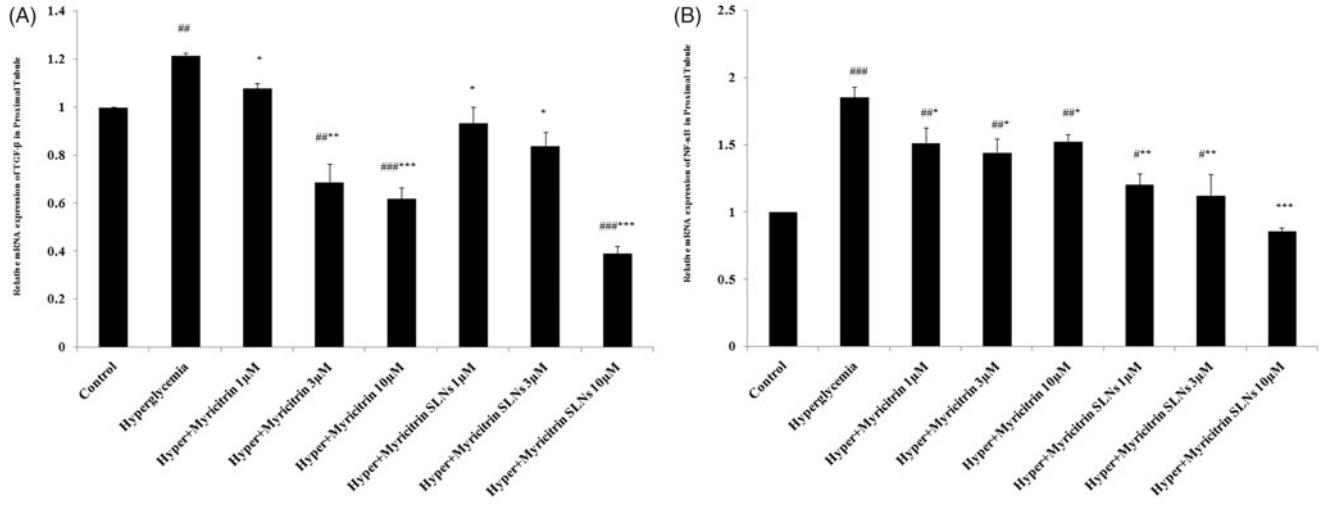

Figure 5. The effects of myricitrin and SLN containing myricitrin on relative mRNA expression of TGF-β and NF-κB in proximal tubules. Data are presented as mean ± SEM; $n = 3$; eight proximal tubules in each cell culture flask; $^{\#}p < .05$, $^{\#\#}p < .01$ and $^{\#\#\#}p < .001$ compared with the control, $^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$ compared with the Hyper (one-way analysis of variance (ANOVA)), followed by *post hoc* least significant difference (LSD) tests). (A) Transforming growth factor-β (TGF-β), (B) Nuclear factor kappa B (NF-κB).

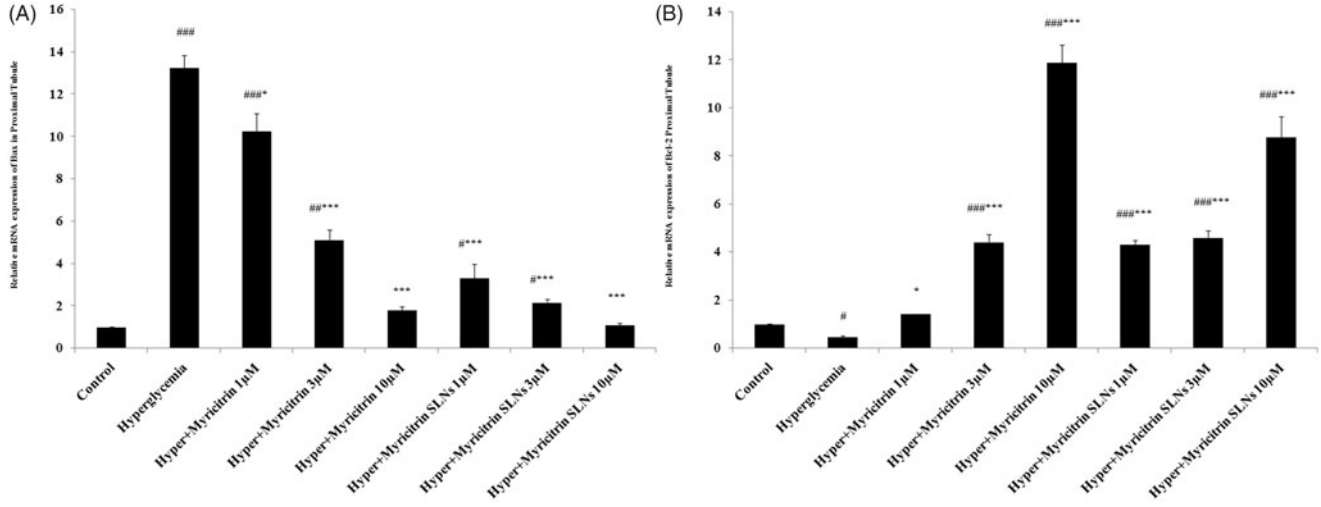

Figure 6. Apoptotic and antiapoptotic relative mRNA expression in proximal tubules. Data are presented as mean ± SEM; $n = 3$; eight proximal tubules in each cell culture flask; $^{\#}p < .05$, $^{\#\#}p < .01$ and $^{\#\#\#}p < .001$ compared with the control, $^{*}p < .05$ and $^{***}p < .001$ compared with the Hyper (one-way analysis of variance (ANOVA)), followed by *post hoc* least significant difference (LSD) tests). (A) Bax, (B) Bcl-2.

in the proximal tubule and its antioxidant defense by decreasing MDA and increasing SOD and TAC levels. TAC consists of both small molecule antioxidants and proteins such as enzyme systems (GSH reductase, catalase, peroxidase, etc.), small molecules (ascorbate, uric acid, GSH, vitamin E, etc.) and proteins (albumin, transferrin, etc.). SOD catalyzes the one-electron dismutation of superoxide into hydrogen peroxide (H_2O_2) and oxygen; CAT, glutathione peroxidase (GPX), glutathione (GSH) and thiol-specific antioxidant enzymes (TSA) convert H_2O_2 into oxygen and water (Peluso *et al.* 2016). Therefore, the results of Myr and its SLN in the present study showed that the increase in SOD resulted in the reduction of superoxide anion and increased H_2O_2; in addition, increased TAC resulted in a decrease in H_2O_2 levels. Due to the increase in TAC and the stability in CAT levels, it could be suggested that the reduction of H_2O_2 could occur by increasing GPX, GSH or TSA levels.

The chronic exposure of proximal tubule cells to high glucose contributes to cellular damage and dysfunction. Prolonged exposure of proximal tubule epithelial cells to the hyperglycemic medium could increase the expression of various pro-inflammatory genes such as TGF-β and NF-κB, thus, inducing kidney injury (Samikkannu *et al.* 2006). TGF-β plays a crucial role in the pathophysiology of diabetic nephropathy in the animal model during hyperglycemia. In one study, it was observed that a high glucose medium increased NF-κB gene expression in the proximal tubules. NF-κB is a ubiquitous gene activated by free radicals and can influence the pathogenesis of diabetic nephropathy (Han *et al.* 2005). Some antioxidant agents, including curcumin, diminish the expression of NF-κB and TGF-β that are closely involved in inflammation (Trujillo *et al.* 2013). Also, hyperglycemic condition reduced the level TAC and SOD and increased MDA level in the rat's kidney along with the activation of TGF-β

and NF-κB signaling pathway (Xu *et al.* 2017). Antioxidant compound administration improved this signaling pathway via increased TAC and SOD levels and decreased lipid peroxidation (Mahmoodnia *et al.* 2017). Hence, consistent with previous studies, the results of the present research revealed that TGF-β and NF-κB gene expression increased in proximal tubules exposed to the hyperglycemic medium for 48 h and that the administration of Myr and SLN containing Myr reduced these pro-inflammatory genes. Accordingly, it could be suggested that this plant-derived antioxidant has anti-inflammatory effects which are more evident in its SLNs and these effects were occurred via increasing TAC and SOD and reducing lipid peroxidation in the proximal tubule.

The destruction of the cell membrane lipids and lipid peroxidation reactions are hazardous for the cell's life and tissues (Park *et al.* 2001). Moreover, renal tubular cell viability is ameliorated by bioflavonoids through their incorporation into membrane lipid bilayers and reduced lipid peroxidation (Ahlenstiel *et al.* 2003). Our data indicated that hyperglycemia reduced proximal tubule viability and that exposure to Myr and SLN containing Myr improved this alteration. Hence, it could be suggested that this antioxidant compound caused this effect through decreased lipid peroxidation and increased SOD and TAC in the proximal tubules. The concentration of MTT depends on the number of viable cells (Ahangarpour *et al.* 2017). Thus, it could be suggested that Myr and SLN containing Myr administration increased the level of MTT in proximal tubules through elevating the number of viable proximal tubule cells.

Pro-apoptotic (Bax) and antiapoptotic (Bcl-2) proteins have an important effect on the apoptotic pathway and the imbalance between these proteins destroys tubular cells death. The main intracellular goal of hyperglycemia and oxidative stress is NF-κB. NF-κB is a factor with redox-sensitive property and an important regulator of antioxidant defense that can begin the transcription of several genes that participate in the inflammatory (TGF-β) and apoptosis such as Bax and Bcl-2 (Kumar *et al.* 2013). One study showed a significant decrease and increase in Bcl-2 and Bax levels, respectively, in proximal tubule cells exposed to high glucose medium (Kim *et al.* 2009). Also, it was revealed that polyphenols reduce apoptosis by decreasing MDA and increasing TAC and SOD. Chen *et al* showed that emodin, as a plant-derived antioxidant, increased Bcl-2 and decreased Bax expression in proximal tubular cells and reduced tubular apoptosis through its antioxidant activity (Chen *et al.* 2017). Therefore, similar to previous studies, hyperglycemia-induced apoptosis in the present research and exposure to Myr and its SLN for 48 h revealed an antiapoptotic effect on the proximal tubules and this improvement were occurred by recover antioxidant defenses. The proximal tubules play the main role in the pathophysiology of the diabetes-induced renal injury. This tissue is the early link to tubulointerstitial inflammation, fibrosis, oxidative stress and renal failure during diabetes. Understanding the molecular mechanisms of these events can help identify new diagnostic biomarkers and therapeutic drugs for human diabetic kidney diseases (Vallon 2011). Therefore, Myr and SLN containing Myr may be useful for the improvement of diabetes-induced proximal tubule complications such as oxidative stress, inflammation and apoptosis in clinical administration.

Conclusion

In conclusion, our results indicated that the hyperglycemic condition induced lipid peroxidation and decreased SOD, TAC, the proximal tubule viability and Bcl-2 level and increased TGF-β, NF-κB and Bax gene expression. In addition, Myr and SLN containing Myr administration revealed antioxidant, anti-inflammatory and antiapoptotic effects in proximal tubules exposed to hyperglycemia by increasing SOD, TAC, the proximal tubule MTT and Bcl-2 gene expression and decreasing MDA level, TGF-β, NF-κB and Bax gene expression. Some of these effects such as anti-inflammatory and antiapoptotic effects were more potent in SLN containing Myr-treated proximal tubule than Myr.

Disclosure statement

No potential conflict of interest was reported by the authors.

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