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Xanthohumol and 8-prenylnaringenin reduce type 2 diabetes–associated oxidative stress by downregulating galectin-3

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Abstract

Background: Galectin-3 (Gal3) expression is associated with accumulation of Advanced Glycation End products (AGE), a common feature in diabetes mellitus (DM). The role of Gal3 in oxidative stress is, however, controversial, being considered in the literature to play either a protective role or exacerbating disease.

Methods: Herein, we examined the interplay between Gal3 and oxidative stress in a high-fat diet -induced type 2 DMC57Bl/6 mice model. Because natural polyphenols are known to play antioxidant and anti-inflammatory roles and to modulate metabolic activity, we further evaluated the effect of xanthohumol and 8-prenylnaringenin polyphenols in this crosstalk.

Results: Gal3 expression was accompanied by 3-nitrotyrosine and AGE production in liver and kidney of diabetic mice compared to healthy animals (fed with standard diet). Oral supplementation with polyphenols decreased the levels of these oxidative biomarkers as evaluated by immunohistochemistry and western blotting. Interestingly, blocking Gal3 by incubating human microvascular endothelial cells with modified citrus pectin increased 3-nitrotyrosine protein expression.

Conclusions: These findings imply that Gal3 overexpression is probably controlling oxidative stress in endothelial cells. In conclusion, our results indicate that supplementation with 8-prenylnaringenin or xanthohumol reverses diabetes-associated oxidation in liver and kidney, and consequently decreases this diabetic biomarker that predispose to cardiovascular complications.

Keywords: advanced glycation end products, diet polyphenols, microvascular endothelial cells, 3-nitrotyrosine, oxidative stress biomarker

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Introduction

According to the International Diabetes Federation in 2015, 415 million people present diabetes mellitus (DM) worldwide.[1] Because of its increasing incidence, DM will be a leading cause of morbidity and mortality in the near future. DM causes multiple serious health complications, demanding a thorough clinical management, which renders this disease already a main burden to the National Care Systems. The diabetic condition is characterized and responsible for alterations in micro and macrovascular beds, by inducing changes in neovascular mechanisms and impairing vascular homeostasis.[2]

One of the conditions associated with these diabetic vascular complications is oxidative stress, which arises from prolonged exposure to elevated blood glucose levels.[3] In addition, it is also associated with a wide number of pathological conditions that coexist in diabetes, such as inflammation and atherosclerosis.[4] In accordance, increased formation of advanced glycation end products (AGEs) plays a role in diabetes progression in many distinct organs. AGEs are responsible for the cross-linking of collagen, tissue stiffness, increased blood pressure, and heart failure among other metabolic complications.[5–7]

It is well established that AGEs bind with high affinity to galectin-3 (Gal3), a member of the highly conserved family of soluble β-galactosidase–binding lectins. Gal3 is thus overexpressed in diabetes,[8] and is also an important modulator in a wide variety of biological activities, namely in immune and inflammatory diseases, in metabolic syndrome and in cancer.[9–11] Accordingly, several studies report Gal3 involvement in diabetic vascular complications[12,13] and predisposition to cardiovascular disease.[14] Interestingly, its function depends on its location and cell damage context. Extracellularly, Gal3 interacts with β-galactosidase residues of extracellular matrix components and with cell surface glycoproteins via the carbohydrate recognition domain.[15,16] Intracellular Gal3, in turn, acts as a pre-mRNA splicing factor, regulating cell cycle by modulating cell proliferation, apoptosis, and differentiation.[17] However, whether Gal3 plays a protective role or, on the contrary, induces disease aggressiveness remains to be determined.

Our group has been investigating the role of natural polyphenols, as preventive and therapeutic agents against many disorders such as cancer, diabetes, and cardiovascular diseases.[18–23] Polyphenols are antioxidant, anti-inflammatory, and often affect vascularization process.[18–20] Recently, we reported that xanthohumol (XN), the major beer-derived polyphenol, modulates inflammation, oxidative stress, and angiogenesis in a type 1 DM Wistar rat skin wound healing model.[23] In addition, 8-prenylnaringenin (8PN) is an XN metabolite with potent phytoestrogen properties, also involved in oxidative stress, inflammation, and angiogenesis,[24] 3 processes imbalanced in diabetes. Using a high-fat diet (HFD)–induced diabetic C57Bl/6 mouse model, we investigated whether XN and 8PN interfered with this AGE-Gal3 crosstalk in kidney and liver of diabetic mice, 2 metabolic organs disturbed in diabetes.

Materials and methods

Cell culture assays

Human Dermal Microvascular Endothelial Cells (HMVEC, ATCC, UK) were cultured in RPMI-1640 medium (Gibco, Carlsbad, California, Life Technologies, 52400-025) supplemented with 10% of fetal bovine serum (Gibco, 10270) and 1% antibiotic/antimycotic (Gibco, Carlsbad, California, Life technologies). The cells were maintained in a humidified chamber with 95% air and 5% CO_2 at 37°C. The cells were used between passages 6 and 9.

For silencing Gal3, cells were treated with 50 mM of Lactose (Merck Millipore, 10039-26-6, Germany) and 1% of modified citrus pectin (Source Naturals, Scotts Valley, California) during 24 hours in incomplete medium.[25]

Cells were fixed in methanol for histological studies. For biochemical studies, cells were cultured in 60 mm plates and total proteins were extracted using Radioimmunoprecipitation assay buffer (RIPA buffer) with protease and phosphatase inhibitors (Sigma, St. Louis, Missouri). Total protein quantitation was carried out using a bicinchoninic acid assay (BCA) kit—Pierce BCA Protein Assay Kit (Thermo Scientific Prod 23225) accordingly to the instructions of manufacturer.

Animals

All research animal experiments were conducted according to accepted standards of humane animal care (Declaration of Helsinki, European Community guidelines, 86/609/EEC), Portuguese Act (129/92), and EU Directive 2010/63/EU for the use of experimental animals. Animal assays were carried out by certified technicians at the animal house.

Six-week old male C57Bl/6 mice strain was used as a model for type 2 DM. Animals were maintained in suitable conditions of temperature and light (20°C–22°C, 12 h light/dark cycle) and subjected to a specific HFD for 20 weeks. The control group was fed with a standard diet. Diabetic animals were fed with HFD and in their beverage, 0.1% ethanol (DM-control), 8-prenylnaringenin (DM-8PN), or xanthohumol (DM-XN). Both polyphenols were dissolved in ethanol (0.1% final concentration). Six animals were studied per group.

Tissue preparation

Murine liver and kidney were dissected and total proteins were extracted using RIPA lysis buffer with protease and phosphatase inhibitors (Sigma, St. Louis, Missouri). Quantitation of total protein was carried out using a BCA kit—Pierce BCA Protein Assay Kit (Thermo Scientific Prod 23225) according to the instructions of manufacturer. Tissues were fixed and embedded in paraffin.

Tissue sections were used for immunohistochemistry.

Immunohistochemistry assays

Paraffin-embedded tissues sections (4 μm thick) were deparaffinized and allowed to react with anti-3-nitrotyrosine (3-NT) primary antibody overnight (Merck Millipore, AB5411, Germany) and subsequent incubation with secondary antibody (Santa Cruz, Dallas, Texas, sc-45101). Tissue sections also react with anti-Gal3 antibody (eBioscience 14–5301) by heat antigenic recovery. Endogenous peroxidase activity was blocked with incubation with 3% hydrogen peroxide in methanol and nonspecific binding was blocked with incubation with 10% rabbit serum in bovine serum albumin. The slides were incubated with the secondary antibody (DAKO, E046801-2, Denmark).

Immunoreactivity was visualized with Avidin-Biotin reaction and revelation was performed using DAB (3,3-diaminobenzidine) HRP substrate (Abcam, ab94665, UK).

All stained specimens were visualized under Nikon Eclipse 501 optical microscope. The images were compiled in Photoscape software (v3.7) later uniformed in ImageJ software (v1.48). Staining quantification was performed in CellProfiler (v.2.1.1).

Western blotting assays

Tissue samples were homogenized with dithiothreitol-reducing agent and loading buffer. Fifteen micrograms of tissue proteins were separated by 10% Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Hybond nitrocellulose membrane (Amersham, Arlington, VA). The membrane was blocked using 5% BSA solution overnight, and subsequently probed with rat anti-Gal3 (eBioscience 14-5301) and horseradish peroxidase–conjugated anti-rat IgG (Santa Cruz Biotechnology, Dallas, Texas, goat anti-rat IgG-CFL 488). Immunoreactive bands were then visualized using a chemiluminescent detection system (ECL) (Bio-Rad, Berkeley, California cat170-5060) according to manufacturer's instructions. β-Actin was used as loading control (Sigma, St. Louis, Missouri).

Band intensity was quantified using the ChemiDoc MP and processed in software ImageLab (V5.1).

AGE quantification assay

AGE intrinsic fluorescence (AGE-FL) was measured in triplicate at a standard concentration of 1 μg/μL in PBS buffer using

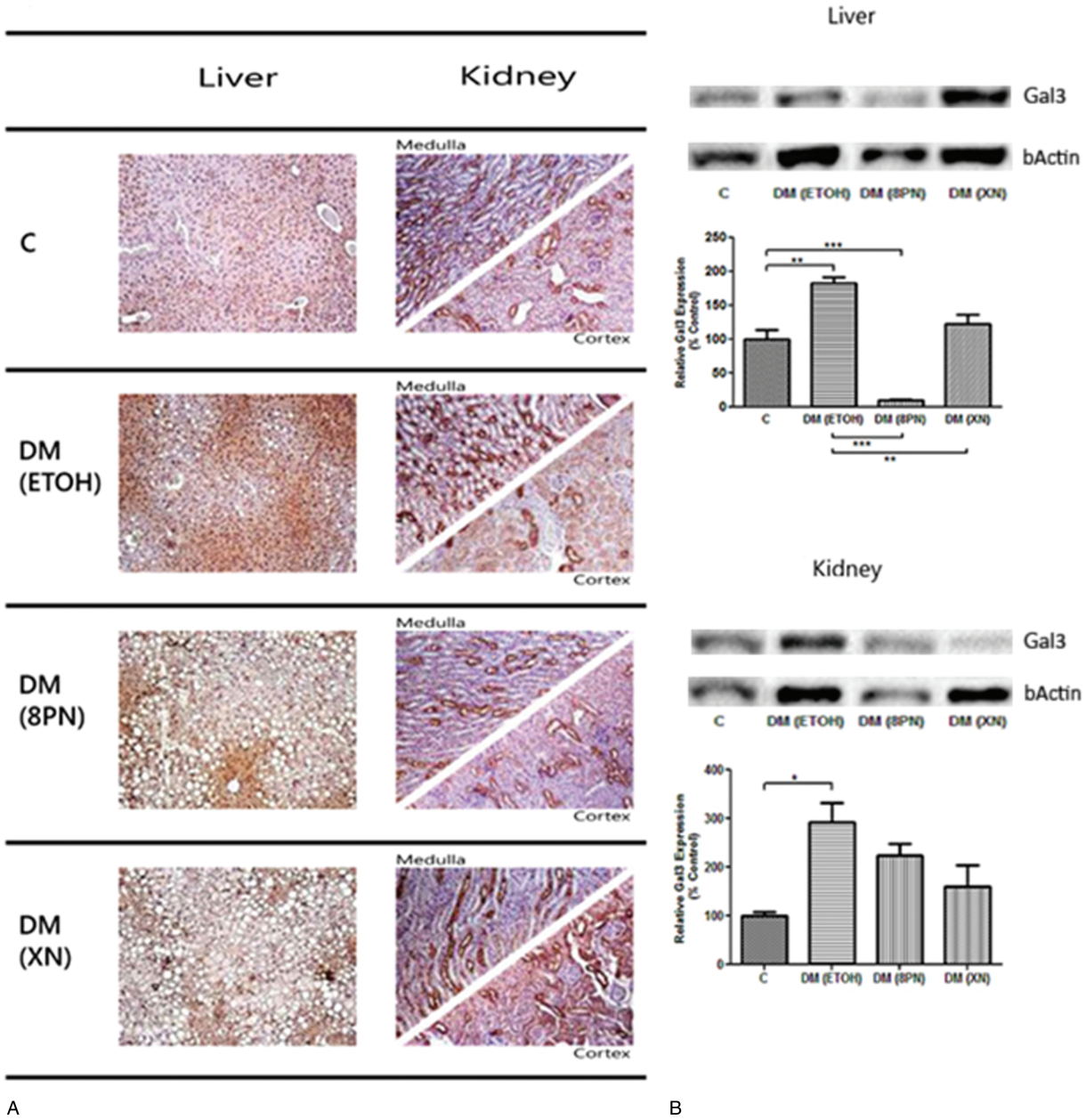

Figure 1. A, Galectin-3 immunostaining in liver and kidney medulla and cortex. Representative images are shown. C, healthy control; DM (ETOH), type 2 diabetic animal; DM (8PN), diabetic animal supplemented with 8PN; DM (XN), diabetic animal supplemented with XN. Magnification 100×. B, Western blot for galectin-3 expression in liver and kidney. β-Actin expression was used as loading control. A representative blot is shown. Graphs represent band intensity mean values. $^{*}\rho < 0.05$ versus control; $^{**}\rho < 0.001$ versus control; $^{***}\rho < 0.0001$ versus control. DM = diabetes mellitus; 8PN = 8-prenylnaringenin; XN = xanthohumol.

Synergy Mx Monochromator-Based Multi-mode Microplate Reader (BioTek, Winooski, Vermont) in black microplate (Eppendorf, Hamburg, Germany). Total AGE-FL was measured at 360/10 nm excitation and 440/10 nm emission.

Statistical analyses

Categorical variables were expressed as percentages by normalization to the control.

Data were presented as mean values and range of values of standard deviation. Statistical differences were analyzed by 1-way analysis of variance and the post-test Newman-Keuls. The value of ($\rho < 0.05$) was taken as statistically significant. All statistical analyses were performed with GraphPad Prism 5.03 software.

Results

8PN and XN reduced Gal 3 overexpression in DM liver and kidney

We first examined the expression of Gal3 in liver and kidney of DM mice. Gal3 immunostaining was increased in diabetic animals when compared to normal (control) animals (Fig. 1). A significant overexpression was evident in diabetic liver particularly along the central vein, in the sinusoids and in the Kupffer

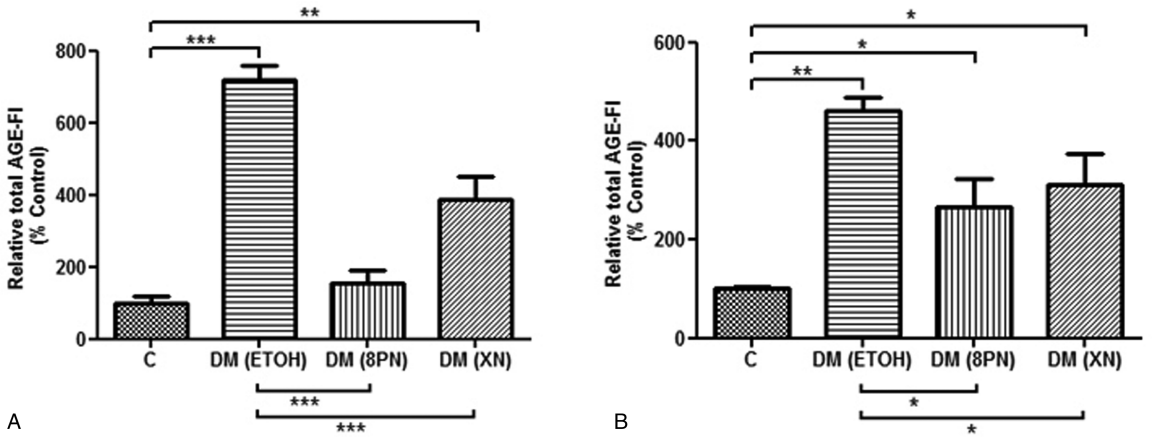

Figure 2. Analyses of intrinsic fluorescence AGE in liver and kidney. Graphs represent the percentage of total AGE-FI relative to control. C, healthy control; DM (ETOH), type 2 diabetic animal; DM (8PN), diabetic animal supplemented with 8PN; DM (XN), diabetic animal supplemented with XN. $^{*}\rho < 0.05$ versus control; $^{**}\rho < 0.001$ versus control; $^{***}\rho < 0.0001$ versus control. AGE = Advanced Glycation End products; DM = diabetes mellitus; FI = Flourescence; 8PN = 8-prenylnaringenin; XN = xanthohumol.

cells (Fig. 1A). However, treatment with polyphenols resulted in decreased Gal3 expression (Fig. 1A and B). As observed by western blotting, XN consumption led to a Gal3 expression decrease toward control values. A drastic downregulation of Gal3 expression was observed in liver of diabetic mice fed with 8PN (Fig. 1B).

Renal Gal3 expression was also increased in renal collecting medullary and cortical tubules in diabetic mice, exhibiting a weak expression in the glomeruli and proximal and distal cortex tubules (Fig. 1A). Strikingly, whenever animals were supplemented with 8PN or XN, Gal3 expression was reduced to values identical to healthy animals (Fig. 1B). Expression variance was mainly located in the external cortical labyrinth (Fig. 1A).

8PN and XN reduced oxidative stress in DM liver and kidney

We next determined the presence of AGE in liver and kidney of diabetic mice with or without polyphenol supplementation. AGE profile was identical to the one observed in Gal3 expression. In comparison to healthy animals, DM mice presented a significant increase in AGE accumulation in liver and kidney, which was reduced by polyphenols supplementation (Fig. 2). In the liver, the consumption of 8PN, but not XN, decreased the accumulation of AGE to values identical to the control group, whereas in kidney accumulation of AGE was significantly reduced by both polyphenols (Fig. 2).

Protein nitration is a feature of oxidative stress often present in diabetes. Therefore, the presence of 3-NT in liver and kidney of diabetic animals was then addressed by immunohistochemistry. Expression of 3-NT found in DM liver and kidney cortex was higher in HFD in comparison to controls (Fig. 3A and B). Moreover, a correlation between 3-NT expression and the presence Gal3 in both organs was observed in these organs (Figs. 1 and 3A). The presence of both polyphenols in diabetic animals resulted in a reduced 3-NT expression to control values in liver (Fig. 3A). In contrast, supplementation of diabetic animals with 8PN decreased 3-NT expression to control values in renal cortex, whereas XN effect was not significant (Fig. 3B). Expression of 3-NT was not evident in kidney medulla of diabetic animals either in the presence or absence of polyphenols.

Gal3 protects vascular endothelial cells against oxidative stress

Our in vivo findings revealed a positive correlation between the presence of Gal3 and oxidative stress both examined by the presence of AGE products and of 3-NT expression. In order to examine whether Gal3 is actually protecting cells against oxidative agents or on the other hand, it is just accompanying oxidative stress, we next silenced this lectin using 1% modified citrus pectin (MCP) and 50 mM lactose, and assessed 3-NT expression in vitro. Because Gal3 was not present in significant amounts in parenchymal cells, but it rather is overexpressed in endothelial cells, HMVECs were used in the next functional experiments. Incubation of HMVECs with MCP and lactose for 24 hours resulted in a 50% decrease in Gal3 expression (Fig. 4B), confirming the antagonizing effects of MCP in Gal3. Interestingly, Gal3 inhibition resulted in increased 3-NT expression (Fig. 4C). These findings indicate that this lectin prevents cell oxidative stress. Moreover, Gal3 abrogation (Gal3[−]) resulted in a decrease in the number of cells, as well as a reduction in cell-cell adhesion (Fig. 4A).

Discussion and conclusions

The current study showed that AGE products and 3NT expression, 2 features of oxidative stress are exacerbated in liver and kidney of type 2 diabetic mice. Our findings further indicate that the presence of oxidative stress conditions is strongly correlated with Gal3 overexpression in both organs of these animals, since whenever diabetic animals were fed with 8PN or XN polyphenols, Gal3 was downregulated and oxidative stress conditions reversed. These effects were more evident when animals were fed with 8PN, resulting in levels similar to those of nondiabetic (healthy) animals.

Expression of Gal3 was not present in hepatocytes of healthy mice (control). However, in agreement with previous studies in other hepatic disorders like cirrhosis and hepatocellular carcinoma,[26] diabetic animals expressed this lectin, especially along the central vein. Expression of 3NT was also higher in central-lobular cells than in perilobular ones, implying that Gal3 is mainly present in the regions exhibiting increased oxidative stress.

The expression of Gal3 in the kidney has an irregular distribution as well. No major difference in Gal3 immunostaining

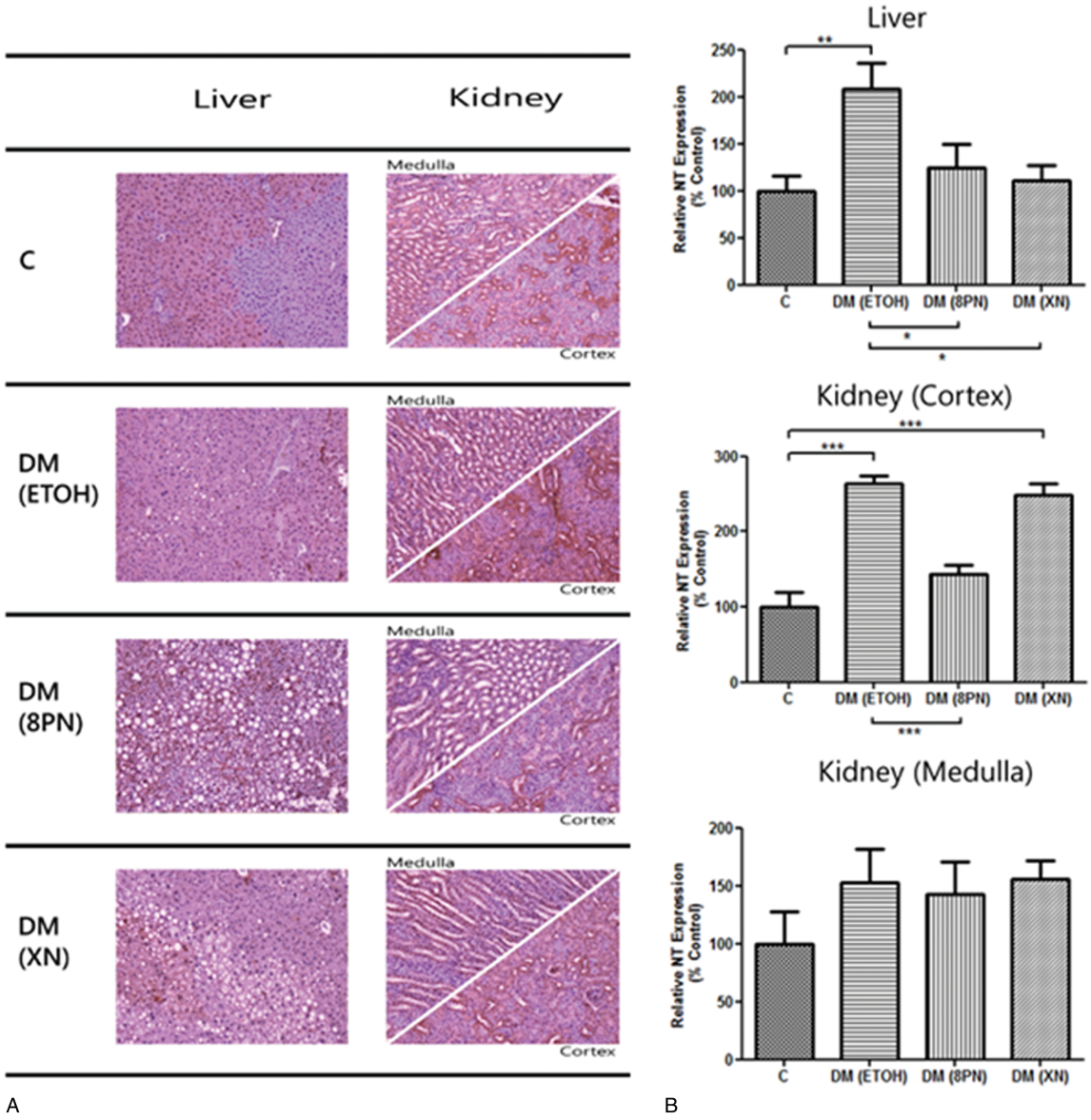

Figure 3. A, Immunostaining for 3-nitrotyrosine residues in liver and kidney medulla and cortex. Representative images are shown. Magnification 100×. B, Quantification of immunostaining for 3-nitrotyrosine residues in liver and kidney cortex and medulla. Graphs represent band intensity mean values. $^{*}\rho < 0.05$ versus control; $^{**}\rho < 0.001$ versus control; $^{***}\rho < 0.0001$ versus control. C, healthy control; DM (ETOH), type 2 diabetic animal; DM (8PN), diabetic animal supplemented with 8PN; DM (XN), diabetic animal supplemented with XN. DM = diabetes mellitus; 8PN = 8-prenylnaringenin; XN = xanthohumol.

(and 3NT) was observed among the distinct groups of mice in the medulla. Remarkably, expression of Gal3 was significantly increased in the cortex of diabetic animals. Accompanying this increase, renal cortex of diabetic mice further presented augmented AGE content and 3NT expression. Supplementation with 8PN and XN polyphenols reduced Gal3 expression in kidney cortex, as well as reduced AGE products and 3NT immunostaining.

Gal3 is a multifunctional protein that works as a broad-spectrum modifier, and although it has been reported to be present in a wide range of pathological situations, its role is still controversial. This lectin has been considered a "bad guy," acting like a biomarker of mortality and morbidity in heart failure. On the contrary, Gal3 can also be the "good guy," regarding its participation in the response to type 2 DM and in modulating the immune/inflammatory system.[27]

Natural polyphenols are well-known antioxidant compounds. Therefore, the findings that 8PN and XN reduce AGE and 3-NT expression in diabetic liver and kidney is expected. However, the observed Gal3 downregulation by these 2 polyphenols could be a consequence of their antioxidative effect, or inversely, 8PN and XN could exert their antioxidant effects through downregulation of this lectin. This was elucidated by our in vitro assay, in which Gal3 lectin was blocked by MCP, a pectin-derived agent known to bind to Gal3 carbohydrate recognition domain, preventing its role.[25] Antagonizing Gal3 activity in HMVECs, a microvascular endothelial cell culture, prone to endothelial dysfunction caused by high exposure to oxidative stress, we were able to show a significant increase in tyrosine nitration expression. This implies that Gal3 is controlling oxidative stress in this diabetic experimental setting.

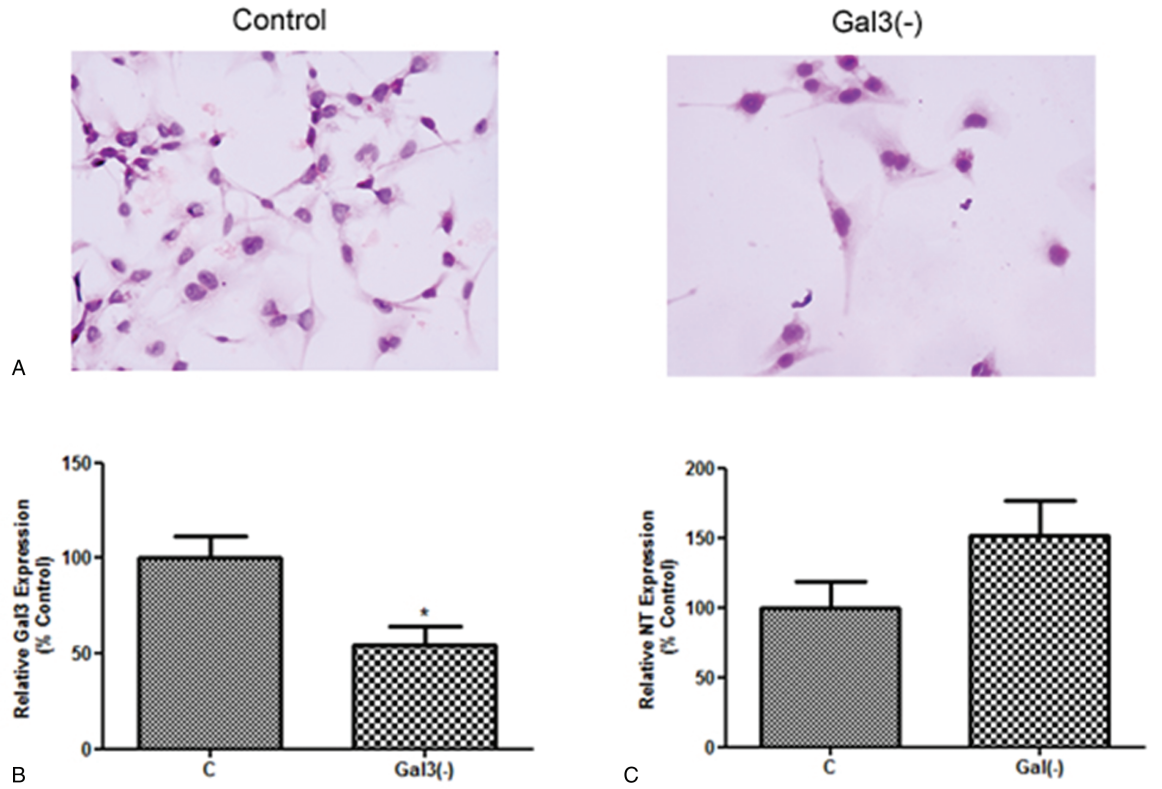

Figure 4. A, HE staining of HMVEC cell culture untreated (Control) or incubated with modified citrus pectin (MCP) (Gal3[−]). Magnification 40×. Western blot analyses of (B) galectin-3; and (C) 3-nitrotyrosine in HMVEC cell culture. Graphs represent band intensity mean values relative to loading control (β−actin). $^{*}\rho < 0.05$ versus control. Gal3 = galectin-3; NT = nitrotyrosine.

In conclusion, the present study reveals that Gal3 is overexpressed in liver and kidney of diabetic mice. Gal3 correlates with oxidative stress, as measured by the presence of AGE products and 3NT. Diabetic animals fed with polyphenols (particularly 8PN) reduce these effects to healthy control levels, probably by decreasing oxidative stress. Nevertheless, future metabolic studies are needed to confirm the effects of these polyphenols as potential therapeutic targets against diabetes.

Acknowledgments

None

Conflicts of interest

The authors report no conflicts of interest.

Author contributions

Contributors: Conception and Design: RC, CR, and RS
Acquisition of Data: CL, AC, and RC
Technical support: IR, SG, AC, and PC
Analysis and interpretation of data: CL, RC, JG, CR, and RS
Drafting the article and revising it for intellectual content: CL, CR, and RS
Final approval of the completed article: CL, RC, IR, AC, PC, SG, JG, CR, and RS

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