Quercetin Treatment Ameliorates Inflammation and Fibrosis in Mice with Nonalcoholic Steatohepatitis

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Abstract

We investigated whether quercetin protects from steatosis and limits the expression of proinflammatory and fibrogenic genes in C57BL/6J mice with nonalcoholic steatohepatitis (NASH) induced by feeding a methionine-choline–deficient (MCD) diet. Quercetin (50 mg/kg) was given by oral route daily. Mice were randomly divided into 4 groups that received for 2 or 4 wk: the control diet plus vehicle, control diet plus quercetin, MCD diet plus vehicle, and MCD diet plus quercetin. At both 2 and 4 wk, feeding the MCD diet resulted in liver steatosis, inflammatory cell accumulation, oxidative stress evaluated by the concentration of TBARS, and fibrosis evidenced by the staining of α-smooth muscle actin-positive cells in the liver. At both 2 and 4 wk, the MCD diet induced an increase in the mRNA levels of Il6, Tnf, Ptgs2, and Hmgb1 and increased the protein concentrations of Toll-like receptor-4, c-Jun terminal kinase, and p65 NFκB subunit compared with control rats. Feeding the mice the MCD diet also triggered an increase of Col1a1, Col3a1, Plod3, Tgfb1, Smad3, Smad7, Pdgfb, Ctgf, Areg, Mmp9, and Timp1 mRNA levels. These effects were totally or partially prevented by treatment with quercetin. The data obtained suggest that attenuation of multiple profibrotic and proinflammatory gene pathways contributes to the beneficial effects of quercetin in mice with MCD diet-induced steatohepatitis. J. Nutr. doi: 10.3945/jn.112.165274.

Introduction

Nonalcoholic fatty liver disease has a high prevalence in the general population and can evolve into nonalcoholic steatohepatitis (NASH)\(^1\), cirrhosis, and complications such as liver failure and hepatocellular carcinoma (1). The pathophysiology of NASH is still not fully understood, and available treatments are not entirely satisfactory. However, therapies that limit hepatic injury and the related occurrence of inflammation and fibrosis are particularly appealing for this condition.

Both biologically active lipid peroxidation products and proinflammatory cytokines such as IL6 and TNFα act together to trigger the diverse hepatic lesions of NASH by inducing hepatic inflammation and fibrosis that eventually lead to end-stage liver disease (2). Inflammation is a key parameter in the progression of hepatic steatosis (3), and studies have shown that the Toll-like receptor (TLR)-4 signaling pathway is pivotal to the pathogenic effect of the proinflammatory response in NASH (4). After acute liver injury, many mediators, including TGFβ and platelet-derived growth factor (PDGF), synergistically enhance collagen synthesis through both Smad and non-Smad signals, which crosstalk with the c-Jun terminal kinase (JNK) pathway at multiple levels to promote liver fibrosis (5). The interaction of cytokines/growth factors with their receptors initiates different signaling pathways, leading to the activation of multiple transcriptional factors such as NFκB, which also has a role in liver fibrogenesis (6).

Animal studies have demonstrated the protective effects of different polyphenolic compounds such as green tea catechins (7), genistein (8), curcumin (9), total flavonoids from Litsea coreana (10), or a combination of anthocyanins, flavonols, and derivatives of phenolic acids (11) in experimental models of NASH. It is also known that the mixture of flavonoids, silymarin, has some beneficial effects in nonalcoholic fatty liver disease patients (12). Quercetin (3,5,7,3–4-pentahydroxy flavone) is a...
flavonoid found in the human diet that has been shown to possess a wide range of activities in the prevention of common diseases (13,14). We previously reported a role for quercetin in fighting the deleterious effects of reactive oxygen species in isolated liver cells (15,16) and its capacity to reduce inflammation and fibrosis in experimental models of liver injury (17–19). Quercetin has been reported to reduce plasma oxidized LDL concentrations in overweight participants (20). Moreover, it was recently shown in mice that chronic dietary intake of quercetin alleviates hepatic fat accumulation associated with the consumption of a Western-style diet (21), and it is known that quercetin ameliorates hepatic and metabolic changes in rats consuming a high-carbohydrate, high-fat diet (22). However, no information is available on the possible modulation by quercetin of liver inflammation and/or fibrosis in experimental steatohepatitis. Therefore, the present study was undertaken to test the hypothesis that quercetin may protect from steatosis and limit the expression of proinflammatory and fibrogenic genes in a methionine-choline-deficient (MCD) diet murine model of NASH.

Materials and Methods

Materials. Quercetin was purchased from Sigma Chemical. TaqMan primers and probes for the different studied genes (Supplemental Table 1) were derived from the commercially available TaqMan Gene Expression Assay (Applied Biosystems). Antibodies against p65 NFκB and lamin-B were from Santa Cruz Biotechnology, antibodies against JNK and phospho-JNK were from Cell Signaling Technology, antibodies against TLR-4 were from Abcam, and antibodies against GAPDH were from Sigma.

Mice and experimental protocol. The study protocol was approved by the institutional Animal Care Committee of the Hospital de Clínicas Porto Alegre (Brazil) and conformed to the Guide for the Care and Use of Laboratory Animals of the NIH. Male C57BL/6 mice weighing 25 g were provided by the Multidisciplinary Center of Biological Investigation of the University of Campinas (Brazil). Mice consumed food and water ad libitum and were maintained on a 12-h-light/dark cycle under controlled temperature (24°C) and humidity (55%) in the Animal Experimentation Division of the Hospital de Clínicas Porto Alegre. Mice were fed either a diet deficient in methionine and choline (MCD diet) (Supplemental Table 2) or a control diet, which was identical to the MCD diet but supplemented with t-methionine (3 g/kg) and choline chloride (2 g/kg). The diets were prepared by PragSolucões Comércio e Serviços and stored at 4–10°C until used.

Mice were randomly divided into 4 experimental groups (n = 16/group): the control group (CO) received the control diet plus vehicle for 2 or 4 wk, the control + quercetin group (CO+Q) received the control diet for 2 or 4 wk plus 50 mg/kg quercetin, the NASH group (NASH) received the MCD diet for 2 or 4 wk plus vehicle, and an additional group (NASH+Q) received the MCD diet for 2 or 4 wk plus 50 mg/kg quercetin. The vehicle was composed of sodium carboxymethylcellulose 1% and functioned as a carrier for quercetin. The dose of quercetin was chosen based on our previous studies demonstrating that protective effects of this flavonoid in rodent models of liver disease are maximal when administered at 50 mg/kg.d (17,19). After 2 or 4 wk of feeding the diets, mice were weighed and anesthetized by inhalatory isoflurane for later staining. The livers were sectioned and a fraction of their lobes was used for histology, fixed in 10% formaldehyde solution for 24 h, and processed into paraffin blocks for later staining.

Biochemical markers of lipid peroxidation and liver injury. Lipoperoxidation was measured in liver using the TBARS assay (23,24). The serum levels of the enzymes aspartate transaminase (AST) and alanine transaminase (ALT) were measured using standard techniques (ALT and AST Activity Assay kits, Biovision).

Histological analysis. For the microscopic analysis, the liver fragment slides were stained with hematoxylin-eosin and subsequently assessed by a single pathologist who was unaware of the experimental groups. The minimum histological criterion for the diagnosis of NASH was the presence of steatosis associated with hepatic cellular ballooning involving zone 3 and lobular inflammatory infiltrate (25). The scoring system recommended by the Pathology Committee of the NASH Clinical Research Network (26), which semiquantitatively classifies each case as steatosis (0–3), lobular inflammation (0–3), and hepatocellular ballooning (0–2), was used for classification. The unweighted sum of these features of active injury potentially reversible in the short time allowed us to obtain an activity score.

Immunohistochemistry. Immunohistochemistry using polyclonal antibody α-smooth muscle actin (SMA) was performed as a marker of activated hepatic stellate cells (HSC). Immunorepression of α-SMA was studied in sections deparaffinized by xylene and rehydrated with graded alcohols, which were incubated with antibody α-SMA at its working dilution of 1:200 (27). The pathological findings were assessed by a single pathologist who was unaware of the experimental groups. The area of α-SMA staining was analyzed using WinRoof version 5.71 in 10 randomly selected fields/section per specimen.

Real-time qRT-PCR. Total RNA was extracted from liver and reverse transcribed using a High Capacity cDNA Archive kit (Applied Biosystems). cDNA was amplified using a TaqMan Universal PCR Master mix (Applied Biosystems) on a Step One Plus (Applied Biosystems). Relative differences in expression levels were determined using the 2-ΔΔCT method (28). The cycle number at which the transcripts were detectable (CT) was normalized to the number of Gapdh gene detection, referred to as ΔCT.

Western-blot analysis. Western-blot analysis was performed in cytosolic and nuclear extracts prepared from liver homogenates as previously described (29). Lysate proteins were fractionated by SDS-PAGE and measured using the corresponding primary antibodies. Bound antibody was detected by enhanced chemiluminescence. Membrane rehybridization with GAPDH or lamin-B was conducted to check loading accuracy (30). The density of the specific bands was quantified with an imaging densitometer (Scion Image, Scion).

Statistical analysis. Results are expressed as means ± SEM. Data were analyzed using multifactorial ANOVA with a 2 (quercetin) × 2 (NASH) × 2 (time) design. A Welch ANOVA was performed when Levene’s test indicated that variances were unequal. When a significant effect was found, post hoc comparisons were carried out using the Tukey’s honestly significant difference test. Differences among activity scores were identified using the Kruskal-Wallis test on ranks and group comparisons were performed using the nonparametric rank-based Mann-Whitney U-test. Differences were considered significant at P ≤ 0.05. SPSS+ version 15.0 statistical software was used.

Results

Effects of quercetin treatment on liver injury in the MCD diet-induced model of NASH. Administration of the MCD diet to C57BL/6 mice resulted in a classical pathophysiological picture of NASH, with microvesicular and macrovesicular steatosis, indicative of disturbed lipid metabolism, multiple foci of inflammatory cell accumulations in the liver (Fig. 1; Table 1), and increased accumulation of α-SMA (Fig. 2), indicative of fibrosis. Scores for steatosis, inflammation and ballooning, activity score, and α-SMA immunostaining, were significantly greater in the NASH group than in the CO and CO+Q groups at both 2 and 4 wk and did not significantly differ between
time periods except for α-SMA immunostaining. Body and liver weights were significantly lower and the serum levels of ALT and AST and the liver TBARS concentration were higher in the NASH group compared with both CO groups, indicating considerable hepatocellular injury and lipoperoxidation (Table 2).

Although values did not normalize, treatment with quercetin resulted, at both 2 and 4 wk, in lower scores for steatosis, inflammation, and ballooning, a lower activity score, and a reduced area of positive α-SMA liver immunostaining in the NASH+Q group compared with the NASH mice (Fig. 2; Table 1). Serum transaminase activities and the TBARS concentration in the liver were also significantly lower in the NASH+Q mice compared with the NASH group, but no significant effects were observed on body and liver weight (Table 2).

Quercetin inhibits expression of inflammatory-related genes in the MCD diet-induced model of NASH. At 2 and 4 wk, the liver protein concentration of TLR-4 was greater in the NASH group than in the CO and CO+Q groups. The protein concentration was significantly reduced by quercetin at both 2 and 4 wk, whereas it was significantly lower in the NASH+Q group. We observed that liver mRNA levels of Il6, Tnf, and Ptgs2 (cyclooxygenase-2) were significantly elevated in the NASH group compared with the CO and CO+Q groups at 2 and 4 wk. In the NASH+Q group, quercetin treatment significantly prevented the increase in the expression of those molecules at both time points, with values significantly higher at 4 wk compared with 2 wk for both Il6 and Ptgs2 (Table 3).

Quercetin inhibits expression of fibrosis-related genes in the MCD diet-induced model of NASH. At 2 and 4 wk, we identified increased mRNA levels of Col1a1 (collagen Iα1), Col3a1 (collagen IIIα1), and Plod3 (procollagen III) in the NASH group compared with both CO groups. Because TGFβ is the most potent profibrotic cytokine, we investigated its expression and that of related members of the Smad family. Tgfb1, Smad7, and Smad3 were significantly upregulated at 2 and 4 wk in the NASH group compared with the CO and CO+Q groups, reaching a greater value at 2 wk in Tgfb1 and Smad7 and at 4 wk in Smad3. Another mitogen for HSC is Pdgfb, the potent stimulator of extracellular matrix Ctgf and the epidermal growth factor receptor (EGFR) ligand, Areg (amphiregulin), which may contribute to the expression of fibrogenic mediators as well as to the growth and survival of fibrogenic cells. Pdgfb

| TABLE 1 | Effect of an MCD diet and treatment with quercetin on histological scores in C57BL/6J mice | "OFN" |
| Score | Time, wk | CO | CO+Q | NASH | NASH+Q | Kruskall-Wallis | P value |
| Steatosis | 2 | 0 (0–0) | 0 (0–0) | 2 (1–4) | 1 (0–2) | 0.001 |
| | 4 | 0 (0–0) | 0 (0–0) | 3 (2–3) | 1 (0–2) | 0.001 |
| Inflammation | 2 | 0 (0–0) | 0 (0–0) | 2 (0–3) | 1 (0–1) | 0.001 |
| | 4 | 0 (0–0) | 0 (0–0) | 2 (1–3) | 1 (0–2) | 0.001 |
| Ballooning | 2 | 0 (0–0) | 0 (0–0) | 2 (0–2) | 1 (0–2) | 0.001 |
| | 4 | 0 (0–0) | 0 (0–0) | 2 (1–2) | 1 (0–2) | 0.001 |
| Activity score | 2 | 0 (0–0) | 0 (0–0) | 5 (4–9) | 2 (1–5) | 0.001 |
| | 4 | 0 (0–0) | 0 (0–0) | 7 (5–9) | 3 (0–4) | 0.001 |

1 Values are median (range), n = 8. Medians for a variable with superscripts without a common letter differ, P < 0.005. CO, control diet plus vehicle; CO+Q, control diet plus quercetin; MCD, methionine-choline–deficient; NASH, methionine-choline–deficient diet plus vehicle; NASH+Q, methionine-choline–deficient diet plus quercetin.

Quercetin treatment ameliorates inflammation and fibrosis
and Areg were also overexpressed at 2 and 4 wk in the NASH group compared with both CO groups. Values were significantly higher at 4 wk compared with 2 wk for both Areg and Ctgf. Quercetin partially prevented all those effects in the NASH+Q group (Table 3).

The fibrogenic process also involves inhibition of extracellular matrix degradation through an imbalance between metalloproteinases (MMP) and their inhibitors. We found significantly greater Mmp9 expression in liver of the NASH mice compared with the CO and CO+Q groups, with values significantly more elevated at 2 wk. These effects were accompanied by an upregulation of the inhibitor Timp1 (tissue inhibitor of metalloproteinase 1), whose mRNA levels increased more at 4 wk than at 2 wk. In the NASH+Q group, administration of quercetin significantly prevented the MCD diet-induced expression of both Mmp9 and Timp1; Timp1 values were markedly higher at 4 wk compared with 2 wk (Table 3) and resulted in a marked increase of the Mmp9:Timp1 ratio.

Quercetin prevents JNK phosphorylation and NFκB activation in the MCD diet-induced model of NASH. Both JNK phosphorylation and increased nuclear expression of phosphorylated p65 NFκB subunit were observed in NASH mice compared with both CO groups, with p65 values significantly higher at 4 wk compared with 2 wk. Those effects were partially abrogated by quercetin treatment in the NASH+Q group, with significantly lower pJNK values at 4 wk compared with the first time point (Fig. 3).

**Discussion**

The use of a diet deficient in essential amino acids such as methionine and choline is a well-accepted model for inducing NASH, which recapitulates many of the features of this disease in humans, including a histologic picture that mimics that seen in human fibrotic disorders associated with hepatic lipid accumulation and the presence of inflammation and oxidative stress.
However, the MCD diet model is associated with several disadvantages, such as significant weight loss, low serum leptin, and peripheral insulin sensitivity (32), which makes caution necessary when extrapolating findings from MCD-fed mice to the human situation. In our study, following the MCD diet intake, mice consistently developed steatosis, inflammatory cell infiltration, hepatocellular necrosis, and fibrosis, which is in line with previous reports on this nutritional model of NASH (33). Animals treated with quercetin had lower ALT and AST serum levels and TBARS liver concentration from the early stages of the disease as well as reduced inflammation, histological evidence of injury, and signs of fibrosis.

Although the sequence of events, including fat accumulation, inflammation, and fibrosis, is not clearly delimited, inflammation seems to play a leading role in NASH progression (34). Activation of the inflammatory cascade may be induced by a variety of danger signals and the transmembrane protein TLR-4 may play a key role. In MCD diet-fed mice, expression of components of the TLR-4 receptor complex increases (35) and TLR-4 deficiency is protective in knockout mice, resulting in prevention of inflammatory cell infiltration and diminished proinflammatory cytokine production (32). Recent data suggest that TLR-4 signaling plays a pivotal role in the early progression of NASH in mice fed a high-fat diet, in which binding to the nuclear protein Hmgb1 serves as a positive component mediating TLR-4 activation (36). We found that the significantly enhanced expression of Hmgb1 and TLR-4 in liver cells of MCD diet-fed mice was parallel to a release of liver damage markers (AST and ALT) and that these effects were to a great extent prevented by quercetin treatment. Activation of the TLR-4 system results in the production of different liver cytokines, which contributes to the inflammatory process (37). As expected, mRNA levels of Il6 and Tnf were significantly elevated in mice fed the MCD diet. Treatment with quercetin resulted in a significant reduction in the expression of proinflammatory cytokines. Because Hmgb1 release from hepatocytes is an active process regulated by reactive oxygen species (38) and enhanced TLR-4–dependent cytokine expression is probably due to dysregulation of redox signaling (39), antiinflammatory action of quercetin could be at least partly a consequence of its antioxidant effect. Factors such as modulation of nuclear factor (erythroid-derived 2)-like 2 and genes containing the antioxidant response elements (40) may, in turn, be contributing to the partial prevention of oxidative stress induced by the flavonoid.

The sequence of pathogenic events during feeding of the MCD diet involves early lipid accumulation and peroxidation, followed by liver cell injury and inflammation, HSC activation, and upregulation of profibrotic genes. Two interrelated processes that could promote fibrogenesis are oxidative stress and the resultant liberation of cytokines (41). Nevertheless, although inflammation has been suggested as a prerequisite for development of fibrosis (42), the chronology and interdependence of inflammation and fibrosis still needs to be fully understood in NASH. In fact, in vivo fibrosis and inflammation can be independently regulated, because VSL#3 treatment prevents fibrosis in the MCD diet-induced NASH model without signif-

**FIGURE 3** Quercetin reduces MCD diet-induced expression of TLR-4, phosphorylated JNK, and p65 NFκB subunit in livers of C57BL/6J mice. Protein from liver extracts taken at 2 and 4 wk was separated by SDS-PAGE followed by immunoblotting. (A) Representative Western-blot photographs. Equal loading of proteins is illustrated by GAPDH and lamin-B bands. (B) Densitometric quantification of TLR-4, pJNK, and p65. Values are mean ± SEM, n = 8. Significant effects of quercetin (Q), MCD diet (NASH), time (T), Q × T, NASH × T, and Q × NASH × T are shown. CO, control diet plus vehicle; CO+Q, control diet plus quercetin; JNK, c-Jun terminal kinase; MCD, methionine-choline–deficient; NASH group, methionine-choline–deficient diet plus vehicle; NASH+Q, methionine-choline–deficient diet plus quercetin; TLR, Toll-like receptor.

Quercetin treatment ameliorates inflammation and fibrosis
significant changes in markers of inflammation or attenuation of the ongoing steatohepatitis (35).

The most important clinical challenge in NASH is the progression to liver fibrosis, which often leads to liver failure (34). The critical step in the generation of liver fibrosis is the activation of HSC resulting in a progressive change in architecture and function (34). The increased a-SMA expression in the livers of MCD diet-fed mice was significantly diminished by quercetin treatment. Consistent with the histology staining results, RT-PCR analysis revealed decreased expression of Plod3, Col1a1, and Col3a1. These results indicate that quercetin induces antifibrogenic actions related in part to a lower activation of HSC during the course of the fibrogenic process. A similar antifibrogenic action could also be a contributor to the antifibrotic properties of quercetin.

It is known that the expression of amphiregulin increases markedly in liver injury induced by carbon tetrachloride and that amphiregulin-deficient mice develop significantly less collagen accumulation, suggesting that this EGFR ligand plays a nonredundant role in hepatic fibrosis (47). Our data demonstrate that upregulation of Tgfb1 in MCD diet-fed mice was associated with increased expression of Smad3 and that these effects were partially abrogated by quercetin. This signaling pathway is downregulated by Smad7, an antagonist of TGFβ that inhibits phosphorylation of Smad3 (5). In animals fed the MCD diet, we identified an early and marked increase of Smad7 expression that was significantly reduced at 4 wk. Therefore, although the role of Smad7 in NASH development still needs to be fully elucidated, it appears that this negative feedback is not able to limit the fibrogenic evolution of experimental steatohepatitis. PDGF is the predominant mitogen for activated HSC. PDGF was recently reported to be upregulated in rats fed a high-fat diet (45) and the expressions of PDGF may act synergistically with TGFβ to enhance collagen synthesis via Smad pathways (46). Results from the present research suggest that downregulation of Pdgfb could also be a contributor to the antifibrotic properties of quercetin.
prevented by the flavonoid. Thus, our data support the suggestion that the amphiregulin/EGFR signaling system could be a new target in the prevention of liver fibrosis (48).

An additional finding from our study was the reversal by quercetin of changes in the expression of Mmp9 and Timp1 and the increase in the Mmp9 : Timp1 ratio. Increases in MMP and TIMP are common in fibrotic diseases, whereas a decrease in the MMP : TIMP expression ratio is known to be critical in discouraging matrix degradation and remodeling and favors extracellular matrix deposition (27). Consequently, data obtained indicate that reduction of extracellular matrix deposition by quercetin would contribute to the alleviation of liver fibrosis, confirming previous results from MCD mice treated with curcumin (42) or zinc-carnosine chelated compounds (49).

Taken together, our data indicate that quercetin exerts its antifibrogenic action at multiple levels. Data obtained indicate that reduced fibrogenesis is dependent at least in part on the modulation of the profibrogenic signals generated by inflammation and oxidative stress. However, a direct antifibrogenic effect cannot be ruled out. Previous studies have shown that curcumin blocks the activation of critical profibrogenic pathways in rodent animal model of NASH and the fact that in our investigation expression of α-SMA was significantly reduced by quercetin and was associated with reduced expression of Plod3 supports a direct effect on HSC. The recent concept of “multiple parallel hits” might reflect more precisely current knowledge of NASH (50). However, further studies will be required to clarify the antifibrogenic effects of quercetin, including possible direct actions on HSC.

Previous studies identified Nfkb and Jnk as key regulators of early hepatic inflammatory recruitment and liver injury in NASH (51,52). The present research demonstrates that MCD diet feeding induced an early Nfkb activation and treatment with quercetin decreased the nuclear levels of p65. Our results are in accordance with a previous report that green tea extracts inhibit the hepatic inflammatory responses mediated by Nfkb in a rat model of diet-induced obesity (53) and are also in accordance with the beneficial effects of the suppression of Nfkb activation induced by chronic quercetin intake in mice fed a Western-style diet (21). In addition, quercetin elicits a significant reduction in the phosphorylation of Jnk. Attenuation of Jnk activation was previously reported in MCD diet-fed rats treated with Silybum marianum extracts (54) and in mice fed the MCD diet and receiving α-lipoic acid (33). Activation of Nfkb contributes to hepatic fibrogenesis (6) and interruption of Nfkb signaling by curcumin suppresses the expression of the profibrogenic cytokine CTGF in HSC (55) and reduces the severity of experimental fibrosis in mice (56). Jnk stimulation has also been linked to fibrogenesis through PDGF induction of Smad3 (5), and in rats with NASH induced by 8 wk of feeding an MCD diet, adenosine A2a receptor stimulation reduced liver inflammation and fibrosis without affecting hepatic steatosis by inhibiting Jnk (57). Therefore, both Nfkb and Jnk may be important links between the effects of quercetin on oxidative stress, chronic inflammation, and hepatic fibrogenesis (6).

In summary, the data obtained suggest that attenuation of multiple proinflammatory and profibrotic gene pathways contribute to the beneficial effects of quercetin in mice with MCD diet-induced steatohepatitis. In spite of the limitations of this animal model of NASH and the fact that a pharmacological dose of quercetin was tested, further molecular mechanisms and the potential clinical application deserve to be investigated.

Acknowledgments
M.J.T., N.M., and J.G.G. designed the research; E.M., D.V., B.S.M., and J.T. carried out the experiments; E.M., D.V., and B.S.M. analyzed the data; M.J.T. and J.G.G. wrote the paper; and M.J.T. had primary responsibility for final content. All authors read and approved the final manuscript.

Literature Cited

Quercetin treatment ameliorates inflammation and fibrosis 7 of 8