

Effect of pioglitazone, quercetin, and hydroxy citric acid on vascular endothelial growth factor messenger RNA (VEGF mRNA) expression in experimentally induced nonalcoholic steatohepatitis (NASH)*

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Received: 22.04.2014 • Accepted/Published Online: 08.10.2014 • Printed: 30.06.2015

Background/aim: Vascular endothelial growth factor (VEGF) is associated with various ischemic and inflammatory diseases, and plays an important role in the development of liver fibrosis and hepatocarcinogenesis in nonalcoholic steatohepatitis (NASH). In this study, the comparative effect of pioglitazone, quercetin, and hydroxy citric acid on VEGF mRNA in experimentally induced NASH was investigated.

Materials and methods: The experimental protocol consisted of five groups: control, NASH, NASH + pioglitazone, NASH + quercetin, and NASH + hydroxy citric acid. The VEGF mRNA expression was evaluated by reverse transcription polymerase chain reaction (RT-PCR) analysis for all experimental groups, and the levels of VEGF mRNA were quantitatively measured by densitometry.

Results: A higher expression of VEGF mRNA was found in the hepatic cells of rats with experimentally induced NASH compared to the control group. A very mild increase in VEGF mRNA expression was observed in the rats treated with quercetin. In contrast, a mild increase in the expression of VEGF mRNA was observed in the rats treated with pioglitazone and hydroxy citric acid.

Conclusion: Quercetin exhibited an effective inhibition of VEGF mRNA expression, while a lower inhibition of the VEGF mRNA level was observed in the hydroxy citric acid- and the pioglitazone-treated rats.

Key words: Pioglitazone, quercetin, hydroxy citric acid, VEGF mRNA, nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH)

1. Introduction

Vascular endothelial growth factor (VEGF) is an inducer of angiogenesis, which is a hallmark of various ischemic and inflammatory diseases (1). An in vitro study revealed that leptin exerts proangiogenic activity in the presence of VEGF. VEGF plays an important role in the development of liver fibrosis and hepatocarcinogenesis in nonalcoholic steatohepatitis (NASH) (1,2). NASH is an asymptomatic disease belonging to the nonalcoholic fatty liver disease (NAFLD) spectrum, but if it is severe it often leads to cirrhosis of the liver, which is an end-stage liver disease, if not diagnosed and treated properly

(3–6). In our previous studies we have reported the effects of pioglitazone, quercetin, and hydroxy citric acid on the hepatic biomarkers, lipid profile, and lipoproteins in experimentally induced NASH (7,8).

We have studied the comparative effects of pioglitazone, quercetin, and hydroxy citric acid on the status of lipid peroxidation and antioxidants in experimental NASH (9), but very little information is known about the role of VEGF in NASH. The present study explores the comparative effects of pioglitazone, quercetin, and hydroxy citric acid on VEGF mRNA in experimentally induced NASH.

* This work was accepted for a poster presentation at the 20th IFCC - EFLM European Congress of Clinical Chemistry and Laboratory Medicine, and the 45th Congress of the Italian Society of Clinical Biochemistry and Clinical Molecular Biology (SIBioC), which was held on 19–23 May 2013, at the Milano Convention Centre (MiCo, Milan, Italy).

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2. Materials and methods

The experimental model of NASH in rats was established by feeding the animals a high-fat diet for 8 weeks (9,10), and this model was used to conduct a comparative study of the roles of pioglitazone, quercetin, and hydroxy citric acid on various parameters in NASH. Male Wistar rats weighing approximately 250 g were housed in solid-bottomed polypropylene cages under strict veterinary supervision, and maintained in control rooms with a 12-h light/12-h dark cycle. The animals received water and a commercial rat diet, standard diet, or high-fat diet ad libitum according to the experimental protocol. This study conformed to the guiding principles of the Institutional Animal Ethical Committee (IAEC), the Committee for the Purpose of the Control and Supervision of Experiments on Animals (CPCSEA), and the Guide for the Care and Use of Laboratory Animals (IAEC Approval Numbers: 001/006/2010 and 01/007/2011).

The male Wistar rats selected for the study were divided into eight groups as shown in Table 1 (9–11).

After the experimental period, the animals were sacrificed, after 12 h of fasting, by cervical decapitation. Liver tissue samples were collected from the sacrificed animals and stored until reverse transcription polymerase chain reaction (RT-PCR) analysis was conducted, using an Applied Biosystem model PCR machine. The total RNA was isolated from liver samples using a cooling centrifuge (Remi model). The total RNA was used as the template to synthesize complementary DNA (cDNA), with 2.5 units of Moloney murine leukemia virus reverse transcriptase in 10 μ L of buffer containing 10 mM Tris-HCl, pH 8.3, 50 mmol of KCl, 5 mmol of random hexamer, and 1.4 units of ribonuclease inhibitor. The RT was performed at 42 °C for 15 min. The resulting cDNA was used as the template for the subsequent PCR analysis.

The specific primer set for the rat VEGF receptor is shown in Table 2. The PCR was performed in 50 μ L of buffer containing 10 mmol Tris-HCl, pH 8.3, 2 mmol $MgCl_2$, 50 mmol KCl, 0.2 mmol of each deoxyribonucleoside triphosphate, 0.4 μ mol of each primer, and 2 U of Taq DNA

Table 1. Division of the male Wistar rats into experimental groups in this study.

Sl.No	Name of the group	No. of animals in group (n)	Description
1	Group 1; Controls	6	The control rats received the regular standard diet for 8 weeks.
2	Group 2; NASH	6	The rats were fed a high-fat diet for 8 weeks to induce NASH.
3	Group 3; Pioglitazone control	6	These rats were fed the standard diet for 4 weeks, and were then fed the standard diet and intragastrically administered pioglitazone (4 mg/kg. b.wt.; 0.5% methyl cellulose w/v) for the next 4 weeks.
4	Group 4; Quercetin control	6	These rats were fed the standard diet for 4 weeks, and were then fed the standard diet and intragastrically administered quercetin (20 mg/kg. b.wt.) dissolved in 1% DMSO v/v for the next 4 weeks.
5	Group 5; Hydroxy citric acid control	6	These rats were fed the standard diet for 4 weeks, and were then fed the standard diet and intragastrically administered hydroxy citric acid (150 mg/kg. b.wt.) for the next 4 weeks.
6	Group 6; NASH + pioglitazone	6	These rats were fed a high-fat diet for 4 weeks, and were then fed the high-fat diet and intragastrically administered pioglitazone (4 mg/kg. b.wt.; 0.5% methyl cellulose w/v) for the next 4 weeks.
7	Group 7; NASH + quercetin	6	These rats were fed a high-fat diet for 4 weeks, and were then fed the high-fat diet and intragastrically administered quercetin (20 mg/kg. b.wt.) dissolved in 1% DMSO v/v for the next 4 weeks.
8	Group 8; NASH + hydroxy citric acid	6	These rats were fed a high-fat diet for 4 weeks, and were then fed the high-fat diet and intragastrically administered hydroxy citric acid (150 mg/kg. b.wt.) for the next 4 weeks.

Table 2. Specific primer sets for rat liver VEGF.

S. No	mRNA	Sequence	Product length
2	VEGF	5'-CCTGGTGGACATCTTCCAGGAGTACC-3' (sense) 5'-GAAGCTCATCTCTCCTATGTGCTGGC-3' (antisense)	196

polymerase. The reaction conditions for the amplification of the genes of interest are presented in Table 3. Ten-microliter aliquots of the PCR products were subjected to electrophoresis on a 1.25% agarose gel, and the DNA was visualized by ethidium bromide staining. The location and sizes of the products were determined using a 100-bp ladder (Genei Lab, India). The gel was then photographed using a UV transilluminator (Medox Bio Transilluminator model). The quantification of VEGF expression was performed through densitometry, using the Image Master VDS software.

3. Statistical analysis

All data are presented as means \pm standard errors of the mean (SEM). Statistical analysis among groups was performed by one-way analysis of variance (ANOVA), followed by Dunnett's T3 comparison post-hoc test. Differences were considered statistically significant if $P < 0.05$.

4. Results

Histopathological studies revealed the ingestion of the high-fat diet for 8 weeks produces all the prominent characteristics of NASH and the principal histological features of NASH, including steatosis and inflammation, which mimics the NASH in humans (9). Treatment with drugs alone does not cause any deleterious effects. There was inflammation observed, with no fatty degeneration on treatment with pioglitazone (9), and local hepatocyte necrosis with inflammatory collections was seen on treatment with hydroxy citric acid (9). However, hepatocytes appear normal with no obvious fatty or inflammatory changes on treatment with quercetin (9).

VEGF mRNA expression was analyzed by the RT-PCR in all of the experimental groups, as shown in Figure 1. Lane 1 represents the expression of VEGF mRNA in the control group (group 1). Lane 2 represents the expression of VEGF mRNA in the rats with experimentally induced

NASH (group 2). Lane 3 represents the expression of VEGF mRNA in the rats with experimentally induced NASH treated with quercetin (group 7, NASH + quercetin). Lane 4 represents the expression of VEGF mRNA in the rats with experimentally induced NASH treated with pioglitazone (group 6, NASH + pioglitazone). Lane 5 represents the expression of VEGF mRNA in the rats with experimentally induced NASH treated with hydroxy citric acid (group 8, NASH + HCA). A higher expression of VEGF mRNA was observed in the hepatic cells of the rats with experimentally induced NASH (group 2), compared with that observed in the control group (Lane 2, group 2). The quantification of VEGF expression was performed through densitometry, using the ImageMaster VDS software, and the results are shown in Figure 2.

Compared with the rats with experimentally induced NASH (Lane 2, group 2), a very mild increase in the expression of VEGF mRNA was observed in the rats with experimentally induced NASH treated with quercetin (Lane 3, group 7, NASH + quercetin), and mild increases were observed in the rats with experimentally induced NASH treated with pioglitazone (Lane 4; group 6; NASH + pioglitazone) and in the rats with experimentally induced NASH treated with hydroxy citric acid (Lane 5; group 8, NASH + HCA). The drug quercetin showed an effective inhibition of VEGF mRNA expression, as evidenced in Figures 1 and 2 (Lane 3), and a lower inhibition of the VEGF mRNA level was observed in the hydroxy citric acid- and the pioglitazone-treated rats (Lanes 4 and 5).

5. Discussion

The RT-PCR analysis of VEGF mRNA was performed in all of the groups, and the results are presented in Figures 1 and 2. A higher expression of VEGF mRNA was observed in the hepatic cells of rats with experimentally induced NASH (group 2), compared with that found in the control group (Lane 2, group 1). Compared with the rats with experimentally induced NASH, a mild increase in the

Table 3. Reaction conditions for PCR.

cDNA	Initial denaturation °C (min)	Denaturation °C (min)	Annealing °C (min)	Extension °C (min)	Final extension °C (min)	No. of cycles
VEGF	94 (5 min)	94 (1 min)	55 (1 min)	72 (2 min)	72 (3 min)	33

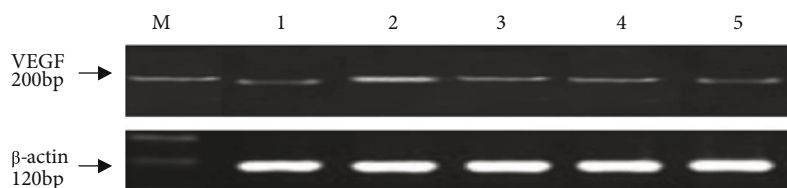


Figure 1. Analysis of vascular endothelial growth factor (VEGF) messenger RNA (VEGF mRNA) expression by reverse transcription polymerase chain reaction (RT-PCR). Lane 1 – Control; Lane 2 – NASH; Lane 3 – NASH + quercetin; Lane 4 – NASH + pioglitazone; Lane 5 – NASH + hydroxy citric acid; Lane M - Marker.

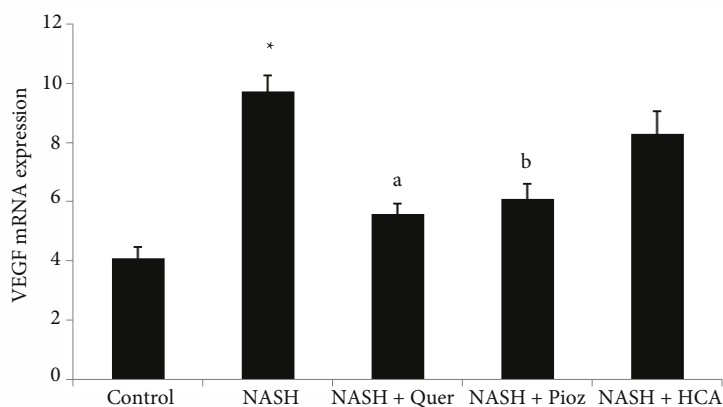


Figure 2. Quantification of VEGF mRNA expression through densitometry using the Image Master VDS software. The values are expressed as the mean \pm standard error of mean (SEM) from six animals in each group; * $P < 0.001$ compared to the control group; ^b $P < 0.01$ compared to the NASH group.

expression of VEGF mRNA was observed in the rats with experimentally induced NASH treated with pioglitazone (Lane 4, group 6, NASH + pioglitazone).

Pioglitazone restores the blood flow and capillary density in ischemic muscle, and this process is associated with increased expression of VEGF (12). VEGF mRNA expression in liver tissue was found to be decreased by pioglitazone treatment, compared with the control group, and this result is concordant with the findings of a previous study conducted in muscles. In contrast, a high dose of pioglitazone suppresses vascular endothelial growth factor (VEGF)-induced endothelial cell proliferation, by suppressing VEGF receptor 1 (Flt-1) and 2 (Flk/KDR) expression in vitro (13). These studies provide supporting evidence for the present findings, which show that pioglitazone treatment decreases the levels of growth factors in liver tissue.

Hydroxy citric acid treatment resulted in a mild increase in the expression of VEGF mRNA in rats with experimentally induced NASH (Lane 5, group 8, NASH + HCA), compared with rats with experimentally induced NASH (Lane 2, group 2). In contrast, quercetin treatment resulted in a very mild increase in the expression of VEGF mRNA in rats with experimentally induced NASH (Lane 3, group 7, NASH + quercetin), compared with rats with experimentally induced NASH (Lane 2, group 2).

VEGF induction is dependent on quercetin-mediated hypoxia-inducible factor-1 (HIF-1) activation. Quercetin delays the appearance of HIF-1 α protein by inhibiting HIF-prolyl hydroxylase (HPH), which is the key enzyme for HIF-1 α hydroxylation and subsequent von Hippel Lindau-dependent HIF-1 α degradation (12). Our data suggest that the clinical effect of quercetin may be partly attributed to the activation of the HIF-1-VEGF angiogenic pathway, through the inhibition of HPH, and that the chelating moieties of quercetin are required for the inhibition of HPH.

Quercetin activates the HIF-1-VEGF angiogenic pathway by inhibiting HIF-prolyl hydroxylase, as determined through a structural analysis of quercetin. Quercetin, which is a flavonol found in several varieties of berries at concentrations of up to 70–80 mg/kg dry wt., has been found to inhibit the growth and stimulate the apoptosis of cancer cells (14).

6. Conclusion

Based on our findings, quercetin exhibits an effective inhibition of VEGF mRNA expression, and a slight inhibition of the VEGF mRNA level was observed in the hydroxy citric acid- and the pioglitazone-treated rats. This study shows the therapeutic value of quercetin, pioglitazone, and hydroxy citric acid.

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