EFFECTS OF MULBERRY HEARTWOOD EXTRACT ON THE GENE EXPRESSION OF NF-kB AND TWO PROINFLAMMATORY CYTOKINES IN A CELL CULTURE MODEL OF OXIDATIVE STRESS

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ABSTRACT

Oxidative stress in adipose tissue is closely associated with low-grade inflammation which can lead to chronic metabolic disorders, particularly obesity-related insulin resistance and type 2 diabetes. In the present study a model of oxidative stress in 3T3-L1 preadipocyte cell culture was used to investigate the effect of Morus nigra tree heartwood ethanol extract on gene expression of nuclear factor kappa B (NF-kB) and the two pro-inflammatory cytokines, tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6). It was estimated that mulberry tree heartwood extract inhibited the expression of NF-kB, TNF-α and IL-6 genes in a concentration depended manner. To our knowledge, these results reveal for the first time the anti-inflammatory potential of the mulberry heartwood extract. This effect could be contributed to the earlier established antioxidative capacity of the extracted compounds.

Key words: Morus nigra; 3T3-L1 preadipocyte cell; oxidative stress; inflammation.

INTRODUCTION

Systemic oxidative stress, defined as a persistent imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses, correlates with metabolic disorders, including insulin resistance, obesity and type 2 diabetes [13]. Increased cytoplasmic ROS contribute to the activation and nuclear translocation of nuclear factor kappa B (NF-kB), which can induce the transcription of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) [7]. Adipose tissue produces large amounts TNF-α and IL-6 and it is a target to study the effects of various biologically active compounds with potential antioxidant activities. TNF-α was the first cytokine to be implicated in the pathogenesis of obesity and insulin resistance [29]. IL-6 was considered to be a stress-induced cytokine with varying effects in different tissues and it is known to be an acute phase protein in the inflammatory response [35].

Morus nigra L. (mulberry tree) is traditionally applied by the folk medicine for various complaints. According to some authors extracts from mulberry leaves exhibit diuretic, hypoglycemic, and hypotensive activities, whereas the fruits and root bark are known to possess antiinflammatory, antitussive, and antipyretic properties [5, 17, 26, 37]. Along with the knowledge about the medicinal properties of the plant, mulberry is known to have also other traditional application – its heartwood is used as a material for barrels manufactured for storage of alcoholic beverages and respectively for their aging. Although the ethanol extracts of mulberry heartwood have specific phytochemical composition [8], to our knowledge their biological effects still remain unrevealed. Moreover, our recent study estimated a high antioxidant activity of the ethanol infusion from heartwood of the plant, strongly correlating with its high polyphenol content [25].

The aim of this study was to determine the ability of ethanol infusion from Morus nigra heartwood to affect the expression of inflammatory cytokines on transcription levels.
MATERIALS AND METHODS

Heartwood processing and extract preparation

Heartwood samples were subjected to fumigation following the popular technology for aging of beverages: the wooden chips were boiled for 10 minutes and then saturated with cold water for 24 hours. Finally, the material was dried for 15 minutes at 150-190°C. Ethanol infusion from *Morus nigra* heartwood was prepared following the traditional recipe for coloring high alcoholic beverages: 2g dried material from heartwood was placed in 1L 40% ethanol for 40 days.

Experimental procedure

Mouse 3T3-L1 preadipocytes were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Lonza), supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin mixture to final concentration of 100U/ml each at 37°C in a humidified chamber containing 5% CO₂. After reaching 80% confluence the cells were collected and seeded in 6 well flasks at density 2x10⁵ cells/well for differents treatments. The *Morus nigra* extract (M) or 40 % EtOOH (Et) were dissolved as follows: 6,25μl, 12,5μl or 25μl to 2 ml in phenol red-free DMEM to a final content in the nutrient medium of 0.3%, 0.6% and 1.25% respectively. For induction of oxidative stress tert-BuOOH (tB) was applied as described earlier [34]. Depending on the treatment, the following groups were defined: Group C (nontreated cells in nutritional medium), Group tB (cells in nutritional medium treated with tert-BuOOH), groups M 0.3/0.6/1.25 and M 0.3+tB/0.6+tB/1.25+tB (cells treated with final concentrations of *M. nigra* extract, respectively 0.3, 0.6 and 1.25 %, without or in presence of oxidant), groups Et 0.3/0.6/1.25 and Et 0.3+tB/0.6+tB/1.25+tB (cells treated with ethanol at final concentrations of 0.3, 0.6 and 1.25 % respectively, without or in the presence of the oxidant). After 24 h- incubation with the respective compounds the cells were harvested for total RNA.

Quantitative real-time PCR analysis

Total RNA was extracted from differentiated 3T3-L1 cells using TRI Reagent according to the manufacturer’s protocol (Ambion). In order to remove contamination of genomic DNA, DNA-ase I treatment was performed using the recommended protocol of the manufacturer (Sigma). Complementary DNA was synthesized using Revertaid™ First Strand Synthesis Kit with oligo (dT)₁₈ primers and RevertAid™ reverse transcriptase (Fermentas). The synthesis reaction was performed on GeneAmp PCR System 9700 (Applied Biosystems). Two-step real-time PCR analysis was performed (ABI PRISM 7500, Applied Biosystems) to estimate gene expression level in cultured cells. Maxima SYBR Green qPCR Kit (Fermentas) was used for sample analysis. The cDNA was amplified using forward and reverse primers of target genes (Table 1) commercially synthesized (Invitrogen Alpha DNA, Canada). Beta-actin was used as endogenous control. All samples were analyzed in triplicates. Gene expression levels were calculated by 2⁻ΔΔCt method [20] and expressed as relative units (RU) mRNA compared to the untreated controls where the level of gene expression of interest was considered to be equal to 1.

Table 1. Sequences of primers used for RT-PCR analysis

<table>
<thead>
<tr>
<th>Genes</th>
<th>Nucleotide sequence</th>
</tr>
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<tbody>
<tr>
<td>β-Actin</td>
<td>F: 5'-ACGGGCAGGCAGGATCCTCAAAGGCTGGAAGAAG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CAAGAGAAGGCTGGAAGAAGG-3'</td>
</tr>
<tr>
<td>NFκB1</td>
<td>F: 5'-ATGGCCAGACGATGATCCCTAC-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-TGTTGACAGTGTTATTTCTGGTG-3'</td>
</tr>
<tr>
<td>TNFa</td>
<td>F: 5'-CCCTCACACTCAGATCATCCTCACT-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GCTACGCAGTGGGCTACAG-3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>F: 5'-GAGTTGTGCAATGGCAATTCGAG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GCAAGTGCACTCAGTGTTCTCAT-3'</td>
</tr>
</tbody>
</table>

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Statistical Analysis
Data are presented as mean ± standard error of mean (SEM). Differences between means of groups were analyzed using Student’s t-test or one-way ANOVA with Dunnett’s multiple comparison test (GraphPad Prism 5.0). Values of P<0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Effect of the oxidant on gene expression
A model of oxidative stress induced in preadipocite cell culture was used with the aim to explore the effects of three concentrations of mulberry heartwood ethanol extract on genes related to inflammatory response. The same concentrations of ethanol or ethanol + oxidant were applied in order to distinguish the effects of the plant-derived active compounds from the effects of ethanol solely. The oxidative stress was induced with tertiary-Butyl hydroperoxide which is commonly used in developing cellular oxidative stress. The mechanisms of its metabolism involves membrane lipid peroxidation and depletion of cellular storage of glutathione [21, 28, 16]. The addition of the oxidant to the nutrition medium (group tB) in our study resulted in a significant increase in mRNA levels of the three tested genes, as compared with the non-treated cells (**p<0.01 vs. group C). Results are summarized in figures 1, 2 and 3.

Expression of NF-kB
None of the applied concentrations of the extract changed the NF-kB expression. Ethanol applied alone stimulated the expression of the gene in concentration dependent manner (*p<0.05).

Upon induction of oxidative stress (groups M+tB) only the highest concentration of the extract significantly inhibited the expression of NF-kB as compared with the two lower concentrations (groups M0.3+tB and M0.6+tB). Furthermore, this inhibitory effect was confirmed in comparison with the cells treated with oxidant alone (group tB) and the same concentration of ethanol with oxidant (Et1.25+tB). It could be suggested that the concentration-dependent manner of the effects is due to the active compounds extracted in the solution from the plant. Data are summarized in figure 1:

![Figure 1](image-url)

**Figure 1.** Expression of NF-kB in preadipocytes, treated with different concentrations of *M. nigra* extract or ethanol in normal medium or under induced oxidative stress. Data are presented as mean±standard error of mean (SEM); *p<0.05, **p<0.01 versus group C, # p<0.01 versus groups M 0.3+tB and M 0.6+tB; †p<0.05 versus group tB; ‡p<0.05 versus group Et 1.25+tB.
NF-kB has been described as a redox-sensitive transcriptional factor since it may be activated by oxidative stress and inhibited by various antioxidants [31]. In a previous study we estimated that the mulberry heartwood extract possesses high antioxidant activity in vitro, correlating with high total polyphenol content [25]. The activation of NF-kB system is associated with chronic pathological conditions including CVD, type 2 diabetes, obesity and neurodegenerative disorders [2, 33, 32] and these conditions are often accompanied by oxidative stress [18, 22]. The results described above revealed the mulberry heartwood extract as a potent NF-kB inhibitor under oxidative stress and thus as a source of active compounds for prophylaxis and treatment of oxidative based-chronic diseases.

Expression of TNFα

The changes of TNFα transcriptional levels are presented in figure 2. Significant increase in mRNA levels of the gene was measured in the cells treated with highest concentration of the extract (M1.25), compared with the two lower concentrations and with the untreated cells (group C). This effect could be attributed rather to the ethanol contented in the extract since ethanol applied alone in all concentrations stimulated TNFα expression (groups Et 0.3, Et 0.6 and Et 1.25). Moreover, the ethanol and the oxidant applied together had a cumulative stimulatory effect on the mRNA levels of the gene (\( \text{**p} < 0.001 \), vs. group Et1.25). This effect of ethanol is not surprising. Several studies reported that the basic mechanism of alcohol-induced cellular damage involves upregulation of proinflammatory cytokines via excessive generation of reactive oxygen species [1, 10, 11].

The highest concentration in the extract, applied in the oxidatively stimulated cells significantly inhibited the expression of TNFα (\( \text{ccc} \ p < 0.001 \), vs. Et1.25+tB). This inhibitory effect of the extract observed under induced oxidative stress is not unexpected, regarding its high polyphenol content. Many authors reported similar effects of the polyphenols or polyphenol-rich extracts on the proinflammatory cytokines (including TNFα) in various experimental models of oxidative stress and inflammation [6, 23, 30, 40, 41].

Adipose tissue is the predominant source of the elevated TNF-α in obesity and it is known as a mediator of obesity-related insulin resistance and type 2 diabetes [12, 36, 38]. Based on the results commented above it could be suggested that the mulberry extract has a potential to ameliorate adipose tissue metabolic disorders.

![Figure 2. Expression of TNFα in preadipocytes, treated with different concentrations of M. nigra extract or ethanol, in normal medium or under induced oxidative stress. Data are presented as mean±standard error of mean (SEM); *p<0.05, **p<0.01 versus group C; a p<0.05 versus group M 0.3; b p<0.05 versus group M 0.6; ccc p<0.001 versus group Et 1.25+tB; ccc p<0.001 versus group Et 1.25.](image-url)
Expression of IL-6

Unexpectedly, the levels of IL-6 mRNA were significantly increased in cells treated with the highest concentration of the extract (M1.25), compared with the two lower concentrations and the controls (fig. 3). This stimulatory effect of the extract could be attributed mainly to the active compounds extracted from the plant than to the ethanol. The results received for the ethanol treated cells (groups Et0.3, Et0.6 and Et1.25) support this suggestion. No changes in mRNA levels were estimated in these groups.

Many of the studies focused on the mulberry extracts from leaves, fruits and root bark demonstrated the potential of the investigated preparations to inhibit proinflammatory cytokins, including IL-6 [9, 19, 24]. However, bearing in mind the specific phytochemical composition of the heartwood as compared to that of other parts of the plant, it could be suggested that along with the polyphenols extracted in the solution, other specific active compounds probably contributed to the effects on cytokines gene expression, including the IL-6 upregulation.

According to the scientific literature, some plant preparations have a potential to activate the healthy immune system by increasing inflammatory and anti-inflammatory cytokines production in vivo and in vitro, suggesting the immunomodulatory properties of the investigated plants [3, 4, 27, 39, 14, 15].

![Graph](image)

**Figure 3.** Expression of IL-6 in preadipocytes, treated with different concentrations of *M. nigra* extract or ethanol, in normal medium or under induced oxidative stress. Data are presented as mean±standard error of mean (SEM); **p<0.01 versus group C; aap<0.01 versus group M 0.3; bpb<0.01 versus group M 0.6; cpc<0.01 versus group Et 1.25

**CONCLUSION**

To our knowledge this is the first investigation of the anti-inflammatory properties of the mulberry heartwood extract. Along with the inhibitory effects on the NF-kB and TNFα gene expression, the mulberry extract stimulated IL-6 mRNA levels. This dual effect reveals the immunomodulatory properties of the investigated extract. Based on the results presented above, we concluded that the studied preparation could have beneficial effects in prophylaxis and therapeutics in oxidative stress-based pathological conditions.

**REFERENCES**


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