Vascular endothelium protective property of curcumin

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ABSTRACT

Objectives: In the present study, we aimed to examine the efficacy of curcumin on endothelial vascular protection on human umbilical vein endothelial cells (HUVEC) cells in the in vitro setting and to determine effective doses.

Materials and methods: Cytotoxic effect and wound healing activity of curcumin on the HUVEC cell line were evaluated and angiogenesis was studied by tube formation assay. The cytotoxic activity of 0.5 to 8 µM curcumin on HUVEC cell line was tested using the WST-1 cell proliferation assay. In wound healing determination, the scratched cells were incubated with the half maximal effective concentration (EC50) dose of curcumin and wounds were monitored by the JuLI™ Br live cell movie analyzer. Wound gaps were measured using the ImageJ software. To determine the angiogenesis, tube formation assay was performed and the results were analyzed.

Results: The 1.77 µM of curcumin increased the cell viability by 150% after 48 h of treatment. According to the wound healing results, after 48 h of incubation, the control group and 1.77 µM curcumin exhibited 47.1% and 43.8% closure, respectively. The mean maximum and minimum tube lengths were found to be 21,858±3,945 and 12,438±3,817 pixel for curcumin, respectively with low fetal bovine serum (FBS) and curcumin with high FBS, respectively.

Conclusion: Our results show that curcumin is a promising endothelial protective agent for HUVEC cell line.

Keywords: Curcumin, cytotoxic effect, endothelium protective, wound healing.

Curcumin ([1E, 6E] -1,7-bis [4-hydroxy-3-methoxyphenyl] hepta-1,6-diene-3,5-dione) is a yellow-orange pigment of a perennial herb turmeric (Curcuma longa and Curcuma spp.) and it is the synonym of diferuloylmethane. It is known as the “solid gold” of India, and it has been used frequently in both medicine and gastronomy since ancient times. The World Health Organization (WHO) recommends a daily intake of curcumin as a coloring agent in foods of 0 to 3 mg/kg. In practice, it is soluble in alkaline or highly acidic solvents, as well as in water-insoluble, but polar and non-polar organic solvents at acidic and neutral pH. In the literature, biological (anti-inflammatory, cyclooxygenase inhibition, protein kinase C and other enzyme inhibitors, antioxidants, radical scavengers, antimicrobials, etc.) and industrial (yellow textile dye, food preservative, etc.) properties has been shown so far. There is also a biological activity on cancers such as liver and cervical cancer, breast cancer, and colorectal cancer.

Endothelium is the inner cellular liner of the blood vessels. It is critical for the homeostasis
and regulation of vascular structure and tone.\textsuperscript{[12]} Endothelial cells are of great importance on the regulation of cardiovascular function.\textsuperscript{[11]} Endothelial cells lose their vascular protective function and become a pro-atherosclerotic structure in cardiovascular diseases such as cerebrovascular disease, peripheral arterial thrombosis, and acute myocardial infarction.\textsuperscript{[12]} Smoking habits, diabetes, high blood pressure, and high serum total cholesterol levels are the risk factors for cardiovascular diseases.\textsuperscript{[13]} In order to protect the endothelium, many natural biological active compounds such as polyphenols, flavonoids, catechin, epicatechin, anthocyanidins and resveratrol have been used.\textsuperscript{[14-19]}

In the present study, we aimed to examine the efficacy of curcumin on endothelial vascular protection on human umbilical vein endothelial cells (HUVEC) cells in the \textit{in vitro} setting and to determine effective doses.

\section*{MATERIALS AND METHODS}

\subsection*{Cell culture}

The current study was designed to evaluate the vascular protector effect of curcumin on HUVEC cells and all experiments were studied in quadruplicates. The HUVEC (ATCC CRL-1730) was obtained from the American Type Culture Collection (Rockville, CT, USA) and cultured in Ham's F12 Medium (Biochrom GmbH, Berlin, Germany) containing 20\% fetal bovine serum (FBS, Cegrogen Biotech GmbH, Stadtallendorf, Germany) and 1\% penicillin/streptomycin (Biochrom GmbH, Berlin, Germany). The cells were incubated in 5\% carbon dioxide (CO\textsubscript{2}) incubator at 37\degree C in humidified air.

\subsection*{Cell proliferation and viability}

The endothelial protective activity of 0.5 to 8.0 \textmu M curcumin (C7727, Sigma-Aldrich, St. Louis, MO, USA) was tested on HUVEC cells. The HUVEC cells were seeded in a quadruplicate in 96-well plate (1x10\textsuperscript{4} cells/well). After 48 h of treatment with curcumin, cell viability was measured via 10 \mu L WST-1 (Version 16 Cell Proliferation Reagent WST-1, Roche Diagnostics, IN, USA). After 2-h incubation with WST-1, the cell viability was measured at 450 nm. Also, the cytotoxic concentration of 2.5 to 50.0 \textmu M curcumin was tested with the same cell viability procedure.

\subsection*{Wound healing assay}

Wound healing assay was applied to measure the migration property.\textsuperscript{[20]} The HUVEC cells were seeded in a six-well plate (30x104 cells/well). After confluency of cells, the wells were scratched via 100 \mu L of pipette tip and the half maximal effective concentration (EC\textsubscript{50}) dose of curcumin was added. The medium was used as the control. The JuLI\textsuperscript{TM} Br live cell movie analyzer (NanoEnTek, JuLI Br04, Hwasung, Korea) was used for monitoring the wounds. The ImageJ version 1.49 software (National Institutes of Health, Bethesda, MD, USA) was used for measuring the wound gaps. Also, the wound healing activity of cytotoxic doses (inhibitory concentration of 50 [IC\textsubscript{50}] and IC\textsubscript{10}) of curcumin was tested via the same protocol.

\subsection*{Tube formation assay}

Tube formation experiment was performed to determine angiogenesis. The matrix was pre-incubated at +4\degree C overnight and seeded in a 24-well plate in 150 \mu L media, reducing the level of growth factor, and incubated for half an hour at 37\degree C. A total of 2.5x10\textsuperscript{4} cells were seeded in each well. Growth media containing 20\% FBS was added as a negative control. Growth media containing 2\% FBS was used as a positive control. The effective concentration of curcumin (1.77 \textmu M) was added to each well. The experiment was repeated three times. Bright field images of capillary-like tubes were taken between 0 and 24 h, and the mean tube length was measured using the ImageJ version 1.49 software (National Institutes of Health, Bethesda, MD, USA).

\subsection*{Statistical analysis}

Statistical analysis was performed using the SPSS version 15.0 software (SPSS Inc., Chicago, IL, USA). Numerical data were expressed in mean ± standard error (SE). A non-parametric Mann-Whitney U test was used to analyze the statistical significance of the results. A \textit{p} value of <0.05 was considered statistically significant.

\section*{RESULTS}

\subsection*{The effect of curcumin of cell proliferation of HUVEC cells}

In order to determine the effective dose of curcumin on vascular endothelial protection, the EC\textsubscript{50} value was calculated and found to be 1.77 \textmu M after 48 h of incubation with curcumin. Also, \textit{in vitro} cytotoxic curcumin doses on HUVEC cells were calculated and IC\textsubscript{50} and IC\textsubscript{10} values of curcumin were found to be 20 \mu M and 10 \mu M, respectively (Figure 1).

\subsection*{Wound healing assay}

After 48 h incubation, both control group and EC\textsubscript{50} dose of curcumin (1.77 \textmu M) exhibited 47.1\%
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Figure 1. Cell viability and proliferation activity of curcumin on HUVEC cells. (a) effective dose of curcumin (b) cytotoxic effect of curcumin (c) effective and inhibitory doses of curcumin.

HUVEC: Human umbilical vein endothelial cell; EC: Effective concentration; IC: Inhibitory concentration

Figure 2. In vitro wound healing activity of curcumin for EC50 dose. The HUVEC cells were seeded in a six-well plate before scratch wounds. Cells were cultured for 48 h and the migration potential was evaluated by measuring the distance of the gap. The images were obtained by the JuLI™ Br and the results were evaluated by ImageJ 0, 24 and 48 h after the scratch.

HUVEC: Human umbilical vein endothelial cell; EC50: Half maximal effective concentration.

Figure 3. In vitro wound healing activity of curcumin for cytotoxic dose. 3 × 10^5 HUVEC cells were seeded in a six-well plate before scratch wounds. Cells were cultured with the IC10 and IC50 doses (10 and 20 µM, respectively) for 48 h and the migration potential was evaluated by measuring the distance of the gap. The images were obtained by JuLI™ Br and the results were evaluated by ImageJ 0, 24 and 48 h after the scratch.

HUVEC: Human umbilical vein endothelial cell; IC10: Inhibitory concentration of 10; IC50: Inhibitory concentration of 50.
and 43.8% closure, respectively (Figure 2). After 48 h of incubation, the control group of the cytotoxic group exhibited 20.1% closure. The IC_{10} dose of curcumin (10 µM) showed 0.7% closure and IC_{50} dose of curcumin (20 µM) showed 43.4% closure (Figure 3).

**Tube formation assay for in vitro angiogenesis**

Tube formation assays was carried out to investigate the efficiency of curcumin with high and low FBS on HUVEC. According to the results, curcumin in the presence of low FBS formed significantly greater tube networks on HUVEC cell line (p<0.05) and tube mean length was found to be 21,858±3,945 pixel. On the other hand, curcumin in the presence of high FBS formed less tube networks on HUVEC cell line (p<0.05) and the mean tube length was found to be 12,438±3,817 pixel (Figure 4a, b).

**DISCUSSION**

In this study, effective and cytotoxic doses of curcumin were determined, migration activity was evaluated by wound healing assay, and angiogenetic efficiency was investigated on the HUVEC cell line. In a study on Montiel-Dávalos et al.,[21] the HUVEC cells were used to investigate whether curcumin could reduce different events related to endothelial activation, cells treated with curcumin, followed by PM10 and TiO_{2}NP. The results showed that curcumin had anti-inflammatory and antioxidant role which could alleviate the activation of endothelial cells through exposure to particulates. Therefore, the authors concluded that curcumin might be useful in the treatment of diseases in which an inflammatory process and endothelial activation are implicated.

By modulating different molecular targets such as inhibition of protein kinases, downregulation...
of expression of growth factors, induction of cell cycle, suppression of transcription factors activation, induction of apoptosis, curcumin exhibits anticancer activity on different cancer cell lines.\[22\] Determination of effective and cytotoxic doses of curcumin on HUVEC is critical for further studies. In this study, effective dose (EC\(_{50}\)) of curcumin was found to be 1.77 \(\mu\)M and IC\(_{50}\) and IC\(_{10}\) doses of curcumin on HUVEC were found to be 20 \(\mu\)M and 10 \(\mu\)M, respectively. After 72 h of incubation with compounds, Koo et al.\[22\] found the IC\(_{50}\) dose of curcumin and its derivatives on HUVEC cell line to be 20 \(\mu\)M by using the methyl-thiazolyl-tetrazolium (MTT) assay.

In the literature, wound healing activity of curcumin has been studied clinically.\[1\] In our study, EC\(_{50}\) dose of curcumin showed 43.8% closure on the HUVEC cell line. According to the study of Koo et al.,\[22\] the HUVEC cell line incubated with curcumin showed almost 50% closure. These results are consistent with our findings.

Angiogenesis is a natural process which refers to generation of new lymph and blood vessel networks from primers. It is crucial for proliferation, metastasis, and reinforcement of oxygen and nutrient on cells.\[23\] In this study, the mean tube length was found to be 21,858\pm3,945 pixel for curcumin in the presence of low FBS. The extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) phosphorylation is of great importance on endothelial cell proliferation and angiogenesis activity. According to the Western Blot analysis results of Koo et al.,\[22\] a derivative of curcumin inhibited angiogenesis more than curcumin. Bai et al.\[24\] also used HUVEC cells to demonstrate that curcumin inhibited angiogenesis by upregulation of micro-ribonucleic acid (microRNA) -1275 and microRNA -1246, suggesting that it was a promising therapy for corneal neovascularization. According to these results, curcumin can inhibit the HUVEC proliferation via upregulation of miR -1275 and miR -1246.\[24\]

The in vitro design is the main limitation of this study, leading to discrepancies in the translation of the information into the in vivo setting, due to lack of metabolism, immune system, and tissue hierarchy.

In conclusion, endothelium is crucial for the regulation and homeostasis of vascular structure, and loss of endothelial cells may lead to development of cardiovascular diseases such as cerebrovascular disease, peripheral arterial thrombosis, and acute myocardial infarction. Therefore, protection of vascular endothelial cells is crucial. Based on our study results, curcumin seems to be very effective, particularly on angiogenesis, and is a promising agent on vascular endothelial cell protection.

**Declaration of conflicting interests**

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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