Green Tea Polyphenols Inhibit Oxidized LDL-induced NF-κB Activation in Human Umbilical Vein Endothelial Cells

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ABSTRACT

Aim: *to examine the anti-inflammatory effects of green tea polyphenols on oxLDL-mediated TNFα expression and NF-κB activation in the human umbilical vein endothelial cells (HUVECs).*

Methods: *we postulate that green tea polyphenols regulate TNF-α gene expression by modulating NF-κB activation through their inhibition effect on IκB Kinase (IKK) activity and as scavenger of free radicals. Pretreatment of green tea polyphenols reduced oxLDL-induced production of proinflammatory cytokine TNF-α and NF-κB activation in dose dependent manner ($p < 0.05$).*

Post hoc comparison test with Mann Whitney between various dosage of green tea polyphenols in inhibition of NF-κB activation showed significant result ($p < 0.05$).

Results: *in TNF-α expression, there was also declined TNF-α productions (p 0.09; 0.2 vs 0.4mg/ml: ns). The effect of green tea polyphenols on TNF-α expression were determined by Mann-Whitney test. There is significant difference between the first dose (0.1mg/ml) vs 0.2mg/ml polyphenols (p=0.009); between 0.1 vs 0.4 mg/ml polyphenols (p=0.009). There was no difference when the dose was increased from 0.2 mg/ml to 0,4 mg/ml polyphenols (0.141). In this study, green tea polyphenols showed significant effects on the inhibition of TNF-α through NF-κB activation pathway in HUVECs with oxidized LDL.*

Conclusion: *green tea polyphenol can be used to prevent endothelial dysfunction, thus, prevent the development of atherosclerosis.*

Key words: *polyphenols, tea, oxidized-LDL, NF-κB, tumor necrosis factor.*

INTRODUCTION

Atherosclerosis is regarded as a dynamic and progressive disease arising from the combination of endothelial dysfunction and inflammation. This is a progressive disease and the underlying cause clinical conditions include coronary heart disease (CHD) and peripheral vascular disease. It is a common cause of morbidity and mortality in developed countries and is therefore a subject of intensive research.[1-5]

Hypercholesterolemia is the main contributing factor to the development of atherosclerosis. It can accumulate in blood vessels and cause changes in endothelial function and structure. LDL is the largest lipoprotein carrier in plasma. Seventy percent of total plasma cholesterol is carried out in LDL. Circulating LDL is sensitive to oxidation. Oxidized lipoproteins are thought to be essential in the initial endothelial insult that precedes lesion formation.[6-9]

Nuclear factor-κB (NF-κB), an oxidative stress sensitive transcription factor, controls the expression of a wide variety of genes active in inflammation that include cytokines (e.g., IL-1, TNFα, IL-8), enzymes (inducible nitric oxide synthase [iNOS]), adhesion molecules and acute phase proteins. These observations suggest that NF-κB is a suitable target to prevent or reduce an inflammatory response. There is increasing interest in the role of nutrients in health and disease. One such nutrient is tea.[10-14]

Epidemiological evidence of linking tea or flavonoid consumption with coronary heart disease or total mortality among healthy adults is conflicting, but tea consumption may have its strongest effect among patients with cardiovascular disease. A recent randomized crossover trial by Sesso showed that short- and long-term black tea consumption could reverse endothelial dysfunction in patients with documented coronary heart disease, providing plausible mechanism for an effect of tea in patients with cardiovascular disease.[15-19]

Epigallocatochin-3-Gallat (EGCG) decreased LPS-induced TNF-α production in the macrophage cell line RAW264.7 and peritoneal macrophages by blocking NF-κB activation.[20] Lin and Lin[21] reported that EGCG inhibited LPS-induced inducible nitric-oxide synthase gene expression in mouse peritoneal macrophages by decreasing the expression of the transcription factor, NF-κB. Ahmad et al. recently showed that green tea

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polyphenols modulate NF-κB in several cancer cell lines, rendering them susceptible to apoptosis. Recently, Pan et al showed that the black tea derivative theaflavin-3,3-digallate and EGCG block the phosphorylation of IκB. In this study, we further examined the anti-inflammatory effects of green tea polyphenols on oxLDL-mediated TNFα expression and NF-κB activation in the HUVECs.[20-24]

METHODS

Reagents

Collagenase, Hank's balance salt sodium, NBS (Newborn calf serum) 20%, gentamycin sulphate, penicillin-streptomycin, sodium hydrogen bicarbonate, HEPES solution, glutamine and all cells growth supplements were purchased from Sigma. M199 from Gibco. Green tea polyphenols were obtained from Sigma (St Louis, MO). Oxidized LDL lipoprotein, Human from Biomedical tech inc. All flavonoids were solubilized by ethanol for culturing with cells; the final culture concentration of ethanol was ≤ 1%.

Primary Culture of Human Umbilical Vein Endothelial Cells (HUVECs)

HUVECs were isolated from fresh umbilical cord obtained at normal deliveries. The umbilical vein was cannulated with 50 ml of PBS to remove any blood, after which the vein was filled with 20cc of collagenase dissolved in PBS and incubated for 8 minutes at 37°C. The collagenase solution was drained from the cord and collected, and the cord was gently flushed with 20 ml of PBS, which was added to the collagenase solution. The cells in these pooled solutions were recovered by centrifugation at 200g for 5 minutes and transferred to 30-mm cultured dishes in M199 containing 20% NBS, antibiotics (penicillins and streptomycins), and 25mg/ml endothelial cell growth supplements (Sigma). On reaching confluency (80%), the cells were used for experiments. Five groups of HUVECs were plated (5 replicates per group). The groups were: a) control group without any treatment; b) group that received 50mg/ml OxLDL; c) group which were incubated with 0,1 mg/ml green tea polyphenols for 2 hours before exposed to 50 mg/ml OxLDL; d) group which were incubated with 0.2 mg/ml green tea polyphenols for 2 hours before exposed to 50 mg/ml OxLDL; e) group which were incubated with 0.4 mg/ml green tea polyphenols for 2 hours before exposed to 50 mg/ml OxLDL. The HUVECs were harvested after 30 minutes exposure of OxLDL to see NF-κB activation and after 24 hours to see TNF-α expression.

Immunocytochemistry

After endothelial cells were thoroughly washed with PBS, cells were incubated for 20 minutes with 10% normal goat serum in PBS to block any non specific binding. After fixed cells were washed twice with PBS, Mouse monoclonal anti p50-p65 human adsorbed/mouse monoclonal TNF-α antibody (Sigma) was added to cells and incubated overnight at 4°C. Cells were washed with PBS and goat antimouse was added as secondary antibody. Images were obtained by inverted microscope with Nikon microscope.

Statistical Analysis

Data were expressed as mean ±SD and mean rank. Differences were examined by Kruskal-Wallis test and post hoc Mann Whitney test for comparisons between groups. Statistical significance was estabilished at $p<0.05$. Statistical analysis was conducted using SPSS software package version 11.5. Each experiments consisted of at least 5 replicates per condition.

RESULTS

In this experiment, after umbilical from fresh deliveries, endothelial cells was isolated and cultured for 3-4 days in cultured dishes. The media was changed every 2 days. Endothelial cell was identified by its hexagonal shaped or cobblestone appearance with eccentric nucleus. After the confluency was achieved, the HUVECs were treated with green tea polyphenols and oxidized LDL.

The data were calculated by counting cells in every 100 endothelial cells that showed brown colored in cytoplasm for TNF-α expression and cells that showed brown colored in both cytoplasm and nucleus for NF-κB activation. Descriptive data were shown as means ± SD. Data for Kruskall Wallis test were shown as mean ranks.

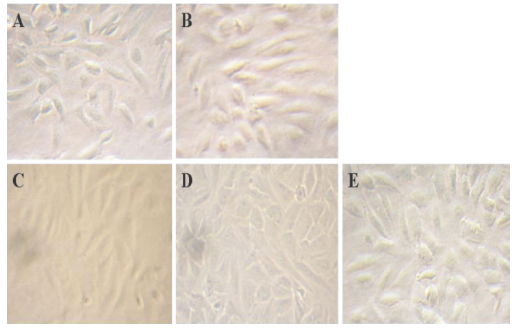

Figure 1. HUVECs culture. A). Control; B). OxLDL; C). OxLDL + 0,1 mg/ml green tea polyphenols; D). OxLDL + 0,2mg/ml green tea polyphenols; E). OxLDL + 0,4mg/ml green tea polyphenols. (Magnification 200x)

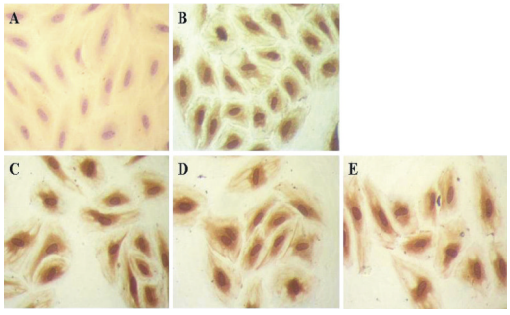

Figure 2. Immunocytochemistry of endothelial cells with monoclonal p50 antibody showed eccentric nucleus and cytoplasm covered in brown color (positive for NF-κB activation) compared with control. A). Control; B). OxLDL; C). OxLDL + 0,1 mg/ml green tea polyphenols; D). OxLDL + 0,2mg/ml green tea polyphenols; E). OxLDL + 0,4mg/ml green tea polyphenols. (Magnification 200x)

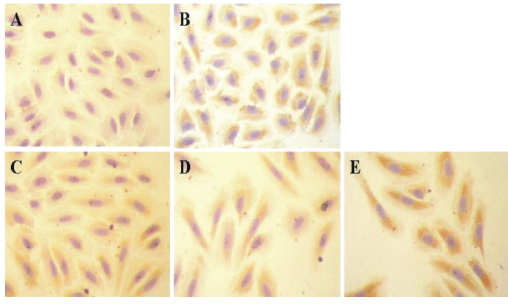

Figure 3. Immunocytochemistry of endothelial cells with monoclonal anti TNF-a antibody showed cytoplasm covered in brown color (positive for TNF-a expression) compared with control. Nucleus is blue. A). control; B). OxLDL; C). OxLDL + 0,1 mg/ml green tea polyphenols; D). OxLDL + 0,2mg/ml green tea polyphenols; E). OxLDL + 0,4mg/ml green tea polyphenols. (Magnification 200x)

Using Kruskal-Wallis test to analyze statistically green tea polyphenols effect on NF-κB activation on HUVECs, the results showed significant result (p=0.000) on all groups. The same results were shown on all groups in green tea polyphenols effects on TNF-α expression with p = 0.000.

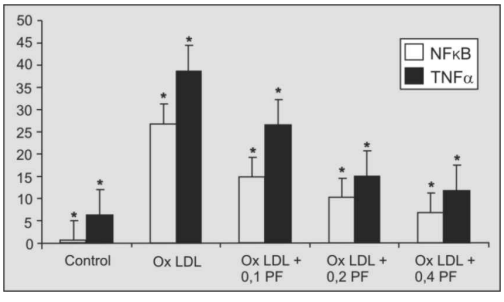

Figure 4. Effect of green tea polyphenols on oxidized LDL-induced NF-κB activation and TNF-α expression in HUVECs. HUVECs were incubated with green tea polyphenols for 2 hours followed by addition of 50mg/ml OxLDL. After 30 minutes, cells were harvested and the data was collected by counting cells with positive staining of NF-κB every 100 cells. Data were expressed as means, n=5, *p<0.05, **p>0.05 (Kruskal-Wallis test)

To compare each dose of green tea polyphenols, we used Mann-Whitney test. The results were shown in figure 5. There is significant difference between the first dose (0.1mg/ml) vs 0.2mg/ml polyphenols (p=0.009); between 0.1 vs 0,4 mg/ml polyphenols (p=0.009). Also in 0.2 vs 0.4 mg/ml polyphenols (0.020). Green tea polyphenols inhibit NF-κB in dose dependent fashion.[25-26]

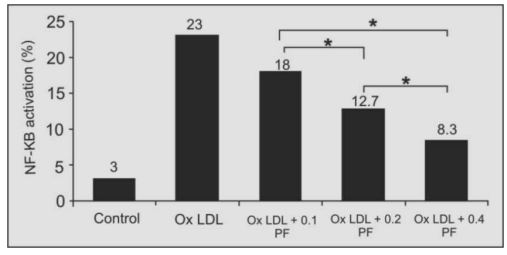

Figure 5. Effect of green tea polyphenols on Oxidized LDL-induced NF-κB activation in HUVECs. HUVECs were incubated with green tea polyphenols for 2 hours followed by addition of 50mg/ml OxLDL. After 30 minutes, cells were harvested and the data was collected by counting cells with positive staining of NF-κB every 100 cells. Data were expressed as mean ranks (Mann Whitney test), n=5, *p<0,05, **p>0,05 (not significant).

The effect of green tea polyphenols on TNF-α expression were determined by Mann-Whitney test. The results were shown in figure 6. There is significant difference between the first dose (0.1mg/ml) vs 0.2mg/ml polyphenols (p=0.009); between 0.1 vs 0.4 mg/ml polyphenols (p=0.009). There was no difference when the dose was increased from 0.2 mg/ml to 0,4 mg/ml polyphenols (0.141).

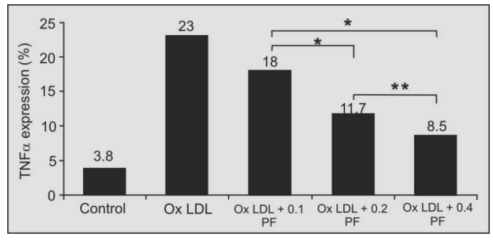

Figure 6. Effect of green tea polyphenols on Oxidized LDL-induced TNF-α expression in HUVECs. HUVECs were incubated with green tea polyphenols for 2 hours followed by addition of 50mg/ml OxLDL. After 24 hours, cells were harvested and the data was collected by counting cells with positive staining of TNF-α every 100 cells. Data were expressed as mean ranks (Mann Whitney test), n=5, *p<0,05, **p > 0,05 (not significant).

DISCUSSION

Our studies support the hypothesis that green tea polyphenols inhibit TNF-α expression by inhibiting NF-κB activation. These results suggest that green tea polyphenols reduce inflammatory responses by attenuating NF-κB activation. The activation of NF- κB

leads to an increase in expression of many genes whose products mediate immune responses. These include proinflammatory cytokines (e.g., IL-1, TNF-α, IL-8), enzymes (e.g., iNOS) and adhesion molecules. The production of IL-1, TNF-α, interleukin-6 (IL-6) and IL-8 are increased in acute and chronic inflammatory processes. Of these, TNFα assumes a pivotal role. Cells of the macrophage/monocyte lineage are the predominate source of TNFα in vivo. The administration of TNFα in physiologically relevant concentrations is sufficient to mediate all of the clinical manifestations of overwhelming inflammation. Clinical studies are now focused on blocking or down-regulating TNFα in chronic inflammatory diseases. Our observations warrant further investigation in the use of green tea polyphenols in the treatment of endothelial dysfunction.

A number of antioxidants such as N-acetyl-L-cysteine (glutathione precursor), dithiocarbamates, vitamin E,and chelators of copper and iron ions have been reported to potently suppress NF-κB activity, suggesting that reactive oxygen species have an intracellular intermediary role. Tea has been shown to have antioxidant effects in both in vitro and in vivo systems.[27] Much of the antioxidant properties of tea arise from the polyphenol fraction. In fact, 78% of the antioxidant potential of green tea extracts are accounted for by polyphenols.[27] Of these, EGCG is the most abundant (40%), and a single cup (100 mL) of green tea contains approximately 50 mg of EGCG. Polyphenols potently scavenge free radicals and are also chain-breaking antioxidants. In comparison to other commonly used antioxidants, green tea polyphenols have at least twice the antioxidant potential of vitamins E or C.

Lin and Lin[22] examined the effect of EGCG on the expression of iNOS in thioglycollate-elicited peritoneal macrophages isolated from BALB/c mice. They showed that doses of 5 and 10 mmol EGCG/L effectively inhibited iNOS expression by blocking NF-κB activation. There are several potential differences in the two studies. These differences include the source of the EGCG extracts, how it was given and the cell models studied. Lin and Lin gave the EGCG with the LPS, whereas we pretreated the cells with green tea polyphenols for 2 h prior to oxidized LDL. Green tea polyphenols showed larger effect than EGCG alone in a study by Yang[20,28] in inhibiting NF-κB activation in intestinal epithelial cell line. Secondly, they studied thioglycolate-elicited peritoneal macrophages, whereas we studied the HUVECs. We want to investigate the role of green tea in the prevention of endothelial dysfunction in atherosclerosis. Endothelial cells initiate the first step where early process of atherosclerosis began. One concern is the time point which is in the study by Lin and Lin chose for detecting NF-κB activity. We examined the effect of green tea polyphenols on oxidized LDL-mediated NF-κB activation at a time point when nuclear NF-κB activity peaks following stimulation (30 minutes), while Lin and Lin measured NF-κB activity 3 hours following stimulation. Third, there was also difference in quantifying results. In the study by Lin and Lin, EMSA was used to determine NF-κB activity. We used semiquantitative the method by using cell counting in HUVECs.

In contrast to our study, Sakagami et al demonstrated that EGCG isolated from Japanese tea potently stimulated IL-1α, IL-1β and TNFα synthesis in cultured human peripheral blood mononuclear cells, but not in several other human cell lines tested. They also observed that EGCG promoted adherence of these cells, though no studies were performed to determine if adhesion molecules were induced. In our study, green tea polyphenols induced a modest decrease in TNF-α expression in the HUVECs. The importance/relevance of these observations and the mechanisms involved remains uncertain, but clearly appear to be cell type or specific cell line. The concentrations used in this study are much higher than those readily available in food sources (tea). This study showed a significant effect of green tea polyphenols in inhibiting NF-κB and subsequently also inhibit TNF-α production. No previous studies had been done in oxLDL-mediated HUVECs to establish the role of green tea polyphenols. Further studies are needed to establish duration of action of green tea polyphenols in inhibiting NF-κB. And whether the inhibition was caused by its effect on IκB or as antioxidant, we need to examine the SOD level and IκB expression. Consequently, even though the result of this study reflected the effect of green tea polyphenols better than EGCG alone, it should be noted that this might still be far away from daily practice. We need to experiment in clinical trial to determine the precise effects of green tea polyphenols in inhibiting atherosclerosis.

CONCLUSION

In this study, we conclude that green tea polyphenols has the ability to inhibit NF-κB activation and TNF-α production, consistent with the previous study by Yang and Lin and Lin. But, we cannot determine whether the effect was caused by the function as antioxidant or as NF-κB inhibitor. The anti-inflammatory mechanism of green tea polyphenols is mediated at least in part through down-regulation of TNFα gene expression

by blocking NF-κB activation. These findings suggest that green tea polyphenols may be effective therapy for prevention of endothelial dysfunction. Our studies demonstrate a mechanism of action that may in part explain the observed health benefits related to green tea consumption. This finding encourages further studies to investigate the pharmacological potential of green tea polyphenols in healthy subjects and treatment of diseases.

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