



RESEARCH ARTICLE

Decaffeinated coffee and green tea extract inhibit foam cell atherosclerosis by lowering inflammation and improving cholesterol influx/efflux balance through upregulation of PPAR γ and miR-155 [version 1; peer review: awaiting peer review]

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Abstract

Background: Foam cells are markers of atherosclerosis and characterise advanced atherosclerotic plaque, stimulated by inflammation caused by high lipid levels in macrophages. The combination of decaffeinated coffee and green tea extract (DCGTE) has been suggested to have a role in foam cell inhibition.

Objective: we investigated the inhibiting role of DCGTE against foam cell formation, through modulation of the inflammation process and cholesterol metabolism in macrophage colony stimulating factor- (M-CSF) and oxidized low-density lipoprotein (oxLDL)-exposed macrophages.

Methods: Coffee and green tea were extracted by filtration and infusion respectively, and underwent decaffeination using active carbon and blanching methods, respectively. Cells were administered 160/160 and 320/320 μ g/ml of DCGTE. Foam cell formation was observed using a light microscope after staining with Oil Red O (ORO), and the accumulation of lipids in macrophages with ELISA. Observations of lipid influx and efflux were determined through semiquantitative cluster differentiation 36 (CD36) and ATP binding cassette transporter A1 (ABCA1) expression through

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immunofluorescence. The inflammation process was quantified using inflammatory/anti-inflammatory markers, e.g., tumor necrosis factor α (TNF α) and interleukin 10 (IL10) with ELISA. Peroxisome proliferator activated response γ (PPAR γ) expression and activity were assessed with PCR and ELISA, respectively. The expression of microRNA 155 (miR-155) was examined using qPCR.

Results: DCGTE at the above concentrations tended to reduce foam cell numbers, significantly inhibited lipid accumulation ($p=0.000$), reduced CD36 expression ($p=0.000$) and TNF α secretion ($p=0.000$) in Raw264.7 exposed to M-CSF 50ng/ml and oxLDL 50 μ g/ml. PPAR γ expression ($p=0.00$) and activity ($p=0.001$), miR-155 relative expression ($p=0.000$), and IL10 production ($p=0.000$) also increased.

Conclusion: DCGTE lowered foam cell numbers, possibly through attenuation of the inflammatory process and improvement of lipid/efflux mechanisms in M-CSF and oxLDL-stimulated Raw264.7 cells, via upregulation of PPAR γ and miR-155. Our results suggest DCGTE may help prevent atherosclerosis-based diseases.

Keywords

coffee, green tea, foam cell, inflammation, miR-155, PPAR γ

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Introduction

Foam cell macrophages are not only hallmarks of early atherosclerosis process, but also drive the progression of atherosclerosis plaque.^{1,2} However, atherosclerosis is the etiologic agent of various cardiovascular diseases that cause high morbidity and mortality worldwide, especially during the ongoing COVID-19 pandemic.^{3–5} The crosstalk interaction between inflammation and lipid metabolism disturbances in macrophages are two key factors that determine foam cell formation.^{6,7}

Upregulation of CD36 expression causes oxLDL endocytosis.^{8,9} Thus, lipid accumulation inside macrophages leads to endoplasmic reticulum (ER) stress, and stimulates the production of inflammatory cytokines through the activation of nuclear factor kappa B (NFκB) signalling and the reduction of anti-inflammatory cytokines. Moreover, the inflammation process gives rise to positive feedback on the increase of oxLDL uptake, thus accumulating the esterified cholesterol content inside the macrophage.^{10,11} On the other hand, proinflammatory cytokines provoke the lysosomal activation and apoptosis signalling that leads to the activation of ubiquitin proteasome signalling, where both processes contribute to the failure of cholesterol efflux from macrophages through ABCA1 degradation.^{12,13} Another significant issue lies in the fact that PPARγ and miR-155 are molecules involved in the dual function of inflammation and the impaired lipid metabolism that inhibits the foam cell formation.

Investigating natural products that may act to reduce foam cell numbers by lowering inflammation and improving the impaired lipid influx/efflux mechanism in macrophages, is an important approach for atherosclerosis prevention. Although many studies have revealed the health benefits of coffee, several reports showed opposite, detrimental results. High caffeine and different roasting levels are two factors that might explained the negative effects of coffee consumption.^{14–20} Based on a previous study, this research tried to optimize the advantages of coffee by performing: (1) light roasting, (2) a decaffeination process, and (3) combination with green tea. Coffee contains several active compounds, mainly chlorogenic acid (CGA), followed by diterpenes (kahweol, cafestol), and trigonelline.²¹ Light-roasted coffee yields the highest antioxidant concentration compared with green coffee or full roasted coffee. On the other hand, green tea has higher catechin levels compared to other tea varieties.²² Moreover, this combination showed a synergistic activity based on a previous study.²³ For the present study, we tried to investigate the anti-inflammatory and modulation of lipid influx/efflux effects of decaffeinated coffee and green tea extract (DCGTE) in suppressing foam cell formation.

Methods

Coffee and green tea extraction

The raw material used in this study was Robusta green coffee beans obtained from the the Dampit area, Malang Regency, Indonesia. The sample used was in the form of powdered green coffee beans that had been roasted at 180°C for 3 minutes (until the first crack of the bean). Three green tea leaves were retrieved from the highest part of trees in the Ciwidey area in Bandung, for the tea extract preparation. The selection of dried green tea was based on final appearance: a rolls shape, a pale green colour, and a sweet scent.

Coffee decaffeination was carried out following the Fischer method.²⁴ The green tea decaffeination process was performed using the blanching method by Liang, 2007,²⁵ whereas green tea and coffee extraction followed the process performed by Vuong *et al.*, 2011, with a water-to-tea/coffee ratio of 20:1 ml/g.²⁶ The optimized boiling time for coffee and tea extraction, boiling water time for tea decaffeination, and addition of agent adsorption of caffeine in coffee decaffeination were decided using response surface methodology (RSM) Box-Behnken design (BBD) in Design Expert 7.1.5 (StatEase) software.

Briefly, the light-roasted ground coffee beans were boiled in mineral water for 10 minutes at 90°C, followed by filtration with fine filter paper. The filtrate then was subjected to the decaffeination process, using activated charcoal for 8 hours at 60°C.²⁷ The tea dried leaves were boiled in mineral water for 5 minutes at 50°C, then filtered using Whatman paper No 1.^{25,28} The filtrate was infused in 90°C water for 30 minutes. Stock solutions were freshly prepared by dissolving the coffee and tea extract (64 mg/ml) and filtering the solution through a 0.22 μm-pore size membrane filter.

Raw 264.7 cell culture

Macrophages from the RAW 264.7 mouse cell line were obtained from ATCC (CVCL_0493). The passage number 10-20 was used in this study. Raw 264.7 cells were cultured in complete medium containing Dulbecco's modified Eagle medium (DMEM) high glucose, Gibco 11965092), supplemented with 10% fetal bovine serum (FBS, Gibco 16000036) and 1% penicillin/streptomycin (P/S, Gibco15140122). The cells were incubated in a humidifier incubator at 37° with 5% CO₂. To induce macrophage type 2 (M2) differentiation and ultimately establish a foam cell model, Raw 264.7 cells were cultured with recombinant mouse M-CSF 50 ng/ml (BioLegend 576406) for 24 hours, then stimulated with 50 μg/ml oxLDL (Thermofisher L34357) for another 24 hours. DCGTE was administered at 2 different doses (160/160 and

320/320 µg/ml). The cells were divided into 4 groups, K, K+, P1, P2 as follows: complete medium + 50 ng/ml M-CSF (K), complete medium + 50 ng/ml M-CSF + 50 µg/ml oxLDL (K+); complete medium + 50 ng/ml M-CSF + 50 µg/ml oxLDL + 160/160 µg/ml DCGTE (P1); complete medium + 50 ng/ml M-CSF + 50 µg/ml oxLDL + 320/320 µg/ml DCGTE (P2).

Oil red O staining

Briefly, the cells were cultured at 15,000 cells/well in 24-well plates placed with a coverslip until reaching 60-70% confluency. The medium was discarded and cells were rinsed twice with PBS, then fixed in 10% paraformaldehyde for 10 minutes. The cells were washed with phosphate buffer saline (PBS) twice (for 3 minutes) again, and were incubated with 60% isopropanol for 30 minutes to facilitate the staining of neutral lipids. The cells were stained with filtered Oil Red O (ORO) solution (Sigma-Aldrich, MAK194) at 37°C for 30 minutes in darkness. The cells were washed with 60% isopropanol for 5 seconds, followed by 5 repeated washing steps with PBS. Positively stained (red cytoplasm) cells were macrophage-derived foam cells, which were observed via light microscope (Olympus Bx51), photographed with Optilab Advance Plus MTN016, and analysed using Optilab Viewer v3.0 software.²⁹

Lipid absorbance quantification

100% 2-propanol was added to the ORO-stained plates (500 µl per well on the 24-well plates). The plates were incubated for 40 minutes at room temperature on an orbital shaker in darkness. 200 µl eluates were transferred to a clear 96-well microtiter plate (polystyrene). As a control, 3 microtiter plate wells were filled with 200 µl of 2-propanol. Absorption was measured in duplicates at 492 nm.³⁰

CD36 and ABCA1 expression

The cells were pipetted into a 24-well plate with a 15,000 cells/well initial cell density. Cultured cells were stained by plating the cells on glass coverslips. After treatment, the cells were washed 3 times with PBS and fixed with 4% paraformaldehyde for 30 minutes at room temperature. The cells were washed twice with PBS (three minutes), blocked with 2% bovine serum albumin (BSA) for 1 hour at room temperature, and incubated with anti-CD36 monoclonal antibody 1:150 (Santa Cruz, Cat. No. sc3709) and Rabbit antiphospho-ABCA1 antibody 1:200 (Bioss, Cat. No. bs-12956R) at 4°C overnight. The cells were washed 3 times with PBS and incubated with anti-rabbit IgG antibody rhodamine conjugated (Rockland Cat. No. 6111002-0500, RRID: AB_11181881) and Fab Goat anti-mouse IgG antibody FITC (Rockland Cat. No. 710-1202; RRID: AB_218843) for 1.5 hours in light-shielded conditions. Nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI; BioLegend Cat. No. 422801 ID = BLG2181) for five minutes in the dark. After three washes with PBS, coverslips were mounted with glycerol. The cells were visualized using an Olympus IX71 Fluorescent Microscope at 40× magnification, and then photographed with Olympus Cell Sens software version 3.2. The pixel intensities in the cell cytoplasm that reflecting the expression levels were quantified using Image J (Fiji) software and presented as percentages of CD36 or ABCA1 expression, then compared statistically.

Tumor Necrosis Factor (TNF) α and Interleukin (IL)10 expression

RAW 264.7 cells were seeded in 24-well plates at 1.5·10⁴ cells/well to get confluency 80-100% in 5th day. Media were collected and centrifuged at 2500 rpm for 7 min. TNF α and IL10 content of the media was then determined by a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) using the Mouse TNF α ELISA Kit (BT Lab E0117Mo) and mouse IL10 ELISA kit (BT Lab E002Mo) according to the manufacturer's instructions in ELISA reader BioTek ELx808 using Gen5 reader software.

miR-155 expression

This procedure was followed in the following steps: isolation of total RNA, cDNA synthesis, and Real-Time PCR. Total RNA from cells was extracted by using the miRVana (Ambion Thermofisher AM1560) reagent kit according to the manufacturer's protocol.³¹ Reverse transcription polymerase chain reaction (RT-PCR) was conducted in a single process using an RT-PCR kit from Qiagen (miRCURY LNA RT kit 33430): incubation for 60 minutes at 42°C, followed by incubation for 5 minutes at 95°C to heat-inactivate the reverse transcriptases, and cooling at 4°C.

The RT-qPCR was run using a PCR kit (Qiagen miRCURY LNA SyBR Green PCR kit 33435) in a Bio-Rad CFX. The primer sequences for miR-155 amplification were: F 5' TGC GGT TAA TGC TAA TTG TGA TA 3'; R 5' GTG CAG GGT CCG AGG T 3'. Normalisation of miR-155 expression was done using U6 as a housekeeping gene. Each PCR amplification was performed under the following conditions: PCR initial heat activation for two minutes at 95°C, denaturation at 95°C for 10 minutes, combined annealing/extension at 56°C for 60 seconds through 40 cycles.

Purification of cellular nuclear extracts

The cells were grown to 80% confluency on a 100 mm plate dish culture. After the cells were washed with PBS twice, they were scraped into a 15 ml conical tube. The cells were centrifuged for 5 minutes at 1000 rpm. The nuclear protein

isolation was done using a nuclear extraction kit from ABCAM ab113474. The pellet was resuspended in 100 μ L 1X pre-extraction buffer, supplemented with dithiothreitol (DTT) and protease inhibitor cocktail (PIC) per 10^6 cells, then incubated on ice for 10 minutes. The pellet was vortexed vigorously for 10 seconds and centrifuged for 1 minute at 12,000 rpm. The nuclear pellet was added with two volumes of extraction buffer containing DTT and PIC at about 10 μ L per 10^6 cells, then were incubated on ice for 15 minutes and vortexed (five seconds) every 3 minutes. The last step was centrifugation of the suspension for 10 minutes at 14,000 rpm at 4°C. The supernatant contained protein nuclear extract.

PPAR γ expression and activity

Primers were designed according to the sequences in GenBank as follows: β -actin: F: 5' TGA GAG GGA AAT CGT GCG TGA CAT 3', R: 5' ACC GCT CAT TGC CGA TAG TGA TGA 3'; PPAR γ : F: 5' AAC TGC AGG GTG AAA CTC TGG GAG ATT CTC C-3', R: 5' GGA TTC AGC AAC CAT TGG GTC AGC TCT3'. Standard 25 μ L-solution polymerase chain reaction (PCR) with 2 μ L of cDNA after reverse transcriptase reaction was performed, with the following parameters: initial heat activation at 95°C for two seconds, denaturation process at 95°C for 40 seconds (three cycles), annealing for 40 seconds at 57°C (three cycles), 55°C (three cycles), 52°C (29 cycles), extension for 45 seconds at 72°C with TaKaRa Ex Taq Hot Start Version (TaKaRa, Japan), in a MJ Research PTC-200 Peltier Thermal Cycler. The PCR reaction product (10 μ L) was run through 1.5% agarose gel by electrophoresis. Densitometric quantification of the band intensities was conducted using Image J (Fiji) software.

PPAR γ activity was analysed using the PPAR γ Transcription Factor Assay Kit (ab133101, Abcam, MA, USA) according to the manufacturer's protocol. Briefly, the nuclear protein extracts were collected as described earlier and added to a 96-well plate containing a specific double stranded DNA (dsDNA) sequence, including the peroxisome proliferator response element (PPRE) at the bottom of the wells. Then, briefly, the primary antibody was added to each well, followed by goat anti-rabbit HRP, and developing and stop solutions supplied in the kit, respectively. PPAR γ activity was detected using a BioTek ELx808 ELISA reader, connected with a Gen5 3.0 software at OD 450 nm.

Statistical analysis

Data were expressed as mean \pm standard error (SE). All parameters were measured in at least three independent experiments (triplicate). One-way analyses of variance (ANOVA) were performed. The significant statistical difference threshold was defined for $p < 0.05$.

Results

The decaffeinated coffee and green tea extract inhibit foam cell formation

First, we investigated the effects of the decaffeinated coffee and tea extract (DCGTE) on Raw 264.7 macrophages treated with 50 ng/ml M-CSF and 50 μ g/ml ox-LDL. The assessment of foam cells characteristics was performed using ORO staining, followed by measuring the absorbance to quantify the lipid content in the cell. Raw 264.7 cells exposed to DCGTE at a concentration of 160/160 and 320/320 μ g/ml elicited an augmentation in foam cell numbers and lipid accumulation at 24 hours compared to the positive control (50 μ g/ml oxLDL) ($p = 0.000^{**}$, Figure 1).

The decaffeinated coffee and green tea extract reduced CD36 and increased ABCA1 expression in ox-LDL stimulated macrophages

Foam cells could be formed due to a dysregulation of the lipid metabolism, which was determined by the continuous uptake of ox-LDL and failure in lipid efflux from macrophages.^{1,2,6} The endocytosis of ox-LDL is mainly regulated by CD36,^{8,9} whereas ABCA1 plays a significant role in cholesterol efflux.^{32,33} Thus, this study aimed to investigate the effect of the extract in the cells examined using the immunofluorescence technique. Figure 2 depicts the CD36 and ABCA1 expression after being incubated with the extract for 24 hours in M-CSF and ox-LDL-stimulated Raw 264.7. One-way ANOVA tests showed significant differences in CD36 ($p = 0.000^{**}$) and ABCA1 ($p = 0.000^{**}$) expression in DCGTE-exposed samples compared to positive control (K+). Therefore, we concluded that the administration of decaffeinated coffee and green tea extract could suppress CD36 and induce ABCA1 expression in Raw 264.7 cells.

The decaffeinated coffee and green tea extract attenuated TNF α and enhanced IL10 secretion in ox-LDL-stimulated macrophages

The inflammation process mediates the increase in CD36 expression¹¹ and degradation of ABCA1.^{34,35} Hence, this current study also tried to investigate the effect of the extract in inhibiting the inflammation process, by examining TNF α and IL10 production. Figure 3 shows a reduced TNF α concentration in the group administered with 160/160 μ g/ml and 320/320 μ g/ml extracts compared to control ($p = 0.012$; $p = 0.000$). Consistently with the result from inflammatory cytokine production, the data shows that IL10, an anti-inflammatory cytokine, was higher in groups treated with the 160/160 μ g/ml and 320/320 μ g/ml extracts compared to control ($p = 0.002$; $p = 0.000$). Therefore, we concluded that administration of DCGTE at concentrations of 160/160 and 320/320 μ g/ml gradually inhibited TNF α and stimulated IL10 production in Raw 264.7 cells.

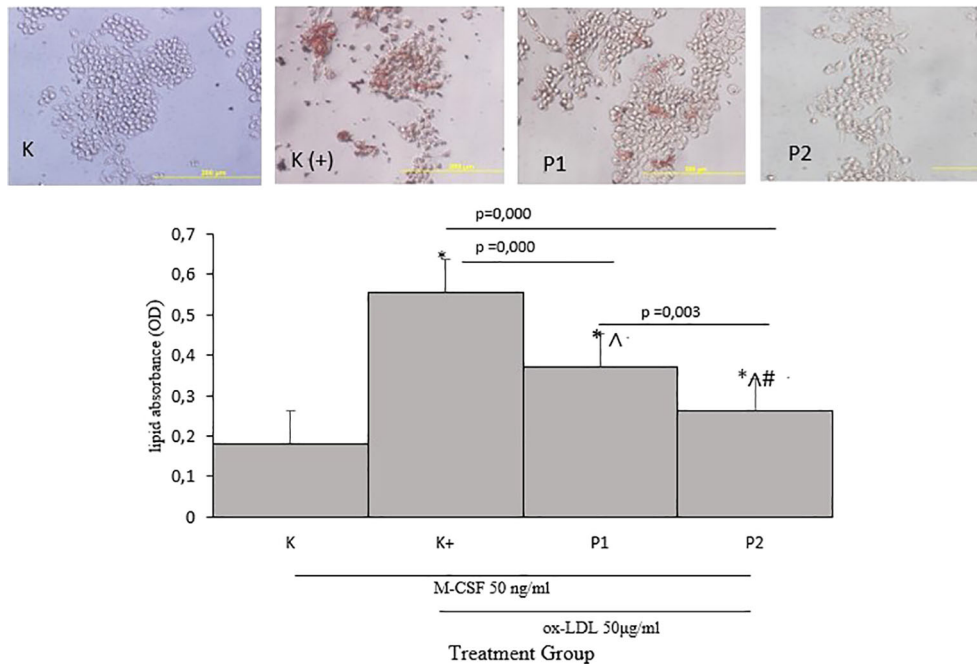


Figure 1. Effect of DCGTE on foam cell number and lipid content in oxLDL-stimulated Raw 264.7 cells. The foam cell was characterized by red color in cytoplasmic part observed with microscope Olympus BX51 200 \times magnification. * $p < 0.05$, relative to C, \wedge $p < 0.05$ relative to C(+), # $p < 0.05$ relative to P1. Culture groups: complete medium + 50 ng/ml M-CSF (K); complete medium + 50 ng/ml M-CSF + 50 μ g/ml oxLDL (K+); complete medium + 50 ng/ml M-CSF + 50 μ g/ml oxLDL + 160/160 μ g/ml DCGTE (P1); complete medium + 50 ng/ml M-CSF + 50 μ g/ml oxLDL + 320/320 μ g/ml DCGTE (P2).

The decaffeinated coffee and green tea extract increased mRNA and PPAR γ activity in ox-LDL-stimulated macrophages

The signalling pathway that mediates DCGTE is also explored in this present study. PPAR γ is an antiatherogenic transcription factor that contributes to the suppression of foam cell formation.³⁶ Figure 4 demonstrates that both the expression and activity of PPAR γ , after cells were administered with DCGTE at 160/160 and 320/320 μ g/ml were increased compared to the K+ group ($p = 0.000^{**}$ and $p = 0.001^{**}$). Additionally, PPAR γ expression and activity showed their lowest level in the K (+) group.

The decaffeinated coffee and green tea extract reduced the miR-155 expression in ox-LDL-stimulated Raw 264.7

Recent reports showed the importance of miRNA in post-transcriptional gene expression regulation.^{37–39} miR-155 plays a significant role in inflammation. In addition, miR-155 has been proven to target CD36, SOCS1, and Vav3 based on *in silico* results.³⁴ Regarding this function, this study explored the effect of DCGTE on miR-155 expression, and results can be seen in Figure 5. The results demonstrated a significant increase in relative miR-155 expression in cells treated with 160/160 and 320/320 μ g/ml DCGTE compared to positive control (K+) ($p = 0.000^{**}$). Surprisingly, the data showed the expression of miR-155 higher in positive control (K+) ($p = 0.000^{**}$) compared to the control group.

Discussion

Our accumulated evidence suggests that inflammation drives the formation of atherosclerotic plaques by disrupting the cholesterol metabolism inside macrophages.^{40,41} The inflammation process could be characterized by the balance production of inflammatory and anti-inflammatory cytokines in macrophages. TNF α is a proinflammatory cytokine that has an important role in the formation of atherosclerotic foam cells.^{42,43} A study using differentiated macrophages from THP-1 exposed to very low-density lipoprotein (VLDL), then incubated with IL-1 β and TNF α for 24 hours, showed an increase of intracellular cholesterol and triglyceride retention compared to control. On the other hand, IL-10 as an anti-inflammatory cytokine also contributes to the whole atherosclerosis process, including foam cell formation. Over-expression of IL-10 in macrophages derived from bone marrow low density lipoprotein knockout (LDLR $^{-/-}$) mice showed inhibition of atherosclerosis by reducing cholesteryl ester accumulation in atherosclerotic sites.⁴⁴ Atherosclerotic plaques of patients with a history of coronary heart diseases have been shown to express IL-10. IL-10 inhibits macrophage

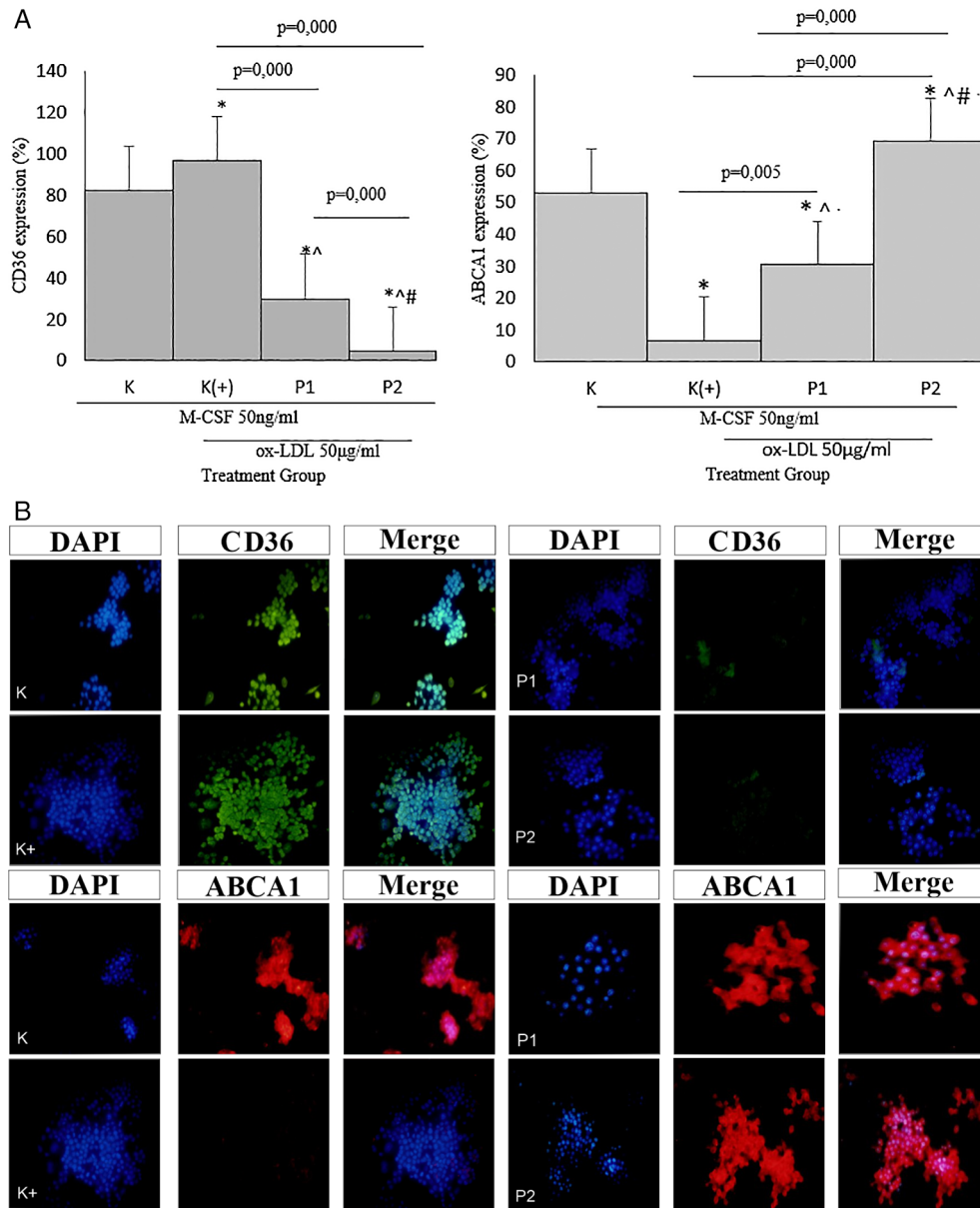


Figure 2. Effects of DCGTE on CD36 and ABCA1 expression in oxLDL-stimulated macrophages. The blue colour indicated nuclear staining, green for CD36 with FITC, red for ABCA1 with rhodamin fluorochrome. The expression was observed using a fluorescent microscope 400× magnification and quantify with Image J as percentage/200 cells. * p < 0.05, relative to C, ^ p < 0.05 relative to C(+), # p < 0.05 relative to P1. Culture groups: complete medium + 50 ng/ml M-CSF (K); complete medium + 50 ng/ml M-CSF + 50 µg/ml oxLDL (K+); complete medium + 50 ng/ml M-CSF + 50 µg/ml oxLDL + 160/160 µg/ml DCGTE (P1); complete medium + 50 ng/ml M-CSF + 50 µg/ml oxLDL + 320/320 µg/ml DCGTE (P2).

apoptosis and TNFα production, which is mediated by the inactivation of the NF-κB transcription factor.⁴⁵ Furthermore, macrophage apoptosis inhibition causes caspase expression decrease, and causes the COP9 signalosome complex (CSN) to remain intact, thus protecting the ABCA1 protein from ubiquitination.^{46,47} Moreover, experiments with primary macrophages indicated that IL-10 directly stimulated the efflux of cholesterol by activating the PPARγ-LXR-ABCA1/ABCG1 pathway.⁴⁴

In this study, we demonstrated the beneficial effects of DCGTE using Raw 264.7 macrophages under M-CSF-induced M2 differentiation and oxLDL-induced foam cells. The suggested mechanisms involved in foam cell inhibition by DCGTE act by suppressing inflammation, thus driving cholesterol influx and efflux in macrophages. A 24-hour

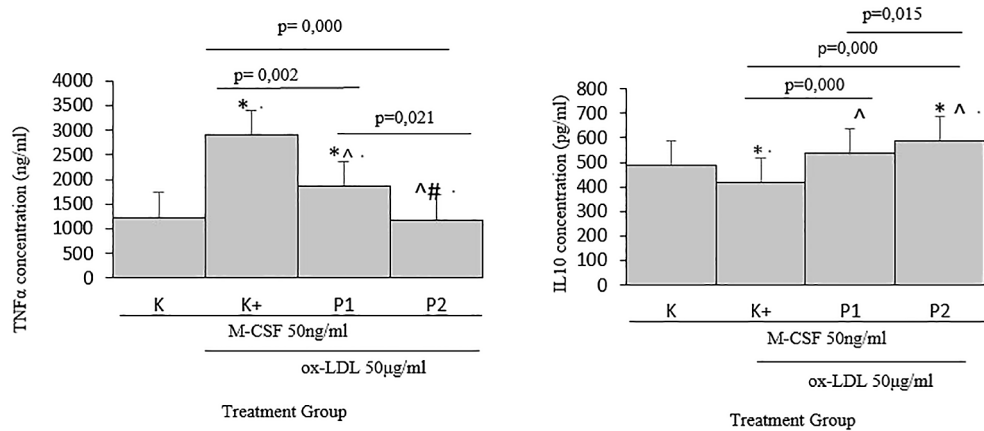


Figure 3. DCGTE effect on inflammation process. On the 4th day after seeding, the cell medium was taken and analyzed for cytokine protein content by ELISA. * $p < 0.05$, relative to K, \wedge $p < 0.05$ relative to K(+), # $p < 0.05$ relative to P1. Culture groups: complete medium + 50 ng/ml M-CSF (K); complete medium + 50 ng/ml M-CSF + 50 μ g/ml oxLDL (K+); complete medium + 50 ng/ml M-CSF + 50 μ g/ml oxLDL + 160/160 μ g/ml DCGTE (P1); complete medium + 50 ng/ml M-CSF + 50 μ g/ml oxLDL + 320/320 μ g/ml DCGTE (P2).

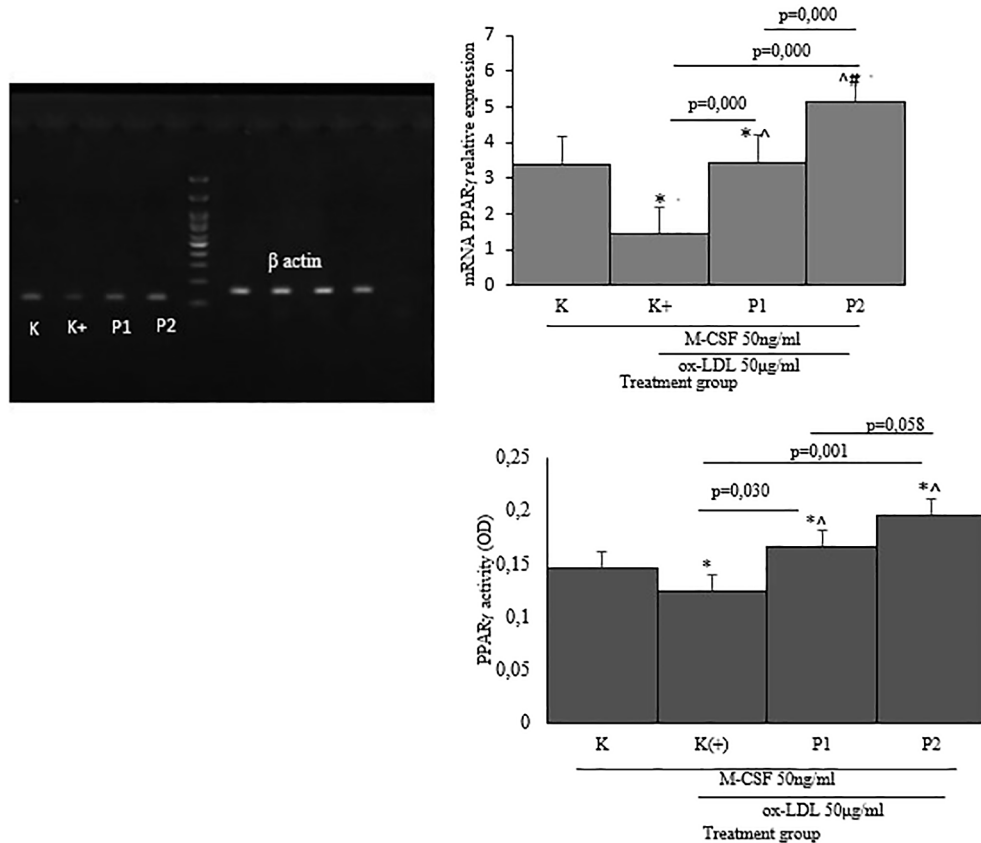


Figure 4. DCGTE increased PPAR γ expression and activity. The mRNA expressions of PPAR γ were analyzed using PCR. The reactions were loaded onto an agarose gel, resolved by size via electrophoresis, and visualized with ethidium bromide. The bands were quantified using Image J and normalized with β actin. PPAR γ activity was analysed using ELISA. * $p < 0.05$, relative to K, \wedge $p < 0.05$ relative to K(+), # $p < 0.05$ relative to P1. Culture groups: complete medium + 50 ng/ml M-CSF (K); complete medium + 50 ng/ml M-CSF + 50 μ g/ml oxLDL (K+); complete medium + 50 ng/ml M-CSF + 50 μ g/ml oxLDL + 160/160 μ g/ml DCGTE (P1); complete medium + 50 ng/ml M-CSF + 50 μ g/ml oxLDL + 320/320 μ g/ml DCGTE (P2).

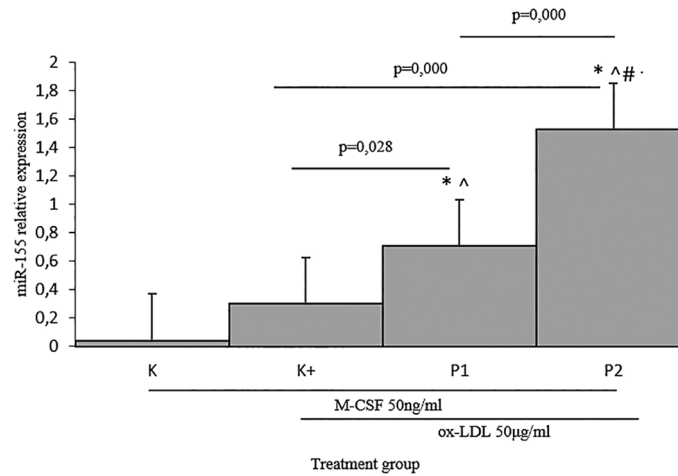


Figure 5. DCGTE increased the miR-155 expression. On the 4th day after seeding, the RNA of the cells was extracted, converted into cDNA and followed by qPCR. The house keeping gene was U6. The expressions were quantified using Livax Method $2^{-\Delta(\Delta Ct)}$. * $p < 0.05$, relative to K, ^ $p < 0.05$ relative to K(+), # $p < 0.05$ relative to P1. Culture groups: complete medium + 50 ng/ml M-CSF (K); complete medium + 50 ng/ml M-CSF + 50 µg/ml oxLDL (K+); complete medium + 50 ng/ml M-CSF + 50 µg/ml oxLDL + 160/160 µg/ml DCGTE (P1); complete medium + 50 ng/ml M-CSF + 50 µg/ml oxLDL + 320/320 µg/ml DCGTE (P2).

incubation of macrophages with 160/160 and 320/320 µg/ml DCGTE significantly reduced TNF α and increased IL-10 production in oxLDL-stimulated macrophages. Interestingly, there was also significant reduction of foam cell numbers, CD36 expression, and enhancement of ABCA1 in DCGTE- exposed sample groups compared to control. Our study was the first to explore the effect of DCGTE in foam cell atherosclerosis. These findings were consistent with data from several studies investigating the effect of green tea extract or catechin as the main active ingredients in green tea on foam cell formation. Green tea extract has been shown to effectively inhibit lipid peroxidation.⁴⁸ Epigallo catechin gallate (EGCG), the main active compound from green tea, upregulated ABCA1 expression through the suppression of TNF α production and NF κ B activity in macrophage-derived foam cells.⁴⁹ Another supporting evidence related to our findings came from a few reports exploring the effect of coffee in atherosclerosis. Coffee was found to enhance the cholesterol efflux based on *in vitro*, *in vivo* and human studies.⁵⁰ Another report showed that supplementation with CGA, the main secondary metabolite of coffee, suppressed oxLDL-induced lipid accumulation from RAW 264.7 cells and decreased LDL and inflammatory markers from apolipoprotein E knockout (ApoE $^{-/-}$) mice.⁵¹

The more complex mechanism underlying DCGTE-driven foam cell inhibition was also investigated in this study. PPAR γ is an antiatherogenic transcription factor that plays an important role in the atherosclerosis process.³⁶ Our findings showed an increase of PPAR γ expression and activity, after administration of 160/160 and 320/320 µg/ml DCGTE. PPAR γ has an anti-inflammatory property and affects lipid efflux processes directly or indirectly. PPAR γ -deficient macrophages displayed an increased pro-inflammatory phenotype upon long-term LPS stimulation, characterized by an elevated production of the pro-inflammatory cytokines TNF α , IL-1 β , IL-6, IL-12, and a reduced production of anti-inflammatory cytokine IL-10 compared to PPAR γ wild-type cells. Moreover, PPAR γ -deficient macrophages showed impaired phagocytosis.⁵² In macrophage lipid metabolism processes, PPAR γ directly increases the expression of ABCA1, which was confirmed by our report. These results were consistent with data from another study showing that CGA significantly increased the mRNA levels and transcriptional activity of PPAR γ , as well as downstream mRNA LXR α , ABCA1 and ABCG1 signaling levels.⁵¹

Interestingly, PPAR γ also directly increased CD36 expression.¹ Based on other studies, we suggest that CD36 expression is not determined solely by PPAR γ expression. However, there are other factors that affect CD36 expression, such as the presence of IL-34, and ER stress.^{10,11} In addition to a direct effect, PPAR γ may indirectly work on influx and efflux mechanisms by modulating the inflammatory process. One study reported a suppression of TNF α promoter activity after myocyte cells were stimulated with lipopolysaccharide in a luciferase assay. Otherwise, no change in TNF α promoter activity was observed in NF κ B knockdown and TNF α reporter-transfected cells. Consistently, Bernardo *et al.*, 2021 reported in his study that the administration of the PPAR γ antagonist GW9662 significantly prevented phosphorylation of ERK1/2, as well as TNF α .⁵³

A second possible driver of foam cell inhibition is the presence of other molecules that might affect CD36 and ABCA1 expression, inflammatory and anti-inflammatory cytokines production. Our data support the antiatherogenic role of

miR-155. This finding supports our previous *in silico* study, that suggested miR-155 as a potential candidate molecule to inhibit foam cell establishment.³⁴ The results showed a significant increase of relative miR-155 expression in DCGTE-treated groups compared to control, hence potentially explaining the decrease of CD36 expression, TNF α and the increase of ABCA1 and IL-10, ultimately decreasing foam cell formation. The role of miR-155 in mediating the action of this coffee and tea combination is probably due to its direct action on lipid fluxes, or indirectly through the inflammatory process. The direct action of miR-155 was on 3' untranslated region (UTR) CD36 mRNA. The indirect action of miR-155 in suppressing foam cell formation was revealed by several studies on the action of miR-155 on inflammatory markers. miR-155 targets and degrades calcium-regulated heat-stable protein 1 (CARHSP1) which causes a decrease in the stability of TNF α mRNA.⁵⁴ Inflammatory macrophage production (TNF α and IL-6) was increased in miR-155 inhibitor--transfected macrophages due to the upregulation of p38 and Janus kinase (JNK), extracellular signal related kinase (ERK), mitogen activated protein kinase kinase kinase (MAP 3K) signaling pathway. The transfection of miR-155 mimic showed the opposite results in oxLDL-stimulated macrophages.⁵⁵ The third target was the action of miR-155 on the suppressor of cytokine signaling 1 (SOCS1). SOCS1 was expected to be an E3 ligase, part of a ubiquitin protease system, which causes the degradation of ABCA1.^{34,56} Chang *et al.*, 2020 and Li *et al.*, 2015 demonstrated that SOCS1 was the direct target of miR-155-5p, by performing a luciferase reporter assay using HEK293 cells.

Many papers have revealed that various natural products exert cardio-protective effects, either as functional food or source of phytopharmacology products.^{59,60} It has been widely accepted that coffee and tea have many benefits as hypolipidemic, anti-inflammatory, and antioxidant agents.^{61,62} However, some controversy remains regarding the cardiovascular and non-cardiovascular side effects of caffeine components in coffee and tea.^{14,15,27,63} The decaffeination process is used to minimize conflicting results. Interestingly, our findings were the first to reveal the combination of decaffeinated coffee and green tea extract could reduce foam cell numbers. However, this study has several limitations. The signaling pathway showing the interaction between PPAR γ , miR-155, CD36, ABCA expression, TNF α , and IL-10 could not directly be proven. Existing guidelines on atherosclerosis management mainly include the use of statin and pioglitazone.^{64,65} Unfortunately, the present study design did not compare the efficacy of statin or pioglitazone administration in inhibiting foam cell formation. Therefore, considering the limitations, extensive efforts are required to develop this extract combination as nutraceutical food for the prevention of atherosclerosis.

Conclusions

These findings suggest that DCGTE confers a protection against the formation of foam cell formation by regulation of cholesterol hemostasis and inflammation. Hence, a DCGTE combination could potentially be used as an agent to prevent atherosclerosis.

Data availability statement

Underlying data

Figshare: the supplementary data.xlsx, <https://doi.org/10.6084/m9.figshare.16664983.v9>.⁶⁶

This project contains the following underlying data:

- Final untuk F1000.xlsx (dataset containing the optimized extraction time, decaffeination time, water decaffeination temperature for coffee and green tea, lipid absorbance, CD36 and ABCA1 expressions, PPAR γ gene expression, PPAR γ activity, relative miR-155 expression, TNF α production and IL-10 production measures)

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0/) (CC-BY 4.0).

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