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Pomegranate punicalagin inhibits the foam cells formation in Raw264.7 macrophages

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Abstract

In order to reveal the inhibitory effect of pomegranate punicalagin on macrophage foam cell formation and its possible mechanism, Raw264.7 macrophages were as the experimental carrier to detect the effect of punicalagin on macrophage cholesterol accumulation and outflow, and evaluate the regulation of punicalagin on cholesterol accumulation related genes CD36, PPARγ and outflow related genes ABCA1/G1, LXRα by Western-Blot method. The results showed that punicalagin can inhibit the cholesterol accumulation in raw264.7 macrophages due to restraining the PPARγ-CD36 expression, and meanwhile it could also promote macrophage cholesterol efflux by regulating LXRα-ABCA1/G1 pathway.

Keywords: pomegranate punicalagin; Raw264.7 macrophage; cholesterol efflux; foam cell; lipid accumulation.

Practical Application: This study provides a theoretical basis for the use of pomegranate peel polyphenols and pomegranate glycosides in food and medical treatment, provides a reference for the comprehensive development and utilization of pomegranate peel resources, and makes a certain contribution to reducing resource waste and the emission of environmental pollutants.

1 Introduction

Pomegranate is a kind of fruits which can make medicine and food. In recent years, it has been widely concerned by researchers for its rich nutrition and extensive medicinal value. The polyphenols in pomegranate are very rich and diverse, and there are many kinds of polyphenols detected from pomegranate, mainly including pomegranate punicalagin(PC), ellagic acid, gallic acid, chlorogenic acid, caffeic acid, quercetin, ferulic acid, rutin, catechin, epicatechin (Chen et al., 2021). PC is the content-highest ingredients of pomegranate peel polyphenols (Liu et al., 2022). The PC content in different varieties pomegranate is 39.80~121.5 mg/(g.peel). As one of the main active components of pomegranate polyphenols, PC is easily absorbed by the human body, and can be decomposed into ellagic acid and urolithin under the action of human enzymes. And it has strong antioxidant (Ivelina & Dasha, 2020), anti-inflammatory, antibacterial, anti-tumor, anti-atherosclerotic and other physiological activities (Dion, 2023).

Atherosclerosis(AS) is still the major premature death cause in the developed world. Macrophage foam cell formation is a key pathological feature of early atherosclerosis occurrence (Fowler et al., 1985; Majdalawieh & Ro, 2014). Many previous studies have demonstrated the prevention and reversal of foam cell formation by limiting cholesterol intake and promoting intracellular cholesterol efflux is a new target for the prevention of AS occurrence (Wang et al., 2015; Yu et al., 2013).

Numerous research results show that PC has the effects of anti-atherosclerosis (Huwait et al., 2022). Barrett showed that PC and pomegranate juice can affect the triglyceride biosynthesis of macrophages by inhibiting DGAT1 activity, and PC can inhibit J774A 1. accumulation of lipid droplets in macrophages and inhibition of cholesterol synthesis of macrophages to resist the destruction of cholesterol balance (Fowler et al., 1985; Majdalawieh & Ro, 2014). Moreover, pomegranate peel polyphenols have been proved to promote the macrophages cholesterol outflow by regulating LXR α- ABCA1/SR-B1 cell signal pathway, and inhibit the formation of foam cells to achieve the purpose of anti-AS (Zhao et al., 2016). How does PC affect foam cells? In this paper, we will study the effect of PC on the cholesterol accumulation, efflux in macrophages and their molecular mechanisms.

2 Materials and methods

2.1 Materials

Punicalagin were obtained from Sigma (USA). Raw264.7 cells were stored in our researcher laboratory. Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were bought from Gibco (USA). MTT and oil red O reagent were supplied by Sigma. Human ox-LDL (2 mg/mL) was purchased from Yiyuan Biotechnologies (Guangzhou, China). CD36 (BS7861), ABCA1 (BS60011), ABCG1 (BS5596), LXRα (BS6785) and PPARγ (BS1587) antibodies was provided by Bioworld Technology, Inc. β-actin antibody (CW0096) was offered by CWBIO Technology (China). The cholesterol assay kits were from Applygen Biotechnologies (Beijing, China).

2.2 Cell culture

RAW264.7 macrophages were maintained in DMEM supplemented with 10% FBS at 37 °C in the 5% CO_2 environment. The raw264.7 macrophages were collected at the logarithmic growth phase to use in various tests (Lee et al., 2019).

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2.3 Cytotoxicity determination

Cytotoxicity of PC were determined by MTT assay. The raw264.7 macrophages were seeded in 96-well culture plates. After treatment with various concentrations of PC for 24 h, the culture medium was removed and replaced by 0.5 mg/mL MTT. After 4h, 100 μL DMSO per hole was added to replace MTT, and incubated at 37 °C for 10 min, then the absorbance was determined at 490 nm to quantitate cellular viability. Attention shall be paid to the setting of zero adjustment holes (including culture medium, MTT and DMSO) and blank control holes (cells, culture medium, MTT and DMSO), with 5 parallel holes for each group.

2.4 Oil red O staining

Raw264.7 macrophages were inoculated into the 6-well plates at the appropriate concentration. When grown to 70-80% confluence, cells were pretreated for 1 h by PC at indicated concentrations, and then exposed to ox-LDL (60 μg/mL) for 24h.Then cells were fixed with 4% paraformaldehyde for 10 min at room temperature, washed twice with PBS, and stained with 0.3% oil red O solution for 20 min. The staining results were observed and photographed under the microscope.

2.5 Measurement of intracellular total cholesterol

The raw264.7 macrophages were inoculated into the 6 well plates at the appropriate concentration. After 24h, the culture was replaced by the fresh culture including PC and ox-LDL (60 μg/mL) in indicated concentration for 24h. Cells were harvested to determine the total cholesterol according to the construction of the intracellular total cholesterol kit (E1015). The data were expressed with mg total cholesterol per g protein.

2.6 Quantification of cellular cholesterol efflux

Raw264.7 cells were cultured as described above. After treated with PC and exposed to 60 μg/mL ox-LDL for 24 h, the cells were replaced with 2 mL serum-free phenol red containing 10 μg/mL apoA-1 or 50 μg/mL HDL for 24 h. The medium was collected and centrifuged to determine the total cholesterol of the medium according to operation guides (E1005).

2.7 Western-blot analysis

After treated by PC and exposed to 60 μg/mL ox-LDL for 24 h, the cells were collected and lysed for western-blot experiments. Protein concentrations were determined by BCA protein assay kit. The equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membrane. The membrane was blocked by 5% skim milk for 2 h and then incubated overnight with the ABCA1, ABCG1, PPARγ, LXRα, CD36 antibodies. After washed, the membranes were incubated with the HRP-conjugated secondary antibody, and adequately contacted with ECL substrate mixture, and conducted with UVP ChemiDoc-IT 510 imaging system.

2.8 Statistical analysis

All data were expressed as means ± standard deviation (means ± SD), and all experiments were performed in triplicate. Data differences and the correlation analysis were assessed by SPSS 26. $p<0.05$ is considered significant.

3 Results

3.1 Effects of PC on raw264.7 macrophage viability

The effects of PC on raw264.7 macrophage viability were determined by MTT assay. As shown in Figure 1, the viability of raw264.7 macrophages was not significantly decreased after treated with PC at the concentration within 5-25 μmol/L. However,50 and 100 μmol/L PC can significantly reduce cell survival rate($p<0.05$). In the 50 μmol/L PC group, the cell survival rate was 92.58%, and in the 100 μmol/L PC group, the number of cells decreased to 82.80%. The results showed that low concentration of PC is non-toxic to cells.

3.2 PC decrease ox-LDL-induced cholesterol accumulation

The accumulation of ox-LDL in macrophages induces foam cell formation and promotes the development of AS (Wu et al., 2015). To investigate whether PC can inhibit the foam cell formation or not, the oil red O staining and intracellular cholesterol measurement were used to estimate the lipid accumulations in raw264.7 macrophages.

As shown in Table 1 and Figure 2, supplementation with ox-LDL resulted in foam cell formation with an enormous accumulation of cholesterol in raw264.7 macrophages, and the ox-LDL-induced intracellular droplets were reduced by PC in a dose-dependent manner. In Table 1, the intracellular total cholesterol content decreased significantly by 16.59%, 27.21% and 24.95% in 10, 25 and 50 μg/mL PC groups(vs foam cell group, $p<0.05$), and there is no significant difference between the 25 and 50 μmol/L doses. This showed PC can reduce the cholesterol accumulation in foam cells within the concentration range of the test.

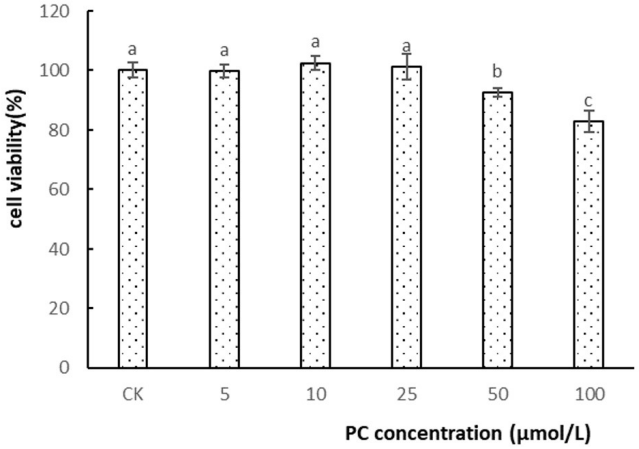

Figure 1. Effects of PC on cell viability of raw264.7 macrophage. CK: macrophage control. Note: different letters represent significant differences between groups($p<0.05$),the same letter represent no significant difference ($p>0.05$).

3.3 PC reduce the CD36 and PPARγ protein expression

Scavenger receptor CD36, expressed in macrophages, is one of the important main receptors involved in internalizing ox-LDL and the formation of foam cells (Podrez et al., 2002). PPARγ is related to cholesterol intake. Lipids entering cells can activate PPARγ, PPARγ also interact with retinol X receptor (RXR-α) to stimulate CD36, and form CD36-ox-LDL-PPAR γ- CD36, a vicious cycle, which accelerates the formation of foam cells (Zhao, 2017). Therefore, We examined the CD36 and PPARγ protein expression changes under the treatment of PC, and the results were shown in Figure 3. The results showed that 25 and 50 μmol/L of PC down-regulated the CD36 and PPARγ protein expression very significantly with dose-dependence. This suggested that PC reduces cholesterol accumulation maybe by regulating PPAR γ- CD36 path.

Table 1. Effect of PC on intracellular cholesterol accumulation.

Group	intracellular cholesterol content (mg/g protein)
ox-LDL	31.82 ± 0.27 a
PC-10	26.54 ± 0.07 b
PC-25	23.16 ± 0.39 c
PC-50	23.88 ± 0.30 c

Notes: ox-LDL: ox-LDL treatment group; PC-10: ox-LDL+10 mol/LPC; PC-25: ox-LDL+25 mol/LPC; PC-50: ox-LDL+50 mol/LPC. different letters represent significant differences between groups($p<0.05$),the same letter represent no significant difference ($p>0.05$).

The correlation analysis of PC and PPARγ, CD36, cholesterol intake was shown in Figure 4. It can be seen that the correlation between cholesterol accumulation and PPARγ, CD36 is greater than 0.85 ($p<0.05$), and there is a strong negative correlation between PC and PPARγ, CD36 expression with the correlation coefficient less than -0.82 ($p<0.05$), indicating that the reduction of cholesterol accumulation by PC is closely related to its down-regulation of PPARγ and CD36 expression.

3.4 PC promote cholesterol efflux from raw264.7 macrophages

In addition to regulating cholesterol intake, cholesterol outflow is also a key link to maintain the cholesterol balance in macrophages. Promoting intracellular cholesterol efflux can also inhibit the foam cell formation and atherosclerotic plaque occurrence. ABCA1 and ABCG1 are involved in the reverse transport of intracellular cholesterol. Phospholipids and cholesterol mediated by ABCA1 actively flow from cells to apoA-1, and cholesterol mediated by ABCG1 flows from cells to HDL (Hu et al., 2022; Ogura, 2022). HDL and apoA-1 mediated cholesterol efflux experiments were carried out to study whether PC can promote the cholesterol efflux from foam cells induced by ox-LDL. The results were shown in Table 2. In the HDL mediated cholesterol efflux experiment, The cholesterol efflux rates in the 10, 25 and 50 μmol/L PC groups were increased by 14.67%, 22.79% and 30.85% compared to the control. Similarly, PC also could promote ApoA-1 mediated cholesterol efflux

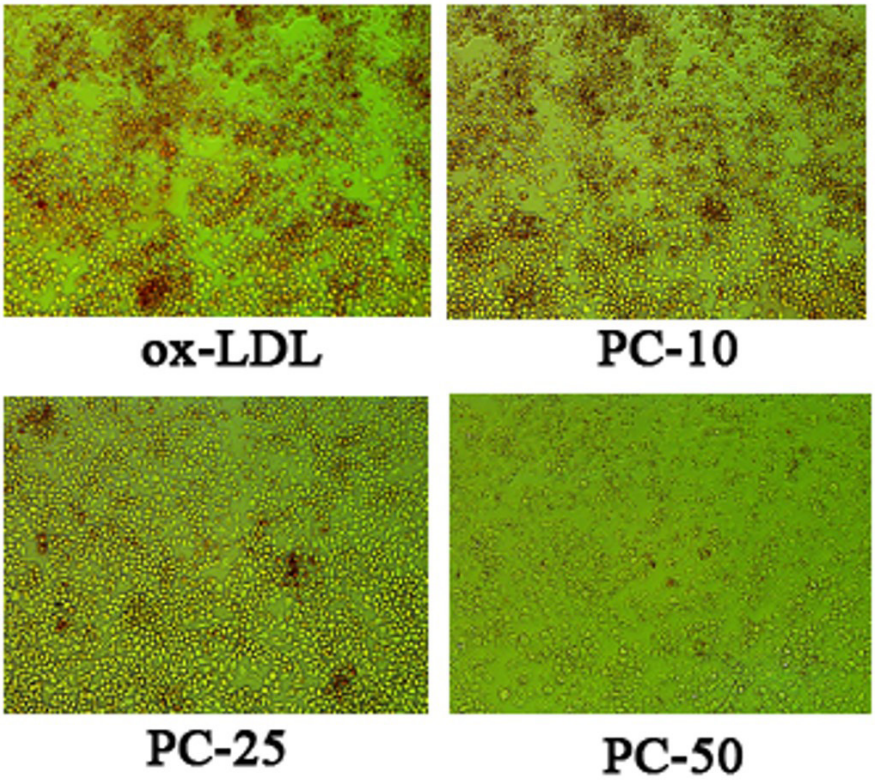

Figure 2. Lipid droplets observed by oil red O staining in mouse RAW264.7 foam cell. Notes: ox-LDL: ox-LDL treatment group, PC-10: ox-LDL+10 mol/LPC, PC-25: ox-LDL+25 mol/LPC, PC-50: ox-LDL+50 mol/LPC.

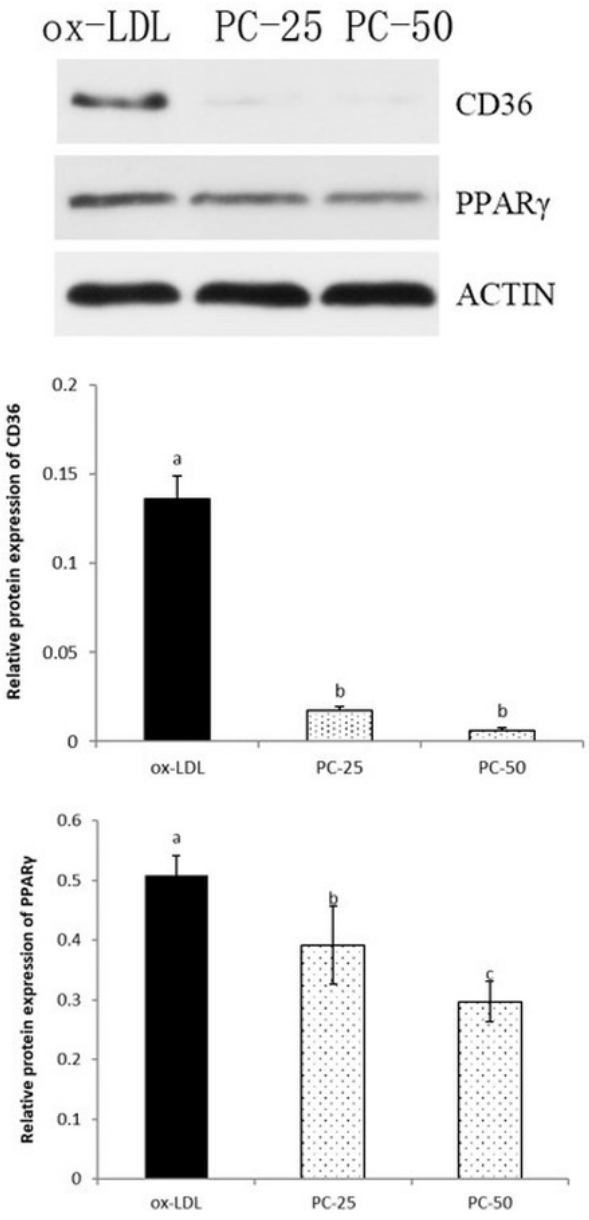

Figure 3. Effects of PC on CD36 and PPARγ protein level in raw264.7 macrophages. Notes: ox-LDL: ox-LDL treatment group, PC-25: ox-LDL+25 mol/LPC, PC-50: ox-LDL+50 mol/LPC. Different letters represent significant differences between groups ($p<0.05$),the same letter represent no significant difference ($p>0.05$).

significantly by 10.82%, 22.94%, 29.75%from macrophages. we can conclude that PC promote cholesterol efflux significantly.

3.5 The effects of PC on the cholesterol efflux-related proteins expression

Some research results showed that activation of LXRα can up-regulate the ABCA1 and ABCG1 gene expression, promote cholesterol efflux from macrophages, transport cholesterol into the blood from HDL to the liver, and finally expel in the form of bile acid or free cholesterol. In order to elucidate the molecular

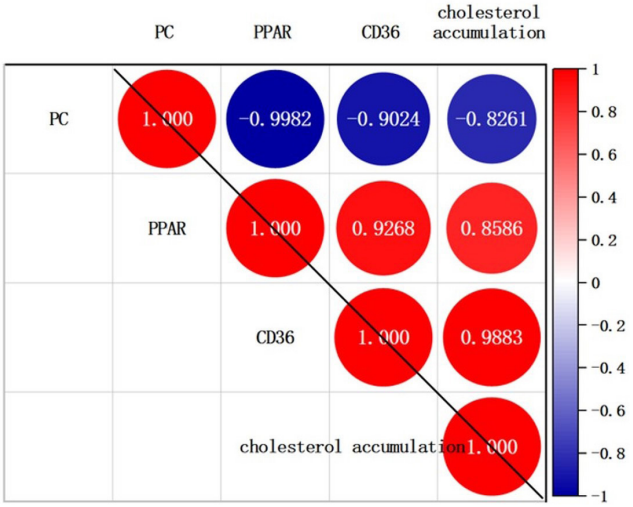

Figure 4. Correlation analysis between PC and PPARγ, CD36, cholesterol accumulation.

Table 2. The effect of PC on HDL and apoA-1 mediated cholesterol efflux in raw264.7 foam cells (mg/g protein).

Group	HDL	apoA-1
ox-LDL	14.39 ± 1.00 c	15.43 ± 0.57 d
PC-10	16.50 ± 0.33 b	17.10 ± 0.76 c
PC-25	17.67 ± 0.63 a	18.97 ± 0.70 b
PC-50	18.83 ± 0.79 a	20.02 ± 0.98 a

Notes: ox-LDL: ox-LDL treatment group; PC-10: ox-LDL+10 mol/LPC; PC-25: ox-LDL+25 mol/LPC; PC-50: ox-LDL+50 mol/LPC. different letters represent significant differences between groups($p<0.05$),the same letter represent no significant difference ($p>0.05$).

mechanism of PC to promote cholesterol efflux, the effects of PC on the expression of LXRα, ABCA1 and ABCG1 were detected by Western Blot, as shown in Figure 5. Compared with macrophage-derived foam cells, the expression of ABCA1 protein was up-regulated under the action of PC. A similar change was found in the ABCG1 and LXRα protein expression. All these indicate PC promotes cholesterol efflux through LXRα-ABCA1/ABCG1 pathway.

The correlation analysis between PC and LXRα, ABCA1, ABCG1,cholesterol efflux were shown in Figure 6. The correlation coefficients between PC and LXRα, ABCA1, cholesterol efflux mediated by apoA-I were all greater than 0.95 ($p<0.05$). The correlation between LXRα, ABCG1 and cholesterol efflux mediated by HDL were all over 0.94 ($p<0.05$). All these indicate LXRα, ABCA1 and ABCG1 are important responsible for cholesterol outflow, and PC application is highly related to these proteins expression and cholesterol outflow.

4 Discussion

Aviram et al. (2000) concluded after people ate pomegranate juice, the susceptibility of LDL to aggregation and retention was reduced, and the serum paraoxonase was increased after

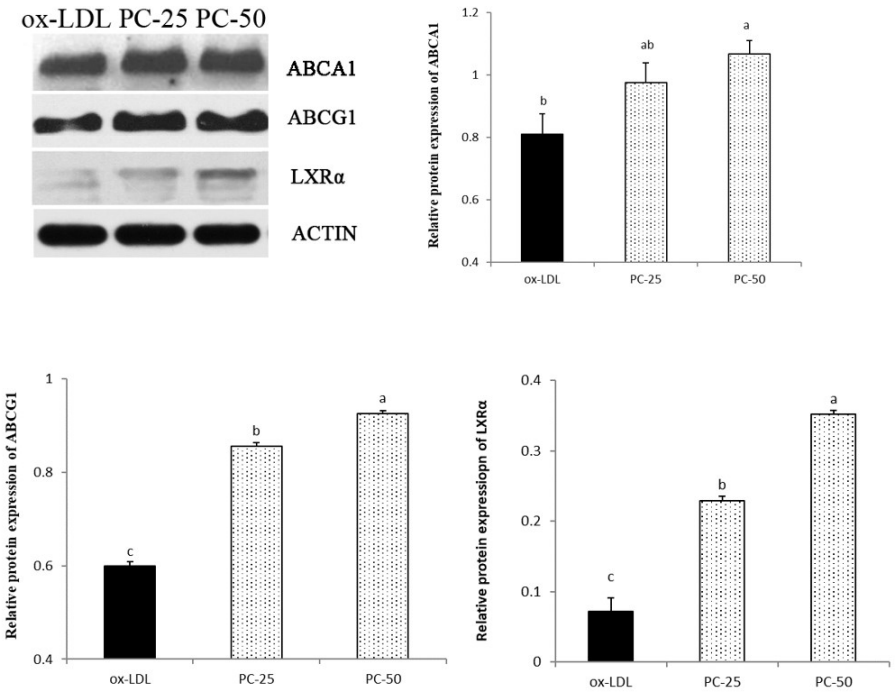

Figure 5. Effect of PC on the gene protein levels related with cholesterol efflux in raw264.7 foam cells derived from macrophages. Notes: ox-LDL: ox-LDL treatment group, PC-25: ox-LDL+25 mol/LPC, PC-50: ox-LDL+50 mol/LPC. Different letters represent significant differences between groups($p<0.05$), the same letter represent no significant difference ($p>0.05$).

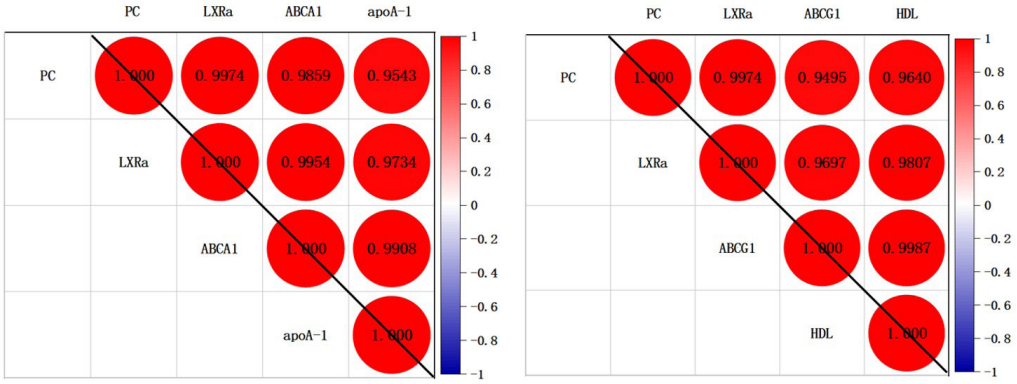

Figure 6. The correlation analysis of between PC and LXRα, ABCA1, ABCG1, cholesterol efflux.

eating pomegranate juice. After atherosclerotic apolipoprotein E-deficiency mice supplemented pomegranate juice, the oxidation of LDL in peritoneal macrophages was reduced by 90%, the size of atherosclerotic lesions was reduced by 44%, and the number of foam cells was also reduced (M et al., 2000). Aviram also evaluated the effects of pomegranate juice on carotid intima-media thickness (CIMT), blood pressure and LDL oxidation in atherosclerotic patients,and the results showed that pomegranate juice can reduce CIMT, blood pressure and LDL oxidation, and play a role in inhibiting the development of atherosclerosis (Aviram et al., 2004). Mira Rosenblat concluded that PC reduced intracellular cholesterol accumulation by preventing cholesterol synthesis, thereby reducing the formation of foam cells (Rosenblat et al., 2013). The effect of PPPs on cholesterol synthesis in L-O_2 cells was found that PPPs, PC and EA reduced the formation of the lipid droplets in liver cells (Jun et al., 2013). Besides, there may be other pathways for the reduction in cholesterol accumulation, and it has been reported that EA can inhibit ox-LDL-mediated LOX-1 expression (Lee et al., 2010). Our previous work showed that PPPs promoted cholesterol efflux by regulating LXRα-ABCA1/SR-B1 (Zhao et al., 2016). These research results all suggest that pomegranate juice, pomegranate polyphenols and their effective ingredients have the effect of anti-atherosclerosis.

Punicalagin was one of the most important ingredients in the pomegranate polyphenols. Recently, there were more and more related studies in recent years, which showed that it could protect and prevent many diseases (Ramlagan et al., 2022). Punicalagin could suppress inflammation through MAPK, JAK/STAT, NF-KB and other signal channels to have an anti-AS effect. In addition, the abnormality in the metabolism of AS was an important factor to AS. Could Punicalagin prevent or delay the occurrence of AS by adjusting the metabolism and promoting the discharge of protein? This paper had carried out relevant research and got certain results. Punicalagin could not only reduce the accumulation of protein in the cells, but also promote the flow of the protein in the macrophage cells. We speculated that the anti-AS effect of Pomegranate was perhaps mainly attributed to punicalagin.

PPARγ can regulate both the cholesterol intake and outflow gene at the same time, PPARγ might play the role of a double-edged sword in the process of foam cell formation (Zhao, 2017). Sin-Hye Park indicated the effect of EA on cholesterol efflux in J774A1 murine macrophages was dependent on PPARγ (Park et al., 2011). Chlorogenic acid was reported to inhibit AS development and reduce TC, TG and LDL-C content in the $ApoE^{-/-}$ mice, and inhibit the formation of ox-LDL induced foam cell, and increase PPARγ, LXRα, ABCA1 and ABCG1 transcription to promote the cholesterol outflow mediated by HDL and apoA-1 in raw264.7 cells (Wu et al., 2014). However, punicalagin decreased the lipid accumulation by downregulating PPARγ and CD36, which indicated that PPARγ was more involved in cholesterol intake in the study. The inconsistency among the studies in PPARγ may be due to differences in cell lines, time, or test substance.

In summary, PC can inhibit the foam cell formation by depressing cholesterol accumulation and promoting cholesterol outflow, these effects may be caused by its multi-targets.

5 Conclusion

In conclusion, our results demonstrated that PC can significantly reduce the cholesterol content in raw264.7 macrophage by down-regulating the PPARγ and CD36 expression. PC can promote the cholesterol efflux from the macrophages by LXRα-ABCA1/G1 pathway. In view of this, PC is the main contribution of pomegranate polyphenols to inhibit the foam cell formation.

Acknowledgements

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