Research Article

Chungtaejeon, a Korean fermented tea, prevents the risk of atherosclerosis in rats fed a high-fat atherogenic diet

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ABSTRACT

OBJECTIVE: Hypercholesterolemia is one of the well-established risk factors for cardiovascular mortality and morbidity in coronary heart disease. The aim of this study was to investigate the anti-atherogenic effect of Chungtaejeon (CTJ, a Korean fermented tea) aqueous extract on proliferation and migration of human aortic smooth muscle cells (HASMCs) in vivo and in vitro.

METHODS: The authors used high-fat atherogenic diet (HFAD) to induce hyperlipidemia in Wistar rats in vivo animal experiments and used HASMCs for in vitro cell experiments. For the in vitro cell experiment, the proliferation of HASMCs was evaluated using the MTT assay. Similarly, the expression of matrix metalloproteinases (MMPs) in HASMCs was measured using gelatin zymography. Antimigratory activity of CTJ was revealed using the wound-healing model and Boyden's chamber assay. In the in vivo experiment, CTJ was administered in three different doses for 20 d from the initiation of the HFAD. After 20 d, the serum lipid profile and total lipid contents in liver were measured.

RESULTS: Treatment with CTJ for 24 h dose-dependently inhibited the proliferation and migration of HASMCs and expression of MMP-2 in HASMCs. The oral administration of CTJ at concentrations of 200 and 400 mg/kg decreased the levels of low-density lipoprotein cholesterol, total serum cholesterol and hepatic cholesterol of HFAD-fed rats.

CONCLUSION: CTJ possessed strong antiproliferative, antimigratory, as well as lipid-lowering activities. Thus, CTJ can be considered as a therapeutic option in the treatment of high-fat diet-induced atherosclerosis.

Keywords: hypercholesterolemia; atherosclerosis; Chungtaejeon; proliferation; migration

(MMPs) to induce proliferation and migration of SMCs has been confirmed by both in vivo and in vitro experimental studies\(^2\). The key role of MMPs in this process is to degrade the elastic lamina barrier of the extracellular matrix (ECM) through its proteolytic activity, leading to pathological conditions such as rheumatoid arthritis, vascular disease and cancer\(^2\). Various studies have reported that the proliferation of SMCs, as well as endothelial cells, is influenced by the surplus expression of MMPs\(^2,3\). Other endogenous molecules that facilitate the proliferation and migration of SMCs are platelet-derived growth factor (PDGF) and tumor necrosis factor-α (TNF-α)\(^6,7\).

Low-density lipoprotein cholesterol (LDL-C), also known as “bad cholesterol”, is actively involved in the progression of numerous diseases, including atherosclerosis, cancer, diabetes mellitus and aging\(^8\). Oxidative modification of LDL, via reactive oxygen or nitrogen species-mediated lipid peroxidation, alters its structure such that oxidized-LDL (ox-LDL) is taken up by LDL scavenger receptors on macrophages, leading to the formation of fatty streak foam cells\(^9\). Several factors such as sedentary lifestyle, consumption of diets rich in cholesterol, and especially LDL, can lead to hypercholesterolemia\(^9\). A recent study has shown that hypercholesterolemia is linked with increased oxidative stress from enhanced lipid peroxidation\(^10\). Increased formation of ox-LDL is a major factor responsible for vascular damage associated with high cholesterol levels\(^12\). The functions of herbal medicines in lowering high cholesterol level are complex. Cholesterol-lowering activity of berberine acts by increasing hepatic LDL receptor mRNA and protein. Similarly, alcoholic extract of Panax ginseng augments antioxidant potency by depleting malondialdehyde levels, as well as elevating erythrocyte superoxide dismutase, a scavenger that reduces high malondialdehyde levels, as well as elevating erythrocyte superoxide dismutase, a scavenger that reduces high cholesterol level are complex. The key role of MMPs in this process is to degrade the elastic lamina barrier of the extracellular matrix (ECM) through its proteolytic activity, leading to pathological conditions such as rheumatoid arthritis, vascular disease and cancer\(^2\).

2 Materials and methods

2.1 Processing of CTJ

Leaves of wild C. sinensis were dried overnight in a well ventilated room. Steaming was carried out 3 to 4 min after removing impurities associated with the leaves. After pulverization, it was kneaded and given a shape with the help of plastic frames having a diameter of 2.5 cm and a thickness of 0.5 cm. These wafers were dried for 2 to 3 d using bamboo baskets. After punching with the help of bamboo needle of diameter 0.2 cm, fermentation was carried out for 7 d using a fermenter. Finally, they were dried at room temperature and relative humidity of 50% for 20 to 30 d. Thus produced CTJ was wrapped in a hand-made traditional Korean paper from mulberry trees and packaged in a case\(^17\).

2.2 Extraction of CTJ

CTJ extract was prepared according to Park et al\(^17\). A total of 12 g of CTJ were boiled in 3 300 mL of water for 3 h; this was repeated twice. The residues were removed by filtration using Whatman’s paper and then the extract was evaporated followed by freeze-drying. The percentage yield of extracts was approximately 12%. In a typical experiment, the extract was dissolved in distilled water to the desired concentrations and used for analysis.

2.3 In vivo toxicity study and dose selection

Before the start of real experiment, an in vivo toxicity study was carried out in male Wistar rats for 3 weeks to identify the safe effective dose. CTJ extract was administered daily at a high dose of 1 g/(kg·d). At the end of study all rats survive with no sign of toxicity, and no alteration in body weight and food consumption pattern. Finally three safe doses of CTJ (100, 200 and 400 mg/ (kg·d)) were selected for the study.

2.4 Administration of diet and treatment

Male Wistar/ST rats (13 weeks old, n=40) were purchased from Central Laboratory Animal Inc., Seoul. Animals were maintained in the Animal Research Center, Mokpo National University, under a strictly controlled environment with alternate light-dark cycle of 12-12 h, 100% fresh HEPA-filtered air, a room temperature of (23±1) °C and humidity of 45%±5%. Animal experiments were conducted after obtaining approval from Mokpo National University-Lab Animal Research Committee (MNU-LARC). Normal basal diet (D12450B rodent diet) was supplied with free access during the 2-week period of acclimation. Rats were then placed in 5 separate enclosures (n=8 rats per enclosure). A normal
group continued receiving rodent diet *ad lib*, while the remaining groups were fed with HFAD (D12492 rodent diet with 60 kCal% fat (Research Diets, Inc. USA). A gastric gavage was used for oral administration of 1 mL of distilled water to normal group (fed with normal basal diet) and control groups (fed with HFAD), while 1 mL of 100, 200 and 400 mg/(kg·d) of CTJ was administered to CTJ100, CTJ200 and CTJ400 groups respectively for 20 d from the day of feeding of HFAD. The diet consumption and body weight were measured at predetermined times. On the final day of the experimental period, rats were fasted overnight and anesthetized with a pentobarbital injection. Blood samples were withdrawn from the inferior vena cava, collected in a vacutainer and centrifuged at 1 600 \( \times \) g for 15 min to obtain the serum. The livers were dissected and rinsed with physiological saline. All samples were stored at –70 °C until use in further experiments.

2.5 Determination of serum and hepatic lipids

Rat serum (20 µL) was added to 3 mL of commercial cholesterol reagent kit (Product code: AM 202-K, ASAN PHARM. Co. LTD, Korea) and vortexed at 37 °C for 5 min. Absorbance of the resultant product was measured using a microplate reader at 500 nm. The total serum cholesterol content was calculated with respect to standard curve for cholesterol, measured according to the manufacturer’s protocol. The level of LDL-C in serum samples was measured using a kit according to Noma *et al* [18]. High-density lipoprotein cholesterol (HDL-C) in the serum was separated according to Warnick *et al* [19], using dextran sulfate and magnesium ions. The HDL-C level in serum was quantified using an enzymatic assay. Finally, the atherogenic index was calculated using the following formula.

Atherogenic index = (Total cholesterol–HDL-C)/HDL-C

To determine total hepatic cholesterol, liver tissue was homogenized in phosphate-buffered saline for 10 min. The extraction of lipid from homogenate was carried out in chloroform and methanol (2:1) for 4 h. The total cholesterol level in liver was determined using an enzymatic cholesterol assay.

2.6 In vitro cell experiment

HASMCs, obtained from the American Type Culture Collection (Manassas, VA, USA), were grown in Dulbecco’s modified Eagle’s medium (Cambrex Inc., USA) supplemented with an antibiotic mixture (penicillin 100 U/mL and streptomycin 100 µg/mL) and 10% fetal bovine serum followed by incubation at 37 °C in a humidified atmosphere with 5% CO₂.

2.7 Proliferation assay

HASMC viability and the PDGF-incuced proliferation were assessed using the thiazolyl blue tetrazolium bromide reagent assay (MTT, Sigma-Aldrich Inc. USA) as described previously [20,21]. Briefly, HASMCs were treated with various doses of CTJ for 24 h. Gelatin zymography were performed in both cell condition medium and cell lysate. SK-Hep-1 cell line was used as negative control.

2.8 Gelatin zymography

Expression of MMPs by HASMCs was assessed by gelatin zymography to check the gelatinolytic activity of MMP-9 and MMP-2, as describe previously [22].

2.9 Migration assay

HASMC migration was evaluated with the scratch wound-healing model and modified Boyden’s chamber assay as describe previously [23].

2.10 Statistical analysis

The results are shown as the mean±standard error of mean from independent experiments (\( n=8 \) in each group for *in vivo* and \( n=3 \) for *in vitro*). Graphs were made using GraphPad Prism software; statistical significance among the groups was determined by one-way analysis of variance followed by Tukey’s multiple comparison test. Values of \( P<0.05 \) were considered statistically significant.

3 Results

3.1 Effects of CTJ on the diet consumption, weight gain and liver weight

There were no significant differences in the food consumption, weight gain and liver weight among different groups (Table 1). All animals survived throughout the experimental period.

3.2 Effects of CTJ on serum and hepatic lipids

Oral administration of CTJ at 200 and 400 mg/kg

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Body weight, diet consumption and liver weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>Dose (mg/kg)</td>
</tr>
<tr>
<td>---------</td>
<td>--------------</td>
</tr>
<tr>
<td>Normal diet</td>
<td>8</td>
</tr>
<tr>
<td>Model control</td>
<td>8</td>
</tr>
<tr>
<td>CTJ-100</td>
<td>100</td>
</tr>
<tr>
<td>CTJ-200</td>
<td>200</td>
</tr>
<tr>
<td>CTJ-400</td>
<td>400</td>
</tr>
</tbody>
</table>

Data are represented as mean±standard error of mean. CTJ: Chungtajeon.
significantly decreased the LDL-C level in comparison to the control ($P<0.05$). Similarly, the total cholesterol levels in serum of the CTJ200 and CTJ400 groups were significantly lower than that in the control (HFAD) group ($P<0.05$, $P<0.01$). However, CTJ elevated the level of HDL-C (Table 2). The effect of CTJ on total cholesterol content in the liver is shown in Table 3. CTJ significantly lowered the total cholesterol content in liver compared to the control ($P<0.05$). The atherogenic index was significantly reduced in the CTJ200 and CTJ400 groups compared with the model group (Figure 1).

### 3.3 Effects of CTJ on proliferation of HASMCs

Figure 2 shows the effects of CTJ on the proliferation of HASMCs induced by PDGF. CTJ at concentrations of 50, 100 and 250 µg/mL had the proliferation rates of 90%±2%, 81%±8% and 53%±10% of control value respectively. However, CTJ at concentration of 500 µg/mL had a cytotoxic effect. The half-maximal inhibitory concentration value of CTJ on HASMCs was calculated to be 290 µg/mL.

#### Table 2 Effects of CTJ on serum LDL-C, HDL-C and total cholesterol levels of HFAD-fed rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>n</th>
<th>LDL-C (mg/dL)</th>
<th>HDL-C (mg/dL)</th>
<th>Total cholesterol (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal diet</td>
<td>8</td>
<td></td>
<td>35.2±0.7</td>
<td>28.5±0.7</td>
<td>67.4±3.2</td>
</tr>
<tr>
<td>Model control</td>
<td>8</td>
<td></td>
<td>53.7±1.2</td>
<td>26.8±1.1</td>
<td>127.5±3.1</td>
</tr>
<tr>
<td>CTJ 100</td>
<td>100</td>
<td>8</td>
<td>50.6±2.5</td>
<td>29.4±0.9</td>
<td>130.8±1.4</td>
</tr>
<tr>
<td>CTJ 200</td>
<td>200</td>
<td>8</td>
<td>45.2±1.4</td>
<td>31.1±1.2</td>
<td>114.8±1.5</td>
</tr>
<tr>
<td>CTJ 400</td>
<td>400</td>
<td>8</td>
<td>44.2±2.1</td>
<td>29.1±2.3</td>
<td>107.4±4.9</td>
</tr>
</tbody>
</table>

Data are represented as mean±standard error of mean. *$P<0.05$, **$P<0.01$, vs model control group. CTJ: Chungtaejeon; HFAD: high-fat atherogenic diet; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol.

#### Table 3 Effects of CTJ on hepatic total cholesterol of HFAD-fed rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>n</th>
<th>Total cholesterol (mg/dL) per gram liver</th>
<th>in liver</th>
<th>in liver of 100 g BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal diet</td>
<td>8</td>
<td></td>
<td>6.5±0.7</td>
<td>77.6±6.7</td>
<td>21.2±3.2</td>
</tr>
<tr>
<td>Model control</td>
<td>8</td>
<td></td>
<td>29.8±1.2</td>
<td>380.7±20.1</td>
<td>117.5±7.1</td>
</tr>
<tr>
<td>CTJ 100</td>
<td>100</td>
<td>8</td>
<td>22.2±2.5</td>
<td>310.5±11.4</td>
<td>81.4±4.4</td>
</tr>
<tr>
<td>CTJ 200</td>
<td>200</td>
<td>8</td>
<td>22.5±1.4</td>
<td>320.4±15.8</td>
<td>84.5±5.5</td>
</tr>
<tr>
<td>CTJ 400</td>
<td>400</td>
<td>8</td>
<td>20.9±2.1</td>
<td>290.2±14.7</td>
<td>67.4±4.9</td>
</tr>
</tbody>
</table>

Hepatic total cholesterol in liver homogenate was determined using cholesterol enzyme kits. Data are represented as mean±standard error of mean. *$P<0.05$, vs model control group. CTJ: Chungtaejeon; HFAD: high-fat atherogenic diet; BW: body weight.

#### Figure 1 Effects of CTJ on atherogenic index of HFAD-fed rats

Atherogenic index was calculated as (total cholesterol–HDL-C)/HDL-C. Data are represented as mean±standard error of mean (n=8). CTJ100: Wistar rats treated with CTJ extract at 100 mg/kg body weight; CTJ200: Wistar rats treated with CTJ extract at 200 mg/kg body weight; CTJ400: Wistar rats treated with CTJ extract at 400 mg/kg body weight. **$P<0.01$, vs control. CTJ: Chungtaejeon; HFAD: high-fat atherogenic diet; HDL-C: high-density lipoprotein cholesterol.

#### Figure 2 Effect of Chungtaejeon on the HASMC proliferation

HASMCs were seeded at a concentration of 1×10⁴ cells/well in a 96-well microplate. CTJ at various concentrations was incubated with HASMCs for 24 h. Absorbance was followed by the measurement of optical density at 540 nm using a microplate reader. **$P<0.01$, vs control (PDGF alone). CTJ: Chungtaejeon; HASMCs: human aortic smooth muscle cells; PDGF: platelet-derived growth factor.
3.4 Effects of CTJ on the expression of MMP-2 and MMP-9

Figure 3 demonstrates the effects of CTJ on the secretion of MMP-2 and MMP-9 in HASMCs. To examine the effects of CTJ on MMP-2 and MMP-9 expression, HASMCs were treated with various concentrations of CTJ for 24 h. The condition medium and cell lysate were subjected to gelatin zymography. CTJ, at concentrations of 50, 100 and 250 µg/mL, showed a dose-dependent inhibition of enzymatic activity on both condition medium (Figure 3A) and cell lysate (Figure 3B).

3.5 Effects of CTJ on the migration of HASMCs

The effects of CTJ on the migration of HASMCs, as shown by a wound-healing assay and Boyden’s chamber assay, are shown in Figure 4. The control group in the wound-healing assay (Figure 4A and 4C) had filled most of the migration zone with migrating HASMCs by 24 h after initial wounding. However, the treatments that received CTJ at concentrations of 100 and 250 µg/mL for 24 h had significantly more inhibited HASMC migration, compared to the control. Similarly, in Boyden’s chamber (Figure 4D), PDGF induced the migration of HASMCs to a significantly higher level whereas treatment of CTJ dose-dependently inhibited the migration of HASMCs (Figure 4B).

4 Discussion

Several factors, such as lack of physical exercise, dietary imbalance (cholesterol-rich food), aging and hypertension are closely associated with atherosclerosis[24]. LDL peroxidation, by unstable free radicals, alters its structure allowing ox-LDL to be engulfed by activated macrophages, endothelial cells and SMCs, leading to the formation of lipid-laden foam cells, which progressively accumulate in intimal layer to initiate atherosclerotic lesions[9]. Therefore, lipid peroxidation is considered to be a major factor for vascular inflammatory disorder, associated with high cholesterol levels[12]. In this study, the preventative effect of aqueous extract of CTJ was investigated in atherosclerotic pathogenesis. The mechanism by which CTJ lowers hypercholesterolemia and inhibits vascular smooth muscle cell proliferation and migration is shown in Figure 5. As shown in our previous study, the DPPH-scavenging anti-oxidant activity and NO-chelating activity of CTJ were 8.91 and 14.32 µg/mL, respectively[21]. Thus, the authors conclude that CTJ has a powerful antioxidant effect by scavenging free-radical molecules, and thereby possibly interrupting the production of oxidative LDL in the serum. In present study, oral administration of CTJ at 200 and 400 mg/kg in rats fed with HFAD lowered the total cholesterol and LDL-C levels in comparison to rats given HFAD only. Polyphenols and tannins present abundantly in CTJ are thought to be responsible for this cholesterol-lowering effect. The hypocholesterolemic effect of C. sinensis on blood lipids has been explored, and various plant polyphenols were shown to reduce serum cholesterol levels at low doses[25]. Phytochemical analysis of C. sinensis has shown the presence of vitamins, polysaccharides, tannins, flavonoids, glycosides and volatile oils[26]. It is likely that active constituents in herbal plants would be modulating different pathways of lipid metabolism[21]. The fall in cholesterol levels indicates changes in pools of cholesterol within the body, possibly due to compensatory mechanisms, such as a decline in resorption of endogenous cholesterol, rise in the rate of secretion into intestinal tract, or both. HDL plays an important role in physiological system, defending against oxidative damage[27,28]. The prominent role of HDL in cholesterol metabolism is to take up and transfer cholesterol from surrounding tissues to the hepatic system, mediated by a process called reverse cholesterol transport. These transferred cholesterol molecules are excreted into the bile and finally to intestine after converting to bile acids. So, the decrease in liver total cholesterol and increase in plasma HDL are marker of the prevention of atheroma development in rats fed with high-fat diet. Low levels of HDL-C in circulation are strongly associated with an elevated risk of cardiovascular diseases[29–31]. In our results, oral administration of CTJ elevated HDL-C levels in Wistar rats. The reduction of
**Figure 4** Effects of CTJ on migration of HASMCs

(A) HASMCs (5×10^5 cells) were seeded in the 6-well plate and migration zone was created by 200 µL tip. Samples were treated for 24 h. Then, the distance of migration zone was measured using image analysis program. (B) The migrated cells were counted in 5 high power fields (200×). (C) Images of wound closure were captured at 0, 12 and 24 h. Distances between the wounds (in μm) for each group are shown in the respective image. (D) Migration of HASMCs was measured by modified Boyden’s chamber assay with PDGF as a migration inducer and the indicated concentrations of CTJ for 6 h treatment using eosin and hematoxylin staining. Values are expressed as mean ± standard error of mean (n = 5). **P < 0.01, vs control (PDGF alone). CTJ: Chungtaejeon; HASMCs: human aortic smooth muscle cells; PDGF: platelet-derived growth factor.
oxidative damage in hypercholesterolemic conditions is the main target of any therapeutic approach to prevent lipid peroxidation\textsuperscript{[32]}. Mechanically, atherosclerosis can be outlined as the complex involvement of macrophages, proliferation and migration of SMCs, as well as the composition and degradation of ECM\textsuperscript{[33]}, SMCs are one of the main cellular components of blood vessel layers and are engaged in vascular pathology\textsuperscript{[34]}. It is well established that SMC proliferation and the consequent production of ECM components contribute to atherosclerotic plaques\textsuperscript{[35]}. Our HASMC in vitro proliferation assay revealed that treatment with CTJ hinders its proliferation. There is substantial experimental support for SMC functional impairment as an element in various blood vascular diseases, including atherosclerosis\textsuperscript{[36]}. MMP-2 is a member of the MMP family that has been extensively researched, and has been found have a prime role in increasing SMC migration, tissue remodeling and cancer cell metastasis\textsuperscript{[37]}. In our study, results of gelatin zymography and wound-healing assays showed that the treatment of CTJ inhibited the expression of MMP-2 and migration of HASMCs respectively. Furthermore, CTJ inhibited the migration of HASMCs in Boyden’s chamber assay. Our results showed that CTJ was successful in elevating serum HDL-C level while diminishing serum total cholesterol, LDL-C and total hepatic cholesterol in HFAD-fed rats. Taken together, our in vitro assays highlighted that CTJ was effective in suppression of proliferation, migration and expression of MMP-2 in HASMCs, while our in vivo results showed that treatment with CTJ improved the imbalance of HDL-C and LDL-C in HFAD-fed rats.

5 Conclusion

It is clear that CTJ extract could be beneficial in the management of cardiovascular diseases in which atherosclerosis plays a major role. This is our second report that provides evidence of CTJ’s anti-atherosclerotic activities. It is worth noting that oral administration of CTJ for 20 d had no noticeable toxic effects on animals in this study, such as reduction of food intake or body weight. The findings of this study provide strong evidence that CTJ should be considered as atherapeutic option for the prevention of high-cholesterol-induced atherosclerosis. Further research should be carried out to explore the detailed mechanism of CTJ to prevention of atherosclerosis.

6 Acknowledgements

This research was supported by the research fund of Mokpo National University in 2014.

7 Conflict of interests

The authors declare that they have no conflict of interests.

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