European Review for Medical and Pharmacological Sciences 2021; 25: 7765-7776

The effect of *per os* colchicine administration in combination with fenofibrate and N-acetylcysteine on triglyceride levels and the development of atherosclerotic lesions in cholesterol-fed rabbits

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Abstract. – OBJECTIVE: Atherosclerosis is a chronic inflammatory disease promoted by pro-inflammatory cytokines produced by NOD-, LRR- and pyrin domain-containing protein 3 (NLRP 3) inflammasome. Colchicine is an anti-inflammatory agent that inhibits inflammasome's action and stabilizes atherosclerotic lesions. N-acetylcysteine (NAC) reduces low-density lipoprotein (LDL) oxidation, metalloproteinase levels, and foam cell count and volume. Fenofibrate also has antioxidant, anti-inflammatory, and anticoagulant properties while also having a beneficial effect on the vasomotor function of the endothelium. The purpose of this study is to investigate the effect of *per os* colchicine administration in combination with fenofibrate and NAC on triglyceride levels and the development of atherosclerotic lesions in cholesterol-fed rabbits.

MATERIALS AND METHODS: Twenty-eight male, 2 months old New Zealand White rabbits were separated into four groups and were fed with different types of diet for 7 weeks: standard, cholesterol 1% w/w, cholesterol 1% w/w plus colchicine 2 mg/kg body weight plus 250 mg/kg body weight/day fenofibrate, and cholesterol 1% w/w plus colchicine 2 mg/kg body weight plus 15 mg/kg body weight/day NAC. Blood samples were drawn from all animals. Lipid profiles were assessed, and interleukin 6 (IL-6) measurements were performed using an enzyme-linked immunosorbent assay (ELISA) kit. Histologic examination was performed on aorta specimens stained with eosin and hematoxylin. Aortic intimal thickness was evaluated using image analysis.

RESULTS: Colchicine administration in combination with fenofibrate or NAC statistically significantly reduced the extent of atherosclerotic lesions in aortic preparations. Co-administration of colchicine with NAC has a stronger anti-atherogenic effect than the colchicine plus fenofibrate regimen. Triglerycide levels were decreased in the colchicine plus fenofibrate group and the colchicine plus NAC group at the end of the experiment ($p < 0.05$), whereas the Cholesterol group had increased levels. A favorable significant lower concentration of IL-6 was detected in the colchicine plus NAC group vs. the other groups.

CONCLUSIONS: In an experimental rabbit model, it appears that colchicine statistically significantly reduces the development of atherosclerosis of the aorta, especially in combination with NAC. Colchicine, as an NLRP3 inflammasome inhibitor, and NAC, as an agent that directly targets IL-6 signaling, can reduce the inflammatory risk. Fenofibrate enhances the attenuating role of colchicine on triglyceride levels. Clinical studies should investigate whether similar effects can be observed in humans.

Key Words:
Colchicine, Myocardial infarction, Inflammation, Atherosclerosis, Coronary, Triglyceride.

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Introduction

Atherosclerosis is associated with significant unfavorable cardiac events, such as myocardial infarction and stroke, the world's two leading causes of death[1]. Atherosclerosis, as a low-grade inflammatory disease, presents an intriguing target for anti-inflammatory medicines[2,3]. These medicines may modulate the inflammatory mechanism responsible for converting a stable to a susceptible plaque, causing thrombosis and increasing the risk of serious clinical outcomes[2,3].

Recent research studies[4-7] indicate that the NOD-like receptor protein 3 (NLRP 3) inflammasome (a cytosolic protein complex triggered by a pathogen or host signals such as cholesterol crystals) increases interleukin (IL)-1 and IL-18 release. These interleukins are known to have a role in the growth and instability of atherosclerotic plaques[8,9]. The activation of the inflammasome is thought to have a role in developing a variety of illnesses, including inflammatory, autoimmune, and metabolic illnesses[10,11]. IL-6 signaling is associated with plaque formation and rupture[12-15]. IL-6 acts as a central hub for the coordination of inflammatory pathways[12-15]. As a result, anti-inflammatory drugs that specifically target IL-6 should also be considered for atherosclerosis[12-15].

Colchicine, a well-known anti-inflammatory agent, acts by attaching to free tubulin dimers and interfering with microtubule polymerization[16]. Recently, numerous investigations[17,18] have been undertaken to determine the potential preventative function of colchicine in atherosclerosis and its harmful consequences. The low-dose colchicine (LoDoCo) study[17] demonstrated that three years of low-dose colchicine therapy in individuals with stable coronary disease decreased cardiovascular events. Additionally, it was observed that short-term therapy with colchicine decreased the production of IL-1 and IL-18 in coronary arteries in individuals with acute coronary syndrome (ACS)[18]. Colchicine exerts one of its anti-inflammatory actions by inhibiting the development of the NLRP3 inflammasome in macrophages and decreasing neutrophil infiltration[19,20].

N-acetylcysteine (NAC) is a cysteine derivative that is less toxic and oxidative than its source[21,22]. NAC is an effective antioxidant and scavenger of oxygen-free radicals in the treatment of oxidative stress-related diseases[21,22]. Its anti-inflammatory activities have been investigated in a few studies, with mixed results[21,22]. NAC exerts its anti-inflammatory impact indirectly by inhibiting nuclear factor kappa-B (NF-κB), which suppresses the subsequent rise of inflammatory biomarkers, or directly by inhibiting several inflammatory biomarkers, including TNF-a[21,22]. Oral NAC supplementation decreases C-reactive protein (CRP) and IL-6 levels in the serum[21,22].

Fibrates are ligands for peroxisome proliferator-activated receptor α (PPARα), a transcription factor belonging to the nuclear receptor superfamily, which mediates their lipid-normalizing effects[23,24]. Fibrates decrease plasma triglycerides and have a positive impact on reducing cardiovascular diseases[23,24]. Fenofibrate, in particular, is not only an anti-hyperlipidemic drug but also shows potent anti-inflammatory activity, which can have an additional impact on the treatment of atherosclerosis[23.24].

The rabbit is the most often used animal model in experimental atherosclerosis research[25-28]. Dietary treatments are often utilized to investigate the positive effects of hypolipidemic and anti-inflammatory medicines on atherosclerotic processes in these animals[25-28]. It is quite simple to induce atherosclerotic lesions in rabbits by feeding them a high-cholesterol diet for 8-12, or even 7 weeks, depending on the amount of cholesterol added to the usual chow[26-29]. Some studies[30,31] have demonstrated that cell proliferation in the thoracic and abdominal aortas and the development of fatty streaks begin early after administration of a hypercholesterolemic diet to rabbits. The events that trigger atherosclerosis are similar to those in humans[31,32]. As a result, we chose the rabbit as the most suitable animal model. The purpose of this study is to investigate the effect of *per os* colchicine administration in combination with fenofibrate and NAC on triglyceride levels and the development of atherosclerotic lesions in cholesterol-fed rabbits. To our knowledge, no study has been conducted on the impact of *per os* colchicine-based combination therapy on the development of atherosclerotic plaques and hypertriglyceridemia. Additionally, we investigated the impact of these combinations on inflammatory indicators, such as IL-6 and CRP levels in New Zealand White rabbits during sustained hypercholesterolemia.

Materials and Methods

Experimental Design

28-month-old male, New Zealand White rabbits (Oryctolagus cuniculus) were separately housed in stainless steel wire-bottom cages

(bodyweight 3.625 ± 0.163 kg, mean ± SD). The animals were donated by an Attica-based farm that breeds rabbits for experimental reasons. The rabbits were housed individually in cages and maintained in a temperature-controlled environment (19 ± 1°C with 50 ± 5% relative humidity) with a 12-hour light/dark cycle (6 am to 6 pm) in an air-conditioned room with 15 air changes per hour. They also had free access to food and tap water. Individuals' daily usage of tap water was monitored during their stay. Throughout the trial period, a veterinarian checked the animals clinically. At each stage of the trial, every precaution was taken to prevent animal harm. The procedures that potentially cause pain or stress to the animals (blood samplings) were conducted under moderate intramuscular ketamine-xylazine sedation. The rabbits were used and treated in line with the European Communities Council Directive of September 22, 2010 (276/33/20.10.2010), and the protocol was authorized by the Athens Prefecture's competent Veterinary Directorate (Approval No. 3231/26.06.2018).

After two weeks of acclimation, animals were randomly divided into four experimental groups. The standard control group's (n = 6) animals were given a commercial rabbit diet. The Cholesterol group (n = 6) had a regular diet supplemented with 1% w/w cholesterol. Animals in the Colchicine + Fenofibrate group (n = 8) received the same cholesterol-enriched diet plus colchicine 2 mg/kg body weight plus 250 mg/kg body weight/day fenofibrate, whereas animals in the Colchicine + NAC group (n=8) were fed cholesterol 1% w/w plus colchicine 2 mg/kg body weight plus 15 mg/kg body weight/day NAC.

The standard rabbit chow (Conigli Svezzamento, Società Italiana Veterinaria Agricola Milano S.P.A., Casalpusterlengo, Lombardy, Italy) contained the following ingredients (w/w): 37% carbohydrates, 16% proteins, 4% fat, 15% fiber, 11% water, 8% ash, and an appropriate mixture of minerals and vitamins for the healthy subsistence of the laboratory animals (added to the premix by the manufacturer). The atherogenic meal was created by dissolving the required cholesterol in diethyl ether (without butylated hydroxytoluene as an inhibitor) and mixing it with rabbit chow. Following ether evaporation, cholesterol food was stored at a temperature of 20°C until used.

Following the practice of the 3Rs (Replacement, Refinement, Reduction), we chose to divide the animals into four experimental groups. The Control group received the fewest interventions, and we decided to maintain the smallest number of animals as possible. However, the results of the Cholesterol group are anticipated to a greater extent, as shown in earlier studies[29]. As a result, a high concentration of 1% w/w cholesterol was added to ordinary rabbit fed with the expectation of increased atherosclerotic lesions. The feeding period was seven weeks, as previous researchers recommended[25]. Colchicine, fenofibrate, and NAC were ground to a powder, and then, dissolved in tap water. Throughout the trial, the four groups of animals had unrestricted access to their food and drinking water. Daily dosages of the three drugs were determined by monitoring daily water consumption and adjusting dilution. A dosage of 2 mg/kg/day was chosen as a high but not deadly dosage since there is evidence that colchicine does not impact the growth of atherosclerotic plaques at low doses[33]. We chose to deliver the medications by drinking water to reduce the stress on the animals induced by injections, which might influence the measured blood markers associated with inflammation.

Blood samples were taken from the central ear artery after overnight fasting at the beginning of the trial, at four and seven weeks of dietary intervention. At seven weeks, the animals were euthanized with an intravenous injection of sodium pentobarbital followed by intramuscular ketamine-xylazine sedation. Their aortas were cut from the arch to the iliac bifurcation, and the perivascular adipose tissue was removed.

Statistical Analysis

Data analysis was performed using Stata/BE 17.0 for Mac (StataCorp, 4905 Lakeway Drive, College Station, TX, USA). Continuous variables were expressed as a mean ± standard deviation (SD). Due to the limited sample size and non-normality of the distributions of interest, non-parametric tests were utilized. The Wilcoxon paired-sample test and Mann-Whitney U test were used for statistical analysis. Benjamini Hochberg's false discovery rate was used to determine pairwise significance levels while accounting for family-wise type I errors in all situations involving multiple comparisons. 0.05 was set as the threshold of statistical significance.

Plasma Analysis

Blood samples were collected for the measurement of total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides, CRP, creatinine, total

protein, gamma-glutamyl transferase (γGT), alkaline phosphatase (ALP), serum glutamic-oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) levels. Samples were stored at -80°C until analysis. Serum IL-6 levels were calculated by enzyme-linked immunosorbent assay (ELISA) using the commercially available Rabbit Interleukin 6 (IL-6) ELISA kit (Biomatik USA, LLC, Catalog No. EKC40248 Wilmington, DE, USA).

Histology and Immunostaining

The isolated aorta was detangled from connective and adipose tissue and longitudinally sliced open. The vessel was fixed to maintain its original dimensions and then immersed for 24 hours in a neutral 10% (v/v) buffered formalin solution. We removed and prepared sections of the ascending, thoracic, and abdominal aortas for paraffin incubation. For further microscopic investigation, paraffin slices of 5 μm thickness of aortas were cut and stained with eosin and hematoxylin. The histologic examination was done by an expert who was blind to the intervention groups.

Morphometry was performed to measure the atheromatous area of each tissue, using the Image J program (version 1.49C, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) to analyze every image. The measurement of the area of atheromatous plaque was expressed in μm. The thickening of the atherosclerotic plaque was calculated with the following formula: initial lumen diameter at the normal aorta (from 5 different points) – final lumen diameter after the development of atherosclerotic plaque (from 5 different points). The average of these points was calculated. The average thickness of the atherosclerotic plaque was calculated in all groups' ascending, thoracic, and abdominal aorta. The Cholesterol, Col + Fen, and Col + NAC groups were compared with the standard control group (paired *t*-test).

Results

Effect on Growth and Body Weight

All rabbits gained weight during the experiment, and no statistical difference was observed between groups receiving the various dietary interventions. Table I summarizes the animals' bodyweight in the four groups at baseline, after 4 and 7 weeks of the study.

Biochemical Measurements

The baseline laboratory measurements of all groups were comparable at the beginning of the experiment (Table I). There were no significant differences between the treatment groups in total cholesterol, LDL, HDL, liver enzymes, creatinine, or body weight (Table I).

At the end of the experiment, Col + Fen group had statistically significantly lower triglyceride levels ($p < 0.05$) than in the beginning, whereas the Cholesterol group had increased triglyceride levels but it is not statistically significant compared to the beginning.

Effect on the Levels of IL-6 and CRP

The measured levels of IL-6 and CRP are presented in Table II. IL-6 levels were significantly increased in the Cholesterol group at the 4th week of the experiment *vs.* baseline ($p < 0.05$), and this significance was also noticed at the 7th week of the experiment ($p < 0.05$ *vs.* baseline). A favorable significant lower concentration of IL-6 was detected in the Col + NAC group *vs.* the other groups.

Serum level of CRP was also profoundly increased in the Cholesterol group, indicating that hyperlipidemia was significantly associated with systemic inflammation. CRP reduction was more prominent in the Col + NAC group (19.63 ± 2.82 mg/dL *vs.* 32.98 ± 3.86 mg/dL, $p < 0.05$), indicating that colchicine plus NAC might have a more potent effect on ameliorating inflammation than colchicine plus fenofibrate, which was independent of lipid-lowering.

Histology

Intima thickness was measured in parts of the ascending, thoracic, and abdominal aorta, and the results are represented in Table III and Figure 1. We observed fatty streaks in the Cholesterol, Col + Fen, and the Col + NAC group, whereas the Standard group did not develop atherosclerotic lesions. The Cholesterol group significantly developed atherosclerosis compared to the treatment groups. Colchicine with NAC resulted in a more pronounced reduction in the extent of atherosclerotic plaques compared to colchicine with fenofibrate.

Discussion

The Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) showed that inhibiting IL-1b lowers cardiovascular event rates without affecting blood pressure or cholesterol

Table I. Bodyweight, lipid profile, liver function tests, and creatinine in the four groups of animals during the entire experimental period†.

	Group	0 weeks	4 weeks	7 weeks
Body weight (kg)	Standard	3.6 ± 0.1	4.3 ± 0.3[a]	4.2 ± 0.2[a,b]
	Cholesterol	3.6 ± 0.2	4.3 ± 0.3[a]	4.3 ± 0.3[a,b]
	Col + Fen	3.5 ± 0.1	4.2 ± 0.2[a]	4.0 ± 0.2[a,b]
	Col + NAC	3.6 ± 0.1	4.1 ± 0.2[a]	4.0 ± 0.2[a,b]
Total cholesterol (mg/dL)	Standard	45.83 ± 10.68	44 ± 6.72	40.66 ± 9.83
	Cholesterol	58.16 ± 19.65	2163.33 ± 675.09[a,c]	2096.66 ± 663.37[a,b,c]
	Col + Fen	40.62 ± 10.15	2496.25 ± 744.96[a,c]	2460 ± 728.08[a,b,c]
	Col + NAC	44.75 ± 10.16	3176.25 ± 515.55[a,c]	3112.5 ± 512.54[a,b,c]
HDL (mg/dL)	Standard	34.33 ± 10.38	33.83 ± 10.14	33.33 ± 9.68
	Cholesterol	36.33 ± 11.05	141.66 ± 44.90	139.83 ± 32.47
	Col + Fen	33.87 ± 10.16	143.75 ± 26.01	143.25 ± 21.58[a,b,c]
	Col + NAC	28.25 ± 4.8	136.25 ± 20.65	132.25 ± 20.6[a,b,c]
LDL (mg/dL)	Standard	20.16 ± 2.85	20.33 ± 2.42	1.33 ± 1.14
	Cholesterol	10.5 ± 10.11	1998.83 ± 653.68	1956.33 ± 212.14
	Col + Fen	26.33 ± 2.56	2337.12 ± 743.1	2356.33 ± 212.14[a,b,c]
	Col + NAC	50.25 ± 12.14	3034.5 ± 493.32	2856.33 ± 212.14[a,b,c]
Triglycerides (mg/dL)	Standard	96.33 ± 14.92	96.66 ± 11.25	94.83 ± 14.26
	Cholesterol	90.83 ± 23.32	114.16 ± 30.72	119 ± 24.37
	Col + Fen	97.25 ± 8.39	56.87 ± 18.88	49.5 ± 14.57[a,b,c,d,f]
	Col + NAC	97.5 ± 16.90	70 ± 14.88	58.25 ± 12.25[a,b,c,d]
SGOT (U/L)	Standard	19.16 ± 5.94	22.33 ± 4.84	23.5 ± 5.99
	Cholesterol	20.16 ± 8.28	22 ± 9.63	20.33 ± 8.71
	Col + Fen	14.25 ± 3.01	21.5 ± 7.5	24.37 ± 7.57
	Col + NAC	20.75 ± 5.84	26.37 ± 2.32	26.12 ± 3.44
SGPT (U/L)	Standard	42.83 ± 12.13	42.83 ± 11.65	43.16 ± 11.82
	Cholesterol	46 ± 23.99	45.5 ± 20.37	46.14 ± 18.66
	Col + Fen	39.87 ± 18.7	42 ± 10.46	42 ± 11.53
	Col + NAC	42.37 ± 15.87	47.62 ± 14.45	45 ± 13.22
γGT (U/L)	Standard	11.33 ± 1.21	10 ± 1.26	9.33 ± 1.21
	Cholesterol	11.33 ± 2.58	10.83 ± 3.43	10.33 ± 3.01
	Col + Fen	11.12 ± 2.03	10.75 ± 1.38	10.12 ± 1.35
	Col + NAC	10.5 ± 1.41	10.12 ± 1.45	9.5 ± 0.92
ALP (U/L)	Standard	141 ± 16.44	137.83 ± 19.13	130.33 ± 21.28
	Cholesterol	145.5 ± 29.18	144.83 ± 27.2	140.66 ± 21.45
	Col + Fen	153.12 ± 17.91	148.75 ± 18.46	146.37 ± 18.1
	Col + NAC	131.12 ± 24.97	137.87 ± 17.65	136.75 ± 14.13
Creatinine (mg/dL)	Standard	1 ± 0.06	1.05 ± 0.08	0.98 ± 0.07
	Cholesterol	1.01 ± 0.14	1.24 ± 0.10	1.18 ± 0.13
	Col + Fen	0.91 ± 0.11	1.05 ± 0.05	1.01 ± 0.08
	Col + NAC	0.91 ± 0.08	1.05 ± 0.07	1.02 ± 0.1

Paired samples Wilcoxon test adjusted with Benjamini-Hochberg procedure.
†Data are presented as mean ± SD, [a]$p < 0.05$ *vs.* baseline, [b]$p < 0.05$ *vs.* 4 weeks, [c]$p < 0.05$ *vs.* Standard group, [d]$p < 0.05$ *vs.* Cholesterol group, [e]$p < 0.05$ *vs.* Col + Fen group, [f]$p < 0.05$ *vs.* Col + NAC.

levels[12]. This study demonstrated the critical role of inflammation in human atherosclerosis and showed the feasibility of targeting pro-inflammatory cytokine pathways[12]. Nonetheless, CANTOS patients receiving both high-intensity statins and canakinumab (a potent IL-1b inhibitor) continue to have a significant risk of recurrent cardiovascular events[12]. Pro-inflammatory cytokine IL-18 and IL-6 signaling may explain part of this residual inflammatory risk[12].

Table II. Levels of IL-6, and CRP in the four groups during the entire experimental period†.

	Group	0 weeks	4 weeks	7 weeks
BIL-6 (pg/mL)	Standard	276.23 ± 42.87	283.59 ± 68.47	275.42 ± 84.23
	Cholesterol	283.83 ± 43.39	295.45 ± 43.66[a]	347.34 ± 78.83[a,b]
	Col + Fen	282.37 ± 46.23	261.13 ± 42.12	238.59 ± 43.87[c,d]
	Col + NAC	292.26 ± 44.18	252.27 ± 46.27	212.27 ± 36.26[c,d,e]
CRP (mg/dL)	Standard	17.53 ± 4.29	18.58 ± 4.50	19.82 ± 3.26
	Cholesterol	17.21 ± 5.45	29.79 ± 4.37	43.26 ± 4.97
	Col + Fen	17.16 ± 4.74	26.46 ± 2.59	32.98 ± 3.86[c,d]
	Col + NAC	17.48 ± 4.62	19.43 ± 3.82[c,d,e]	19.63 ± 2.82[c,d,e]

Paired samples Wilcoxon test adjusted with Benjamini-Hochberg procedure.
†Data are presented as mean ± SD, [a]$p < 0.05$ vs. baseline, [b]$p < 0.05$ *vs.* 4 weeks, [c]$p < 0.05$ *vs.* Standard group, [d]$p < 0.05$ *vs.* Cholesterol group, [e]$p < 0.05$ *vs.* Col + Fen group, [f]$p < 0.05$ *vs.* Col + NAC.

Table III. Intima thickness of different sections of aorta†.

Group	Standard (n = 6)	Cholesterol (n = 6)	Col + Fen (n = 8)	Col + NAC (n = 8)
Ascending aorta	4.18 ± 0.89	39.83 ± 8.08	23.62 ± 8.78	19.5 ± 3.58[a,b,c]
Thoracic aorta	7.59 ± 4.62	41.83 ± 8.08	25.75 ± 4.71	22.5 ± 3.58[a,b,c]
Abdominal aorta	6.26 ± 4.36	34 ± 2.6	21.37 ± 3.29	10.25 ± 5.33[a,b,c]

Paired samples Wilcoxon test adjusted with Benjamini-Hochberg procedure.
†Values are expressed in μm, mean score ± SD, [a]$p < 0.05$ *vs.* standard group, [b]$p < 0.05$ *vs.* Cholesterol group, [c]$p < 0.05$ *vs.* Col + Fen group, [d]$p < 0.05$ *vs.* Col + NAC.

The purpose of this study was to investigate the effect of *per os* colchicine administration in combination with fenofibrate and NAC on triglyceride levels and the development of atherosclerotic lesions in cholesterol-fed rabbits. Colchicine simultaneously inhibits IL-1b and IL-18 as an NLRP3 inflammasome inhibitor. NAC directly targets IL-6 signaling. Colchicine inhibits the progression of aortic atherosclerosis, particularly when combined with NAC, by reducing the inflammatory risk. Fenofibrate enhances the attenuating role of colchicine on triglyceride levels.

Inflammation plays a critical role in the development of atherosclerosis[34,35]. Given colchicine's well-known anti-inflammatory effects, we chose a high cholesterol diet-induced animal model characterized by the early development of atherosclerotic lesions and inflammation after the intervention[36].

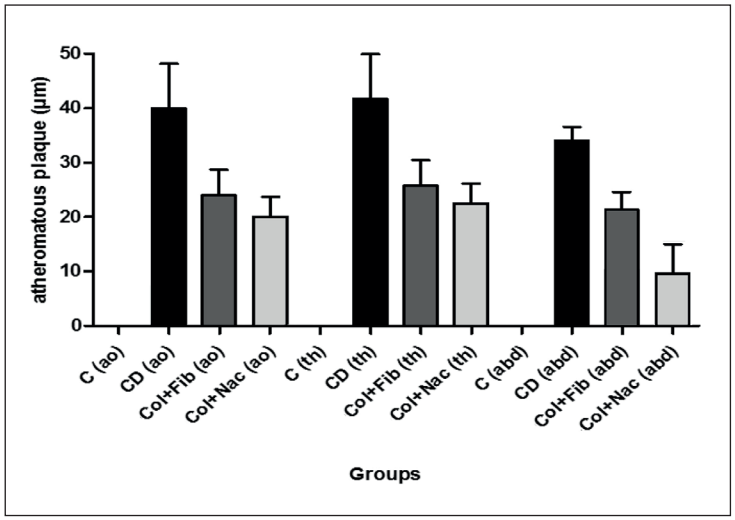

Figure 1. Atheromatous plaque formation in different sections of the aorta in the four groups. C: standard control group; CD: cholesterol diet group; Col + Fib: colchicine and fenofibrate; Col + Nac: colchicine and N-acetylcysteine; ao: ascending aorta; th: thoracic aorta; abd: abdominal aorta.

Colchicine's impact on the progression of atherosclerotic disease has been studied in the past[37]. Colchicine was originally shown to prevent the development of atherosclerotic lesions in rabbits by Hollander et al[37]. A study by Wójcicki et al[38] corroborated the findings above in which the authors injected intraperitoneal colchicine into cholesterol-fed rabbits. On the other hand, colchicine had no impact in the same animal model with pre-existing atherosclerotic lesions or pigs[39,40]. Experimental and clinical studies revealed disparate outcomes when colchicine and stent insertion were administered concurrently, or balloon angioplasty was performed alone[41-45].

NAC's impact on inflammatory biomarkers is also far from an agreement in the scientific community[21]. While many clinical studies showed that oral NAC supplementation might decrease plasma levels of inflammatory biomarkers, others failed to demonstrate any protective effect[21].

PPARα is a critical regulator of lipid and lipoprotein metabolism, influencing plasma lipid risk variables associated with atherosclerosis favorably[23]. Additionally, it is well established that PPARα and especially fenofibrate exerts anti-inflammatory and antithrombotic actions on both the systemic and vascular levels[23]. Additionally, it promotes macrophage lipid homeostasis[23]. The synergistic anti-inflammatory effect of colchicine and fenofibrate, though, has not yet been investigated.

In the rabbit animal model, it is well established that consuming foods high in cholesterol results in atherosclerotic lesions that mimic the early stages of atherosclerosis in humans. The administration of a 1% (w/w) cholesterol diet for seven weeks produced the desired model, as shown by an increase in plasma cholesterol and the presence of typical atherosclerotic lesions in the aorta. Colchicine 2 mg/kg/d is considered a high dosage compared to previous animal trials. When combined with fenofibrate and NAC, it substantially decreased the number of atherosclerotic lesions in aortic preparations. Colchicine with NAC showed a more significant anti-atherogenic impact than colchicine plus fenofibrate. There were no diarrheal symptoms or weight loss noted. Cholesterol crystals have been linked to the activation of the NLRP3 inflammasome, which initiates the inflammatory cascade that contributes to atherosclerosis[13]. Colchicine has been shown to inhibit the activation of the NLRP3 inflammasome by cholesterol crystals. It is intriguing to speculate that the observed benefit in animals with higher cholesterol levels could be due to greater inhibition of the inflammatory pathway by colchicine.

The cholesterol-enriched diet raised total plasma cholesterol and LDL in the treatment groups regarding the rabbits' lipid profile. On the other hand, triglyceride levels decreased statistically significantly in the colchicine and fenofibrate group after the experiment.

In apoE-deficient mice, fibrate-mediated activation of PPARα reduces atherogenesis[23,24]. Fenofibrate, in particular, has anti-atherosclerotic properties beyond its lipid-lowering action[23,24]. PPARα agonists exert anti-inflammatory and antithrombotic effects by directly acting on vascular cells, which may account for these anti-atherosclerotic actions that are not mediated by systemic lipid alterations[23,24]. In endothelial cells and macrophages, PPARα agonists have been found to suppress smooth muscle cell production of IL-6 and reduce cytokine-induced genes, such as vascular cell adhesion molecule 1, matrix metalloproteinase-9 (MMP-9), and tissue factor[23].

Even though the atherogenic function of triglycerides is well established, the mechanism by which colchicine reduces triglyceride levels is unknown[46-50]. Colchicine may function similarly to niacin by increasing apolipoprotein B (apoB) breakdown and decreasing VLDL production. Alternatively, it could act as an antagonist of the bile acid receptor farnesoid X receptor (FXR), mimicking guggulsterone's lipid-lowering action[51,52]. The ethyl ester of eicosapentaenoic acid has been shown to decrease TG levels *via* decreasing apoB and lipoprotein-associated phospholipase A2 levels[53]. Additionally, as King et al[54] demonstrated using a small-molecule inhibitor of DGAT 1, colchicine may block acyl CoA/diacylglycerol acyltransferase (DGAT) 1.

Colchicine had no adverse effect on liver function. Serum liver enzymes SGOT, SGPT, γGT, and ALP did not vary significantly across the groups. Additionally, there were no significant variations in creatinine levels across the groups.

Askari et al[21] showed that oral NAC supplementation decreases blood CRP and IL-6 levels. NAC inhibits NF-κB, a transcription factor required to produce genes implicated in oxidative stress and inflammation, including TNF-α, IL-1, and IL-6[21,22]. NAC inhibits TNF-α activation of NF-κB by inducing structural changes in the TNF-α receptor, thus decreasing the receptor's affinity for this biomarker[21,22]. NAC also promotes the production of glutathione, which functions as a nucleophilic scavenger and antioxidant in the presence of oxidative tissue damage[21,22]. Oxidative stress changes the lymphocyte balance and

stimulates the production of pro-inflammatory biomarkers, such as TNF-, IL-6, and IL-1[21,22].

Despite the injectable version of NAC having a higher anti-inflammatory impact, we selected oral supplementation for our experiment[21]. The intravenous injection of NAC has been associated with serious consequences, such as difficulty breathing, heart and circulation problems, rash, nausea, and vomiting[21]. Thus, notwithstanding certain clinical circumstances, it is recommended that NAC be given orally[21]. Although oral NAC may induce moderate nausea, vomiting, electrolyte abnormalities, and diarrhea, these problems have been limited to high doses of NAC, and as a result, we adjusted the dosage to that end[21]. Additionally, oral NAC is more tolerable due to its classification as a nutraceutical[21].

Colchicine is an anti-inflammatory medication used to treat gout, pericarditis, and familial Mediterranean fever[10]. Colchicine suppresses the inflammasome NLRP3 activation in neutrophils *via* binding to microtubules involved in the spatial organization of mitochondria[10,19]. As a result, colchicine inhibits the generation of inflammatory cytokines[10,19]. Specifically, colchicine's impact on tubulin influences the assembly of the inflammasome and the production of IL-1 and IL-18 by macrophages[16].

Recently, several researchers[10,55-57] tried to associate the effect of colchicine on the levels of IL-18 and the stability of atherosclerotic plaques. IL-18, as a member of the IL-1 family, is regulated by the activation of the NLRP3 inflammasome. It has been proven to play a pro-inflammatory role in the pathogenesis of atherosclerosis[58-61]. Raised serum levels of IL-18 have been associated with increased intima thickness and plaque instability, while administration of IL-18 binding protein in apoE deficient mice reduced the development of atherosclerotic lesions[55-57]. Martinez et al[18] suggested that short-term colchicine treatment reduces local production of IL-18 in ACS patients. The LoDoCo trial supports the fact that colchicine prevents plaques from rupture[17]. The PRIME study also showed that IL-18 might be a biomarker for future coronary events in healthy subjects[62].

Inflammation is a modifiable risk factor[12,14,15]. Blocking particular inflammatory pathways reduces the future risk of cardiovascular events in patients with a high residual inflammatory risk[12,14,15]. Canakinumab, a monoclonal antibody to human IL-1, has little effect on lipid levels or platelet function[12,14,15]. Colchicine was recently demonstrated to reduce future cardiovascular events in stable and unstable coronary artery disease patients by inhibiting the NLRP3 inflammasome[16]. Colchicine is a less expensive drug than canakinumab, with a good safety profile and a low incidence of adverse events, including gastrointestinal symptoms[16]. Within 24-72 hours, it accumulates in granulocytes and monocytes and shows anti-tubulin activity, affecting neutrophil function, as well as a modest impact on the NLRP3 inflammasome[16].

CRP is a downstream hepatic acute-phase reactant that is unlikely to affect atherothrombosis, as shown by the CANTOS trial directly[12,14]. In contrast, IL-6, the main cytokine responsible for hepatic CRP generation, is located at a critical junction in a well-established route connecting inflammation to vascular events[12,14]. Mendelian randomization studies[12,14] have shown an association between a polymorphism in the IL-6 signaling pathway and high sensitivity CRP levels and vascular risk. The CANTOS trial demonstrated that the degree of anti-cytokine benefit in atherothrombosis is directly proportional to the number of inflammation reduced[12,14]. As a result, IL-6 is a significant target for future cardiovascular clinical trials[12,14]. From a diagnostic standpoint, since IL-6 is directly involved in a major causative pathway, its measurement in current clinical practice may provide a higher signal-to-noise ratio than CRP. However, translational evidence comparing the predictive usefulness of IL-6, CRP, and LDL is limited. Additionally, there is a dearth of comparable data among secondary prevention patients using statins, a lipid-lowering treatment with concurrent anti-inflammatory effects[12,14].

The Cardiovascular Inflammation Reduction Trial (CIRT) of 4168 North American atherosclerosis patients showed that LDL, IL-6, and the downstream inflammatory biomarker high sensitivity CRP all continue to predict high cardiovascular risk. The results were consistent despite aggressive contemporary care, including statin therapy, angiotensin-converting enzyme inhibitors, beta-blockers, antithrombotic therapy, and high rates of coronary revascularization[14]. Furthermore, these recent data demonstrated that patients with treated atherosclerosis who retain residual cholesterol and residual inflammatory risk have a high event rate. Thereby, these patients may benefit from additional interventions targeting hyperlipidemia and inflammation, the two critical pathways that drive the development and progression of atherosclerotic cardiovascular disease[14]. In contrast to previous findings on canakinumab and colchicine, randomization to low-dose methotrexate in CIRT did not reduce vascular event rates or changes in IL-6,

high sensitivity CRP, or LDL levels[14]. As a result, these findings provide credence to the emerging hypothesis that combination treatments, which go beyond statins to include further LDL and innate immune activity reductions, may be beneficial in treating atherothrombosis[14].

Combination treatments addressing both pathways have therapeutic benefits that are more than additive, potentially lowering relative risks by 40% or more[14]. Such combination treatments may be aimed at individuals with elevated levels of inflammation and LDL following statin medication, specifically, a subgroup with an abnormally high residual risk of cardiovascular events and all-cause mortality.

Our study simultaneously addresses aggressive lipid-lowering and inflammation inhibiting therapies. The former targets LDL production directly, and the latter targets IL-1, downstream IL-6, or the upstream NLRP3 inflammasome. Additional research with a larger sample size is required to establish the connection between colchicine combination treatment, serum IL-6 levels, and intima thickness.

Study Limitations

Our study has some limitations. The reaction of rabbits to the atherogenic diet varied significantly in this animal model. Given that dyslipidemia is the primary pro-atherosclerotic component in this model, animals with low cholesterol levels may have shown considerably lower atherosclerosis activity and, therefore, a decreased response to drugs.

Colchicine is linked with gastrointestinal adverse effects that impair medication tolerance and absorption when taken orally[16]. As a result, in order to maintain a balance between animal comfort, administration tolerance, a high but not deadly dosage of colchicine, and our laboratory's work schedule, we powdered the drugs for oral administration. Additional research should be conducted to determine the most beneficial dosage and route of administration.

Conclusions

In an experimental rabbit model, it appears that colchicine statistically significantly reduces the development of atherosclerosis of the aorta, especially in combination with NAC. Colchicine as an NLRP3 inflammasome inhibitor and NAC as an agent that directly targets IL-6 signaling can reduce the residual inflammatory risk. Fenofibrate enhances the attenuating role of colchicine on triglyceride levels.

Combination therapy for atherosclerosis may be adequate to address the dual pathways leading to cardiovascular disease and residual risk. Our effective lipid-lowering and anti-inflammatory experimental investigation suggests that future combination treatments using both routes may enhance patient care. Clinical studies might assess whether the combination treatment of colchicine with fenofibrate or NAC has similar effects in humans.

Financial Disclosures

The research work was supported by the Hellenic Foundation for Research and Innovation (HFRI) under the HFRI PhD Fellowship grant (Fellowship Number: 347).

Conflict of Interests

The authors declare that they have no conflict of interest.

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