Effect of a Cholesterol Rich Diet, Recurrent Infection and Possible Treatment Modalities on the Pulmonary Vascular System: An Experimental Study

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ABSTRACT

Objective: *Infection may lead to inflammation, atherosclerosis and thrombotic vascular events. The atherosclerotic effect of hypercholesterolaemia on the vascular system is well-known. However, limited studies were done on the therapeutic and preventative agents. The aim of this study was to investigate the effects of infection and cholesterol rich diet combined with an antibiotic, anti-inflammatory agent and red wine on the pulmonary vascular system.*

Methods: *Fifty-nine rats were evaluated. Six groups were created: Control-Group I (n = 10); infection – Group II (n = 9), infection-cholesterol rich diet – Group III (n = 12), infection-cholesterol rich diet-cefepime – Group IV (n = 11); infection-cholesterol rich diet-diclofenac potassium – Group V (n = 9); infection-cholesterol rich diet and red wine – Group VI (n = 8). Blood samples of rats were collected for cholesterol analysis every month. Sections of central pulmonary arteries were examined for thickness of the intima and medial wall by computerised image analysis.*

Results: *There was a statistically significant difference in serum cholesterol levels and in thickness of the intima between the groups (*p = *0.000). The rest of the groups had more intimal thickening than Group I (*p = *0.000). Group III had thicker intima than Groups IV and V (*p = *0.009,* p = *0.011 respectively). There was no significant difference between the groups in thickness of media (*p = *0.432).*

Conclusion: *Infection and cholesterol rich diet have a synergistic effect on atherosclerosis in pulmonary arteries. However, antibiotics and anti-inflammatory agents could be useful in prevention.*

Key Words: Atherosclerosis, cefepime, diclofenac potassium, pulmonary artery, red wine

Efecto de la Dieta Rica en Colesterol, la Infección Recurrente, y las Posibles Modalidades de Tratamiento Sobre el Sistema Vascular Pulmonar: un Estudio Experimental

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RESUMEN

Objetivo: *La infección puede conducir a inflamación, ateroesclerosis y eventos vasculares trombóticos. El efecto aterosclerótico de la hipercolesterolemia en el sistema vascular es bien conocido. Sin embargo, se hicieron estudios limitados sobre los agentes preventivos y terapéuticos. El objetivo de este estudio fue investigar los efectos de la infección y la dieta rica en colesterol, combinados con agentes antibióticos, anti-inflamatorios, y vino tinto, sobre el sistema vascular pulmonar.*

Métodos: *Cincuenta y nueve ratas fueron evaluadas. Se hicieron seis grupos: grupo-control I (n = 10), grupo-infección II (n = 9), grupo infección-dieta rica en colesterol III (n = 12), grupo-infección-dieta rica en colesterol-cefepima IV (n = 11), grupo-infección-dieta rica en colesterol-diclofenaco potásico V (n = 9), grupo-infección-dieta rica en –vino tinto VI (n = 8). Se tomaron muestras de sangre de ratas para analizar el colesterol cada mes. Se examinaron secciones de las arterias pulmonares centrales para determinar el grosor de la pared íntima y media mediante análisis computarizado de imágenes.*

Resultados: *Hubo una diferencia estadísticamente significativa en los niveles de colesterol en suero y el grosor de la íntima entre los grupos (*p = *0.000). El resto de los grupos tenía más engrosamiento de*

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*la íntima que el grupo I (*p = *0.000). El grupo III tenía una íntima más gruesa que los grupos IV y V (*p = *0,009, p = 0.011 respectivamente). No hubo ninguna diferencia significativa entre los grupos en cuanto al espesor de la media (*p = *0.432).*

Conclusión: *La infección y la dieta rica en colesterol tienen un efecto sinérgico sobre la aterosclerosis en las arterias pulmonares. Sin embargo, los antibióticos y los agentes antiinflamatorios podrían ser útiles para la prevención.*

Palabras claves: Aterosclerosis, cefepima, diclofenaco potásico, arteria pulmonar, vino tinto.

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INTRODUCTION

Several studies have been done on the potential role of infection in the development of atherosclerosis. Most of the studies have focussed on sero-epidemiology which demonstrates an association between atherosclerosis and infection with a specific pathogen, or on the "pro-atherosclerotic" effects of pathogens that directly infect the vessel wall. However, it seems that the association between infection and atherosclerosis is more complex than the hypothesis of the direct infection of the vessel wall by a single pathogen.

In a recent study (1), it was shown that cytomegalovirus infection increased the neo-intimal response of the vessel wall in the absence of direct infection. The vascular effects could be mediated by some humoral mechanism, such as infection-induced circulating cytokines or on autoimmune response. The autoimmune response could be stimulated by the antigens of pathogens, if they contain the peptides similar to those present in host proteins, so the host tissues containing the cross-reacting peptides are attacked.

Studies on the role of infection in atherosclerosis have identified several candidate pathogens such as cytomegalovirus, hepatitis A virus, herpes simplex virus 1 and 2 and *chlamydia pneumonia* (2). The role of multiple pathogens in atherogenesis is also suggested by Wick *et al.* The presence of chronic respiratory, urinary tract, dental and other infections increased the risk of atherosclerosis development (3).

It is well known that recurrent lung infections increase mortality and morbidity by affecting the pulmonary parenchyma and vascular system. Although many pathogens play a role in pulmonary infections, *Pseudomonas aeruginosa* is a commonly encountered agent particularly in patients with cystic fibrosis or bronchiectasis. Recurrent pulmonary infections can lead to the development of pulmonary hypertension directly or indirectly. Pulmonary arteries are resistant to atherosclerosis in the absence of pulmonary hypertension (4), however, in experimental studies, the development of atherosclerosis was shown even in the absence of pulmonary hypertension (5, 6).

The atherosclerotic effect of hypercholesterolaemia on the vascular system has been shown in many experimental studies (7). A previous study found high-grade vascular occlusive lesions in the coronary artery and aorta (8) but little is known about changes in the lung. The aim of this study was to investigate the effects of infection and/or cholesterol rich diet combined with therapeutic and preventive agents such as antibiotic, anti-inflammatory agents and red wine on the pulmonary vascular system.

SUBJECTS AND METHODS

Pseudomonas aeruginosa strain ATCC 1942 which has a stable mucoid phenotype was used as infectious agent. The bacteria were grown at 37°C for 18–24 hours on sheep blood agar and then scraped and resuspended in sterile saline to a density of 5·McFarland standard (OD550 1.25; equivalent to 1.5 x10^9 CFU/mL). The viable count was confirmed by plating serial dilutions on sheep blood agar and counting colonies.

The study was started with 72 Wistar rats (three months-old and pathogen free) which were randomly divided into six groups. However, during the experiment, 13 rats died and the final groups were as follows:

Group I: Control rats which were fed by regular rat chow (n = 10)
Group II: Infected rats which were fed by regular rat chow (n = 9)
Group III: Infected rats fed by cholesterol rich diet (n = 12)
Group IV: Infected rats fed by cholesterol rich diet and treated by cefepime (n = 11)
Group V: Infected rats fed by cholesterol rich diet and treated by diclofenac-potassium (n = 9)
Group VI: Infected rats fed by cholesterol rich diet and red wine (n = 8).

Cholesterol rich diet was obtained by 1% cholesterol supplemented rat chow (Sigma, St Louis, MO63178, USA). The rats in groups II, III, IV, V and VI were infected by intratracheal injection of 0.1 mL (1.5 x 10^9 CFU/ml) of *P aeruginosa* suspension under anaesthesia. The trachea of each rat was surgically explored using titrated intramuscular doses of ketamine hydrochloride (30−100 mg/kg) and xylazine hydrochloride (10−15 mg/kg). The rats in the control group (Group I) were given 0.1 mL saline by intratracheal route. This procedure was repeated six times at four-week intervals. As therapeutic agents, Cefepime was used at 60 mg/kg every eight hours for 2.5 days and therapy was started three hours after bacterial inoculation in Group IV and diclofenac

potassium was used daily as a single 0.5 mg/kg oral dose in Group V. Red wine was added to chow at 5 ml/kg/day. When all rats were anaesthetized, a blood sample was collected from the tail (0.5 mL) for cholesterol analysis every month.

Animal care and processing were performed under strict adherence to the Institutional Animal Care and Use Committee guidelines. Six months after the initial inoculation, all rats were sacrificed to evaluate the pulmonary arteries.

The left and right lungs of rats were removed from the thoracic cavity and histological specimens were fixed overnight by instillation of 10% buffered formalin into the airways and vascular structures. Then, representative cross-sections of the lungs which include the peripheral and the central pulmonary arteries were sampled and embedded in paraffin blocks. Subsequently, 5-µm-thick serial sections were prepared and stained with haematoxylin and eosin for the assessment of vascular morphology. Two experienced pathologists independently reviewed all the samples in a blinded, random fashion from Akdeniz University School of Medicine, Department of Pathology. The areas of the media and the neo-intima of pulmonary arteries in the parenchyma were assessed by computerised image analysis (Samba 2000, Gateway, GP7-450, GW-2K, Ireland). To demonstrate the changes due to atherosclerosis, three slides with maximal pulmonary artery thickness were determined and measured. The average of three measurements for each rat was used in the statistical analysis.

Statistical analyses and graphs were performed using the Statistical Software Package for the Social Sciences, (SPSS Inc, Chicago, IL, USA) version 13.0 for Windows. Serum total cholesterol levels were expressed as mean ± SD. The average of intima measurements, the intima/media ratio and serum total cholesterol levels were evaluated by variance analysis and the *p*-value of 0.05 was considered as significant.

RESULTS

The average of total serum cholesterol levels of the rats in Group I, II, III, IV, V and VI were 72.70 ± 11.63 mmol/L, 75.62 ± 3.52 mmol/L, 90.25 ± 11.28 mmol/L, 75.73 ± 8.68 mmol/L, 111,55 ± 15.92 mmol/L and 81.87 ± 10.11 mmol/L respectively. There was a statistically significant difference in serum cholesterol levels between the groups ($p = 0.000$).

On microscopic examination, Groups I and II exhibited mainly normal pulmonary artery wall structure compared to Groups III, IV, V and VI which included the infected rats fed by cholesterol rich diet. Typical atherosclerotic lesions characterized by the presence of fatty streaks, various proportions of foamy cells, smooth muscle cells and extracellular matrix were determined in pulmonary arteries of Groups III, IV, V and VI (Fig. 1).

The average of intima measurements and the intima/media ratio are given in Table 1. Comparing the thickness of the intima, a significant difference was found between the groups ($p = 0.000$). Groups II, III, IV, V and VI had more intimal thickening than the Group I which was used as control group ($p = 0.000$). Group III which was fed by cholesterol rich diet but did not have any treatment modalities, had thicker intima than Groups IV and V ($p = 0.009$, $p = 0.011$ respectively). There was no statistically significant difference between Groups III and VI ($p = 0.458$).

Significant differences were also shown between the groups by comparing the intima/media ratio ($p = 0.000$). Group III had higher intima/media ratio than Groups IV, V and VI ($p = 0.004$, $p = 0.048$ and $p = 0.05$ respectively). There was no significant difference between the groups in thickness of media ($p = 0.432$).

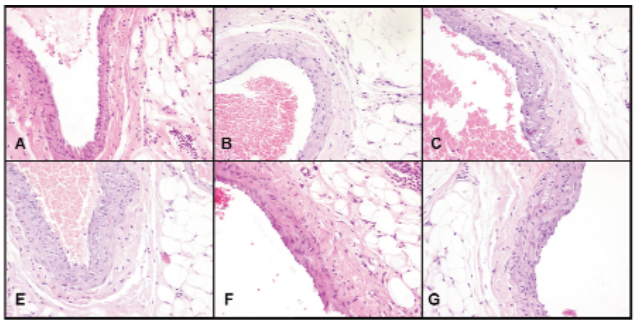

Fig. 1: The sections of pulmonary arteries were shown for **(A)** Group I, **(B)** Group II, **(C)** Group III, **(D)** Group IV, **(E)** Group V and **(F)** Group VI, H&E x 200. Note the typical atherosclerotic lesions characterized by the presence of fatty streaks, various proportions of foamy cells, smooth muscle cells and extracellular matrix in pulmonary arteries of Group III, IV, V and VI.

Table 1: The average of intima measurements and the intima/media ratio were given.

	Intima (µm)	intima/ media ratio
Group I	20.12 ± 7.73	0.34 ± 0.09
Group II	60.93 ± 19.60	1.13 ± 0.43
Group III	68.15 ± 11.04	1.25 ± 0.16
Group IV	46.71 ± 6.81	0.89 ± 0.12
Group V	45.81 ± 19.63	0.96 ± 0.19
Group VI	55.58 ± 18.64	0.96 ± 0.20

DISCUSSION

The initiating factor for atherosclerosis is intimal thickening of the artery and the vascular endothelium plays a central role in the regulation of vascular homeostasis by producing anti-atherogenic factors (9). Endothelial dysfunction contributes to the pathogenesis of atherosclerosis by increasing procoagulant activity, production of cytokines, leukocyte adhesion and decrease in intrinsic fibrinolysis (10). Besides, hyperlipidaemia and hypertension increase, respectively, the amount and rate of lipid transport across the arterial wall and the accumulation of cholesterol in the *tunica intima* promotes proliferation of connective tissues, sclerosis and intimal

thickening in the arteries. Haemorrhage into the vascularised and thickened *tunica intima* provokes further organization and thickening. Mechanical strains on the arterial wall are exaggerated by hypertension and by inducing reparative changes, contribute to intimal thickening (11).

Atherosclerosis is a disease of the large and medium arteries, but later, smaller arteries get involved. Pulmonary artery atherosclerosis is accelerated in patients with atherosclerosis of the systemic arteries and the pathologic lesions associated with hypertensive pulmonary vascular disease (12). Also, the experimental studies have shown that atherosclerotic lesions develop in the pulmonary arteries in animals fed on a cholesterol-rich diet or with spontaneous hypercholesterolaemia (13−16). In the present study, we demonstrated intimal thickening of the pulmonary arteries which was the main sign of atherosclerosis in rats fed with cholesterol-rich diet.

The validity of the hypothesis that infection contributes to atherosclerosis has not been definitively established. Infectious agents may lead to inflammation, atherosclerosis and thrombotic vascular events directly by causing destruction of the arterial wall or endothelial dysfunction (17). On the other hand, the evidence is accumulating that autoimmune responses, perhaps triggered by infection, may be one of the mechanisms contributing to atherogenesis and could explain the indirect effect of infection (18, 19). Besides, some recent studies showed that infection accelerates development of atherosclerosis, but treatment after infectious exposure prevents accelerated development of atherosclerosis. Anderson *et al* have shown that chlamydia pneumonia infection can accelerate atherosclerosis and this can be prevented by treatment with azithromycin in a rabbit model (20). In our study, we showed that if the rats which were infected and fed by cholesterol rich diet got the treatment for infection such as antibiotic and anti-inflammatory agents, they had lower measurements of pulmonary artery wall thickness compared to non-treated rats and these results are consistent with the literature (21).

Red wine has been considered to be protective against atherosclerotic coronary heart disease development, an oxidative stress associated disease. Wine contains polyphenols displaying antioxidant properties and due to this, a general consensus exists, both among the general public and the scientific community, that red wine is an antioxidant beverage. Besides, resveratrol, a polyphenolic constituent of red wine, is known for its anti-atherogenic properties and is thought to be beneficial in reducing the incidence of cardiovascular diseases (22). In a recent study, it was shown that resveratrol appears to be a natural antioxidant that enhances cholesterol efflux, therefore it is a potential natural antioxidant that could be used to prevent and treat cardiovascular diseases (23). In this study, we found a significant difference in the intima/media ratio of pulmonary arteries between the Group III, which included the rats infected and fed by cholesterol rich diet, and Group VI, which included the rats getting the red wine additionally.

In conclusion, infection and a cholesterol rich diet have a synergistic effect on atherosclerosis in pulmonary arteries. However, antibiotics and anti-inflammatory agents could be useful in prevention. Although, a variety of clinical trials utilizing antibiotic and anti-inflammatory agents for the secondary prevention of atherosclerosis have been performed and several pilot studies have shown significant positive clinical effects, thus far, no large randomized trial has confirmed those findings. Therefore, our results suggest an approach to the treatment of atherosclerosis, but still further studies are needed for clinical use.

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