Role of Calcifying Nanoparticle in the Development of Hyperplasia and Vascular Calcification in an Animal Model

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WHAT THIS PAPER ADDS

Vascular calcification is recognized as an independent risk factor that aggravates the prognosis of atherosclerosis. Some aspects of the cause have been elucidated, but others are unclear. Calcifying nanoparticles (NPs) have been detected recently in calcified arterial specimens and are involved in the process of calcification. This study confirms the ability of human-derived calcifying NPs to accelerate hyperplasia and stimulate calcification in previously damaged areas, and opens new lines of research for the prevention and treatment of arterial calcification.

Objective: Calcifying nanoparticles (NPs) have been detected recently in calcified human arterial specimens and are involved in the process of calcification. This study was designed to test the hypothesis that human-derived NPs could worsen the response to arterial endothelial injury and induce vascular calcification.

Methods: The right carotid artery of 24 New Zealand rabbits was injured with an angioplasty balloon. Animals were perfused intravenously with saline (100 mL) during the experiment and divided into three groups: group-A, control; group-B, exposed to NPs (2 mL) obtained from calcified aortic valves; and group-C, exposed to NPs (2 mL) and treated postoperatively with atorvastatin (2.5 mg/kg/24 h). At 30 days, both carotid arteries were removed and examined histologically. Blood measurements were monitored during the study.

Results: The intimal hyperplasia area was significantly larger in the injured right carotid artery compared with the left unoperated carotid artery in all groups. There was no significant variation in medial area between groups. Morphometrically, the intima/media ratio (IMR) was significantly higher in damaged carotids compared with controls. A significant increase of IMR was found in group-B (1.81 ± 0.41) compared with group-A (0.38 ± 0.59; $p = .004$) or group-C (0.89 ± 0.79; $p = .035$). Differences between groups C and A were not significant ($p = .064$). Calcifications were observed in six animals, all of which had been exposed to NPs (4 in group-B, 2 in group-C, $p = .027$). Plasma levels of cholesterol and triglycerides remained stable.

Conclusions: This research confirms the ability of systemic inoculation of human-derived NPs to accelerate hyperplasia and stimulate calcification in localized areas of arteries previously submitted to endothelial damage, while it was harmless in healthy arteries. Atorvastatin was demonstrated to slow down this process.

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INTRODUCTION

Vascular calcification is recognized as an active process associated with inflammation that is an independent risk factor for cardiovascular morbidity and mortality.[1,2] Some aspects of the cause of arterial calcification have been elucidated (e.g. mechanical forces, dysregulation of mineral metabolism, etc.), but it is unclear why some patients suffer from this disorder and others do not. Nanometer scale bacteria-like organisms (nanobacteria) have been detected recently in calcified human arteries and cardiac valves, and are involved in the process of calcification.[3–5]

The term 'nanobacteria' was first used and patented by Kajander and Cifcioglu as the name for very small bacteria-like organisms discovered in a cell culture.[6,7] This new entity is self-replicating, 0.08–0.5 μm in size, and has the capacity to form calcium phosphate minerals under subsaturation levels of calcium and/or phosphate. For this reason, they have been identified as an infectious cause of pathological calcification.[8,9]

At the moment, there is not enough proof that nanobacteria are living organisms, so they are named calcifying

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nanoparticles.[9–11] However, there is evidence from previous experimental studies that seem to confirm a relationship between nanoparticles and the response to arterial damage, including arterial calcification.[12]

This study was designed to test the hypothesis that human-derived nanoparticles (NPs) could worsen the response to arterial endothelium injury and induce vascular calcification in an animal model. As a secondary aim, based on the pleiotropic activity of statins (mainly related to inflammation and atherogenesis[13,14]), the therapeutic effect of atorvastatin in the prevention of such lesions was analyzed.

MATERIALS AND METHODS

NP culture and collection

Human NPs were obtained from calcified aortic valves. Calcified valves were harvested from patients undergoing valve replacement for symptomatic severe aortic stenosis, following the principles outlined in the Declaration of Helsinki. The valves were demineralized, filtered, and cultured according to Bratos-Pérez et al.[5] After 6–8 weeks of culture, the cells were re-suspended with the help of sterile glass pearls and re-inoculated in new Roux flasks in DMEM (Dulbecco's Modified Eagle Medium) supplemented with gamma-irradiated fetal bovine serum at 37 °C, under an atmosphere with 5–10% CO_2. A whitish precipitate appeared at the bottom of the positive test tubes. These strains have been maintained in the laboratory since then, by way of subcultures in the same medium but without fetal bovine serum every 8 weeks. In the successive subcultures, the deposit appeared increasingly quickly and in larger quantities. The sediment was prepared for observation under transmission electron microscopy (TEM) and scanning electron microscopy (SEM), as explained in Bratos-Pérez et al.[5] The observation with TEM showed pleomorphic particles with a size of 0.2–0.5 μm. Observation with SEM showed spherical particles grouped in clusters (Fig. 1).

Once the presence of NPs was confirmed, positive subcultures in Roux flasks were re-suspended with the help of sterile glass pearls, centrifuged, and washed with distilled water. The resultant pellet was washed three times with sterile phosphate-buffered saline (PBS) before preparation for injection into animals. Finally, this solution was re-suspended in 2 mL 0.9% NaCl. This was the final dose used for animal inoculation.

Animals

Twenty-four male New Zealand white rabbits (weighing 2.5–3.5 kg) were used for this study. Animals were fed with a LabDiet rabbit chow (0.25% cholesterol) and water *ad libitum*, and were maintained in individual cages (12 h light/12 h dark cycle). All experiments were conducted in compliance with the 'Principles of Laboratory Animals Care' formulated by the European Council (Directive 2010/63/EU) and approved by the Animal Care and Bioethical Committee of the Medical School of the Valladolid University (Spain).

Animals were divided into three groups: group-A (control), in which 100 mL of isotonic saline solution was perfused during the experiment; group-B (treated), in which a solution of 2 mL NPs was added to 100 mL saline solution; and group-C (therapeutic), in which the animals perfused with a solution of NPs, as in group-B, were treated in the postoperative period with atorvastatin (2.5 mg/kg/24 h).

Surgical procedure

Animals were sedated intramuscularly using a mixture of ketamine (35 mg/kg), xylazine (5 mg/kg), and acepromazine (1 mg/kg). To avoid interferences with the NP solution, antibiotic prophylaxis was not administered. The animals were immobilized in supine position on the operating table. The fur in the neck was clipped with electric shears, and the skin was prepared with iodine solution. After local infiltration of mepivacaine hydrochloride (10 mg/kg), a right laterocervical incision was performed to allow identification of the cervical vessels. The jugular vein was cannulated with 20-gauge Abocath (BD Insyte, BD Vialon Material, Sandy, Utah, USA). A venous blood sample from the vein was obtained (2 mL), and an intravenous infusion of isotonic

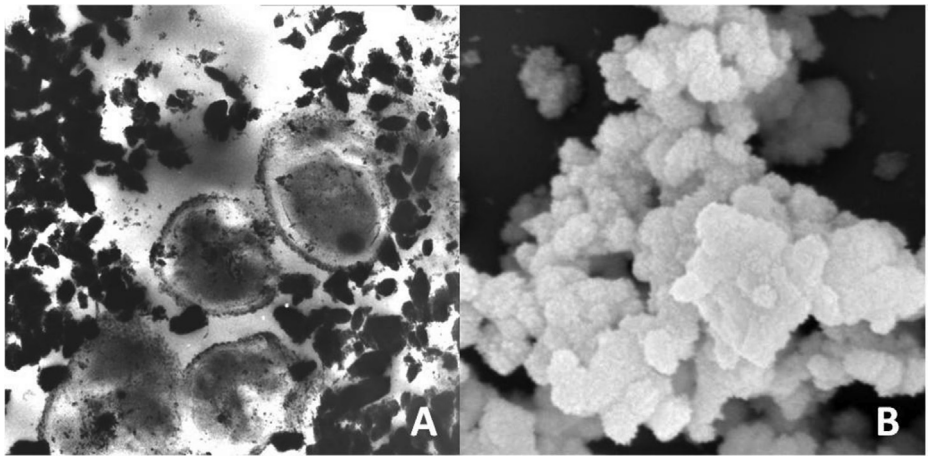

Figure 1. (A) Transmission electron microscopy (TEM) showing pleomorphic particles with a size of 0.2–0.5 μm. (B) Scanning electron microscopy (SEM) showing spherical particles grouped in clusters.