Planimetric and histological study of the aortae in atherosclerotic chickens treated with nifedipine, verapamil and diltiazem


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Summary. Calcium appears to be involved in many of the cellular events which are thought to be important in atherogenesis. Calcium channel blockers have been shown to reduce arterial lipid accumulation in animals without altering serum cholesterol. Avian models of atherosclerosis offer economic and technical advantages over mammalian models.

In this study, we examine the effects of nifedipine, verapamil and diltiazem at clinical and higher doses, on the extent of atherosclerosis of egg-fed chickens. In order to assess the extent of atherosclerosis quantitatively, the aortic lesions of the thoracic and abdominal aorta, aortic arch and supravalvular regions were measured by planimetry. Atherosclerotic lesions were evaluated histologically. Statistically significant reductions in the lipid deposition of the aorta were found in all the treated groups. The extent and distribution of atherosclerotic lesions were decreased in a significant way by verapamil, nifedipine and diltiazem. The higher the dosage used, the higher the regression of the atherosclerotic lesions. At clinical dosage, nifedipine showed the highest decrease of the lesions. In addition, the chicken atherosclerosis model has proved itself useful and very suitable for in vivo drug intervention studies.

Key words: Atherosclerosis, Nifedipine, Verapamil, Diltiazem, Chicken

Introduction

Atherosclerosis and its consequences continue to be the major cause of death in Europe and the United States (Sans et al., 1997; Kesteloot et al., 2002). It appears to develop over much of the lifetime of a patient, and is related to well-known risk factors, especially heredity, hyperlipidemia, hypertension, cigarette smoking and diabetes mellitus (Peeters et al., 2002).

Animals have been used as experimental models in atherosclerosis-related research since the turn of the past century (Narayanaswamy et al., 2000). Avian models of atherosclerosis helped pioneer the study of vascular biology, and offer economic and technical advantages over mammalian models (Wang et al., 1999). The egg-fed chicken is a good animal model for the study of atherosclerosis research (Siller, 1961; Gosling et al., 1969; Valdés, 1976; García Pérez et al., 2002). The chicken is small and suitable for prolonged laboratory investigation; able to develop spontaneous atherosclerosis; capable of producing atherosclerosis after cholesterol feeding with elevated hypercholesterolemia and there is no essential difference between vascular lesions seen in chickens as a result of cholesterol diet and that of atherosclerosis observed in man (Wong, 1975). The rabbit, also free of spontaneous atherosclerosis, is extremely sensitive to lipid-rich diets, but the lesions induced resemble more a xanthomatosis than an atherosclerosis (Hadjiiisky et al., 1991). The monkey and pig, which are phylogenetically close to man, develop spontaneous atherosclerosis exacerbated by lipid-rich diets or other procedures, but the cost and problems of upkeep make these two models inaccessible to most laboratories (Hadjiiisky et al., 1991). The utility of transgenic mouse models of atherosclerosis, such as the apolipoprotein E (apoE)-deficient mouse and the low density lipoprotein (LDL) receptor-negative mouse, for the evaluation of antiatherosclerotic agents has yet to be
determined. Paradoxical observations raise an important issue relating to interpretation of the results of drug intervention studies in genetically-derived mouse models (Bocan, 1998).

Calcium appears to be involved in many of the cellular events, which are thought to be important in atherogenesis (Rouleau et al., 1983; Catapano, 1997; Miller, 2001; Pepine and Handberg, 2001). Compounds which decrease intracellular calcium by reducing calcium influx have been shown to suppress atherogenesis in experimental atherosclerosis (Rouleau et al. 1983, Ginsburg et al. 1983; Catapano, 1997). Hypocalcemic agents, calcium channel blockers and other calcium antagonists have been shown to reduce arterial lipid accumulation in several animals without altering serum cholesterol, blood pressure or heart rate (Kramsch et al. 1980). Calcium antagonists have protective effects against ischaemia and antiatherogenic potential (Zannad, 2000), and may have beneficial effects on the development of atherosclerosis, such as the inhibition of vascular smooth muscle cell proliferation and migration, reduction of calcium influx into the vascular wall, reduction of extracellular matrix synthesis, promotion of uptake and breakdown of low-density lipoproteins, protection of lipoproteins from oxidative modification, maintenance of endothelial cell function, inhibition of platelet activation and reduction of blood pressure (Schachter, 1997). They also appear to have antioxidant effects in addition to their potent vasorelaxant properties (Sobala et al., 2001).

However, there are very few experimental studies comparing different kinds of calcium entry blockers and most of them use much higher doses than the clinical ones (Henry and Bentley, 1981; Ginsburg et al., 1983; Catapano, 1997). Besides, there are no serial studies which focus on the use of calcium entry blockers in avian models of atherosclerosis.

In this work, we examine the effects of three calcium entry blockers (nifedipine, verapamil and diltiazem), at human clinical and higher doses, on the extent of atherosclerosis of chickens fed a high-cholesterol diet. Therefore, new histological and planimetric data concerning reduction in the lipid deposition of the aorta by the use of several calcium channel blockers in chickens are provided in this study.

Materials and methods

Experimental procedure

Eighty male 3-week-old White Leghorn chickens were housed under controlled conditions and randomly assigned to 8 groups and 2 kinds of diet (during the first 3 weeks of life all of them received a standard growing diet). Diet A (normal): a standard grower mash. The weekly amount of it was increased with the age of the animals. Diet B (atherogenic): boiled eggs ad libitum, enriched with vitamins, minerals and fiber. Water was given ad libitum.

Each of the 8 groups consisted of 10 animals which were treated as follows. Group A: normal diet. Group B: atherogenic diet. Group C: atherogenic diet and nifedipine at clinical doses (3 mg/kg/day in two doses). Group D: atherogenic diet and nifedipine at high doses (40 mg/kg/day in two doses). Group E: atherogenic diet and verapamil at clinical doses (4 mg/kg/day in two doses). Group F: atherogenic diet and verapamil at high doses (40 mg/kg/day in two doses). Group G: atherogenic diet and diltiazem at clinical doses (6 mg/kg/day in two doses). Group H: atherogenic diet and diltiazem at high doses (60 mg/kg/day in two doses).

For all experiments, the calcium entry blockers nifedipine, verapamil and diltiazem were prepared weekly. The clinical doses were equivalent to the standard doses of each drug for adult humans. The high doses were 10 times the clinical doses. Animals were weighed weekly, in order to calculate the doses. Water was used for the solution of verapamil and diltiazem; polyethyleneglycol for nifedipine. An ultrasound bath was needed for the complete solution of diltiazem. Nifedipine was protected from light in dark bottles.

Medications (1 ml) were administered (force-fed) daily at 7 a.m. and 7 p.m. for two months (at the end the chickens were 81 days old). Polyethyleneglycol was administered to the control groups as placebo.

Tissue preparation

The chickens were sacrificed after two months of treatment. Traumatic damage to the arterial endothelium and intima was avoided by reducing the duration of the animals’ dissection to less than 15 minutes. The aortae of five animals were removed, cleaned of surrounding tissue, opened and stained for lipid with Sudan III as described by Holman et al. (1958). Briefly, after washing in 70% alcohol for 2 hours, samples were incubated in 0.3% Sudan III in 70% alcohol for 2 days at 37 ºC. Dissected aortas were then washed and the luminal surface of the aortae was photographed by means of a Reprovit (Leitz Wetzlar, Germany) and Gold 100-2-Kodak 5095 film. The atherosclerotic involvement was evaluated in the thoracic and abdominal aortae, aortic arch and supraaortic regions by computerized planimetry (Hewlett-Packard 85-B computer and HP 9111-A digital ultrasonic tracer). The percentage of the sudanophilic area was quantified (sudanophilic area/aortic area).

Samples of five animals of each group were fixed for light and electron microscopy. For light microscopy, samples were fixed in 10% formalin in 0.1M phosphate-buffered saline, pH 7.4 (PBS), for 12 hours. After fixation, they were processed for inclusion in paraffin wax and 5 µm-thick paraffin sections were cut. Sections were stained with haematoxylin and eosin (HE) and Van Giessen stain for elastic tissue. Lesions were graded using a scale of 0 to 3 points, on the basis of similar criteria to Stary et al. (1995), as follows. Type I:
presence of foam cells in the intima layer (0 points); type II: lipid accumulation in the media layer (1 point); type III: extracellular lipids and increase in the elastic layer (2 points); and type IV: lipid core, fibrotic layer and calcium deposits (3 points). Five sections of each studied region were evaluated and quantified following the previous criteria and the mean and standard deviation were calculated. A total parameter of a complete aorta was determined by addition of the mean area of all the regions of the aorta in each experimental group.

Before fixation for light microscopy, small pieces of the atherogenic diet animal (group B) samples were immersed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in alcohol and embedded in epon 812. Ultrathin sections were cut using an Ultracut ultramicrotome and stained with uranyl acetate and lead citrate. Electron microscopy was performed with a Zeiss EM/10cR.

Statistical analysis

Results are expressed as mean ± standard deviation. Statistical significance was evaluated by t test comparisons with the 0.05 confidence level accepted as statistically significant. Differences between groups were evaluated by the Newman-Keuls test.

Results

The percentage of the aortic surface that was lipid-stained in each group is shown in Table 1. A representative aorta from each group is shown in Fig. 1. No Sudan-positive lesions were found in the normal diet-group (A), in contrast with the developed lesions of the atherosclerotic control group (B), which showed an average surface covered by Sudan-stain of 67.2%. Significant differences in the lipid deposition of the total aortic area were also found between group A and the calcium entry blocker-treated groups (C, D, E, F, G and H). Besides, atherosclerotic areas of the treated groups were significantly fewer, statistically, than the atherosclerotic control group (B). The extent and distribution of atherosclerotic lesions, detected by Sudan stain, were not altered in a significant way by the kind of calcium entry blocker or dosage used.

Thoracic aorta atherosclerotic involvement of group B (78.5%) was larger than that of the abdominal aorta (31.1%) and total aorta (67.24%). These differences were also observed in all the treated groups. Statistically significant differences were observed between group A and the other groups, and between group B and the rest of the groups.

Abdominal aorta atherosclerotic involvement was lower, although statistically significant differences were observed for this parameter between group A and groups B, C, D, E and G (no differences with groups F and H), and also between the atherosclerotic control group (B) and the treated groups E, F, G and H.

Light microscopy evaluation demonstrated normal histology of the aorta wall in group A control samples (Fig. 2a), while several grades of lesions were found in the different treated groups. A representative picture of each grade of the lesions is shown in Fig. 2. The quantitative analysis of the samples, following the previously described histological criteria, is summarized in Table 2. Results showed a decrease of the atherogenic lesions in the experimental groups treated with calcium antagonists. An exception was observed in the group at clinical dosages of verapamil, where no significant differences were observed, in reference to group B samples. The decrease of the lesions was dosage dependent, except for the nifedipine groups, where no significant differences were observed between clinical and higher doses (Table 2).

Electron microscopy of group B samples showed type IV lesions. Foam cells, accumulations of lipids in muscular cells and mineral deposits were found (Fig 3).

Table 1. Aortic planimetry in the different groups. Sudan stain (% sudanophilic area/ aorta area, mean±standard deviation).

<table>
<thead>
<tr>
<th>EXPERIMENTAL GROUP</th>
<th>THORACIC AORTA (%)</th>
<th>ABDOMINAL AORTA (%)</th>
<th>TOTAL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.75±0.5</td>
<td>0.5±0.57</td>
<td>0.5±0.57</td>
</tr>
<tr>
<td>B</td>
<td>7.8±0.8</td>
<td>31±6</td>
<td>67±8</td>
</tr>
<tr>
<td>C</td>
<td>58±7</td>
<td>16±4</td>
<td>46±5</td>
</tr>
<tr>
<td>D</td>
<td>54±5</td>
<td>12±2</td>
<td>41±4</td>
</tr>
<tr>
<td>E</td>
<td>57±7</td>
<td>8±2</td>
<td>44±6</td>
</tr>
<tr>
<td>F</td>
<td>59±5</td>
<td>9±2</td>
<td>45±4</td>
</tr>
<tr>
<td>G</td>
<td>48±5</td>
<td>5±1</td>
<td>36±8</td>
</tr>
<tr>
<td>H</td>
<td>52±4</td>
<td>6±2</td>
<td>39±4</td>
</tr>
</tbody>
</table>

Table 2. Quantitative evaluation of the atherosclerotic lesions by means of histological criteria (mean±standard deviation).

<table>
<thead>
<tr>
<th>EXPERIMENTAL GROUP</th>
<th>SUPRAORTIC REGION</th>
<th>AORTIC ARCH</th>
<th>THORACIC AORTA</th>
<th>ABDOMINAL AORTA</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.75±0.25</td>
<td>0.5±0.26</td>
<td>0</td>
<td>0</td>
<td>1.25±0.4</td>
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<tr>
<td>B</td>
<td>2.5±0.26</td>
<td>2.5±0.26</td>
<td>2.25±0.25</td>
<td>1.5±0.5</td>
<td>8.75±0.7</td>
</tr>
<tr>
<td>C</td>
<td>2±0</td>
<td>1.25±0.25</td>
<td>1±0.5</td>
<td>0.75±0.25</td>
<td>5±0.5</td>
</tr>
<tr>
<td>D</td>
<td>1.5±0.5</td>
<td>1±0</td>
<td>0.75±0.25</td>
<td>0</td>
<td>3±0.4</td>
</tr>
<tr>
<td>E</td>
<td>2.5±0.26</td>
<td>1.75±0.22</td>
<td>1.75±0.5</td>
<td>1.5±0.26</td>
<td>7.5±0.5</td>
</tr>
<tr>
<td>F</td>
<td>2.2±0.25</td>
<td>0.75±0.24</td>
<td>0.5±0.5</td>
<td>0.5±0.26</td>
<td>3±0.6</td>
</tr>
<tr>
<td>G</td>
<td>1.2±0.25</td>
<td>1.5±0.26</td>
<td>1.5±0.26</td>
<td>1±0.4</td>
<td>6.25±0.25</td>
</tr>
<tr>
<td>H</td>
<td>1.2±0.25</td>
<td>1.2±0.5</td>
<td>0.75±0.25</td>
<td>0.5±0.2</td>
<td>3.75±0.6</td>
</tr>
</tbody>
</table>
Discussion

Chickens used in this study developed atherosclerosis using an atherogenic diet. Atherogenic lesions were classified into four types by means of histological criteria. The characteristics of the lesions were similar to those described by Stary et al. (1995). We used a simplification of Stary's classification. Type IV lesions were detected in samples of group B. On electron microscopy, accumulations of extracellular lipids, mineral depositions and smooth muscle cells containing lipid droplets were observed. Significant reductions in the lipid deposition of the aorta were found in all the treated groups. The lesions were mainly found in the thoracic aorta. Sudan stain did not detect differences between the dosage used but quantitative

Fig. 1. Sudan-stained aorta of each of the groups (A-H). The dark areas show the atherosclerotic lesions; the pale ones indicate absence of lipid deposits.
histological criteria showed significant differences between clinical and higher dosage. Both dosages significantly decreased the atherosclerosis lesions, but a higher reduction was observed with the higher dosage. No histological differences were observed between the different calcium entry blockers.

Sugano et al. (1988) studied the effects of diltiazem (50 mg, administered by daily intraperitoneal injections) at the end of 10 weeks on the development of atherosclerosis in cholesterol-fed rabbits. They found a reduction of 97% in aortic atheromatous lesions. However, Ginsburg et al. (1983) found only a 37% reduction, using the same drug at a daily oral dosage of 103 mg/kg.

Dihydropyridine calcium antagonists reduce the spread of atherosclerotic lesions in a variable way. Henry and Bentley (1981) tested the effects of orally administered nifedipine (40 mg/day) in rabbits fed a 2% cholesterol diet during 8 weeks, obtaining a 50% reduction in aortic lesions. Willis et al. (1985), in a similar experiment, observed a 69% reduction. Pangiotopoulos and Nayler (1984) used oral nifedipine at a daily dosage of 1 mg/kg, with the same diet and time, obtaining a 26% reduction of sudanophilic aortic

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**Fig. 2.** Haematoxylin-eosin staining. 
**a:** control group, no affection of the aorta wall is observed. 
**b:** type I lesion, slight affection is detected in the intima. 
**c:** type II lesion, note the increase in the accumulation of lipids and structural disorganization begins to be detected. 
**d:** type III lesion. 
**e:** type IV lesion. 
Thickness of the intima and disorganization of the connective tissue is shown. 
**f:** a detail of the accumulations of lipids in the intima is observed (arrows). 
Bar: 
*a-e:* 40 mm; 
*f:* 60 µm.
lesions. Oral administration of nicardipine at dosages of 40 mg/kg twice daily for 8 weeks reduced plaque area by 75% (Willis et al., 1985). Isradipine (0.3 mg/kg/day) reduced aortic lesions by 31% (Henry, 1983).

Verapamil and similar calcium antagonists reduce the spread of atherosclerotic lesions, depending on the route of administration. Blumlein et al. (1985), administered subcutaneous verapamil (1.5 mg/kg/day) to rabbits for 10 weeks, obtaining less severe atherosclerosis in their aortae (reduction of 54%). Rouleau et al. (1983), obtained a 66% reduction in the aortic lesions in cholesterol-fed rabbits treated with oral, (8 mg/kg/day) and parenteral, (0.5 mg/kg/day) verapamil, but the reduction of atherosclerosis was not statistically significant in the treatment with only oral verapamil. Pangiotopoulus and Nyler (1984) reported that verapamil (80 mg/kg/day) induced a 48% reduction in aortic lesions. Sievers et al. (1987) showed a 26% reduction in cholesterol-fed rabbits treated with daily oral verapamil (2 gr/l of water), but no reduction was found in the animals that were treated for several weeks after receiving a high-cholesterol diet. Catapano (1997) reported a 28% reduction in rabbits treated with oral anipamil (10 mg/kg/day).

The reduction in the percentage of lipid-stained aorta was statistically significant for the treated groups (C, 29%; D, 35%; E, 32%; G, 44% and H, 35%), but was lower than that obtained by other authors (Henry and Bentley (1981), 50%; Willis et al. (1985), 75%, or Sugano et al. (1988), 97%), although our results are similar to those obtained by several authors who used clinical dosages in their experiments (Henry and Bentley (1981), 31%; Pangiotopoulus and Nyler (1984), 26%, or Catapano (1997), 28%). These differences could be explained by the different routes used for drug administration and dosages. It should be noted that we used high doses but only ten times higher than the clinical ones, and the calcium entry blockers were always orally administered.

The mechanism by which calcium antagonists exert their anti-atherosclerotic activity is not clear. One of the early stages in the pathogenesis of atherosclerosis is the accumulation of cholesterol in the arterial wall (Catapano, 1997). Calcium antagonists reduce aortic cholesterol, but this effect is not mediated by a reduction in plasma lipid concentrations (García Pérez, 2002). However, several studies using cultured cells indicate that calcium antagonists modify cellular lipid metabolism in these cells of the arterial wall (Catapano, 1997).

It may be concluded therefore, that nifedipine, verapamil and diltiazem induced statistically significant reductions (30-40%) in the lipid deposition of the aorta of chickens fed a high-cholesterol diet. Quantitative histological analysis showed significant differences between clinical and higher dosages of calcium entry blockers. The higher the dosage of the calcium antagonist, the higher the degree of atherosclerotic regression. Therefore, the improvement of the

![Fig. 3. Electron microscopy of group B atherogenic sample. a: macrophage foam cell, b: lipid droplets (lp) are observed in the smooth muscle cells, c: mineral deposits in the lesions. Bar: 0.9 µm.](image-url)
atherogenic lesions was dosage-dependent. Nifedipine was the calcium antagonist that caused the highest reduction at clinical dosage. In addition, the chicken atherosclerosis model proved itself useful and very suitable for in vivo drug intervention studies.

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