Effect of Acetylsalicylic Acid on Pulmonary Arteriosclerosis Induced by a One-Year *Dirofilaria Immitis* Infection

Clarence A. Rawlings, James C. Keith, Jr., and Robert G. Schaub

The ability of aspirin to block arteriosclerosis that developed in response to chronic, low-level injury to pulmonary arteries was evaluated in 21 dogs during their 1-year infection with *Dirofilaria immitis*. Three groups, with seven dogs in each group, were studied before and after sustained injury produced by the transplantation of 28 adult *Dirofilaria immitis* into each dog. Group A received no treatment and served as controls; Group B received no treatment for 6 months and then received 7 mg/kg of aspirin daily for 6 months; Group C received 7 mg/kg of aspirin daily for the entire year. The pulmonary arterial response was evaluated by hemodynamic and arteriographic studies at 6 and 12 months and by scanning electron microscopy at the end of the 12-month study. All groups developed a similar, mild pulmonary hypertension. The arteriographic changes of dilation and flow obstruction were worse in Groups A and B than in Group C at 6 months, and at 12 months both Groups B and C were less obstructed than Group A. Scanning electron microscopy revealed large, complex myointimal proliferations in Group A, whereas the two aspirin-treated groups had smaller, less complex lesions that covered a much smaller surface area. We concluded that: 1) aspirin markedly reduced the microscopic and macroscopic arteriosclerosis in Groups B and C; 2) aspirin in Group B not only arrested further development but also permitted resolution of arteriosclerosis while the arteries were still being injured. (Arteriosclerosis 5:355–365, July/August 1985)

Many experimental models have been used for study and attempted modification of arteriosclerosis development in response to vascular injury. Examples include dietary regimens to increase plasma lipid levels, removal of endothelial cells by balloon catheters, air-drying of exposed vascular surfaces, homocystine infusion, viral infections, vascular grafting, and parasite infection. Each vascular injury model has advantages, along with sufficient disadvantages to cause caution in relating the experimental results to human atherosclerosis.

Some models are acute and produce extensive damage as compared to the apparently prolonged, low-grade insults in humans. Variations in these injury models have probably contributed to the differing conclusions from experiments evaluating the effects of platelet-active drugs on arteriosclerosis development.

Our chronic injury model of *Dirofilaria immitis* (DI) infection produces long-term, low-level endothelial damage. The severity of the endothelial damage and thrombosis is limited and can be graded according to the duration of the infection and the number of parasites introduced. Baseline studies can be performed before injury and repeated at regular intervals after heartworm infection. From 3 to 4 days after DI adults are transplanted into the central venous system of dogs, the pulmonary arteries have an increased permeability to Evan’s blue dye-tagged albumin, endothelial swelling and fenestration, widened endothelial junctions, and focal areas of endothelial denudation. Activated platelets and leukocytes adhere to the damaged arterial surface and medial smooth muscle cells can be seen migrating toward the arterial lumen.
Between 3 to 4 weeks after infection, the pulmonary arteries have developed proliferative intimal lesions consisting of smooth muscle cells and collagen.\textsuperscript{10-12} These villous lesions are covered by endothelial-like cells. Pulmonary hypertension and the arteriographic changes of dilation and aneurysms can develop in the pulmonary arteries within months of DI infection.\textsuperscript{14-15} The chronic, low-grade vascular insult of the live DI can be removed by killing the DI with thiacectarsamide. After a brief period of thromboembolism,\textsuperscript{18,17} the pulmonary hypertension, arteriographic lesions, and myointimal proliferation undergo marked resolution.\textsuperscript{10,14,15} The primary differences between this model and human atherosclerosis appear to be the site of injury (pulmonary arteries versus systemic arteries), the absence of intramural ischemia (due to an apparently good bronchial arterial supply), and the absence of cholesterol types of lesions (due to the dietary and metabolic differences between dogs and humans).

Since the intimal proliferation appears to be related to the endothelial damage and platelet-leukocyte adhesion, the use of aspirin as a platelet-inhibitor might impede lesion development. In a previous short-term study using dogs infected for 30 days with DI, the aspirin-treated dogs developed less endothelial damage, less platelet adhesion, and smaller and less complex intimal lesions than nontreated control dogs.\textsuperscript{18,19} In the current study, the vascular insult was increased by extending the duration of DI infection to 1 year and by using three times as many DI per dog. In addition to determining whether aspirin was beneficial in arresting myointimal proliferation, we performed another experiment to see if the lesions would be arrested or would regress. A third group of dogs was permitted to develop lesions for 6 months; then the dogs were treated with aspirin.

**Methods**

Three groups of seven dogs each were studied before and after DI infection. The dogs were determined to be healthy and free of pre-existing cardiovascular pulmonary disease by physical examinations, Knott's test for circulating DI microfilaria, thoracic radiographs, pulmonary arteriograms, and pulmonary hemodynamic studies.\textsuperscript{14-16} Following these baseline studies, each dog was anesthetized with thiamylal sodium induction (16-20 mg/kg) and halothane maintenance in order to surgically introduce 28 adult DI into an external jugular vein. After transplantation, the dogs were randomly assigned to one of three groups. Group A received no treatment and were the controls. Group B received no treatment for 6 months and then received 7 mg/kg of aspirin orally daily for 6 months. Group C received 7 mg/kg of aspirin orally daily for the entire year. The research protocol and the use of dogs in these studies were approved by the Laboratory Animal Use Committee and the Research Committee of the College of Veterinary Medicine. The protocol and procedures were in accord with the guidelines of the University of Georgia and the College of Veterinary Medicine.

At 6 months (before start of aspirin treatment in Group B) and at 12 months (before termination), pulmonary hemodynamic studies and arteriography were performed on each dog during pentobarbital anesthesia (30 mg/kg, supplemented to maintain surgical anesthesia). The trachea was intubated and cathereters were surgically positioned into the aorta and pulmonary artery via the carotid and jugular vessels, respectively. Catheter positions were verified by fluoroscopy and pressure recordings. Previously described techniques were used to record direct and mean pulmonary arterial and aortic pressures, cardiac output by indocyanine green dye dilution, and arterial blood gases.\textsuperscript{14} The cardiac index was calculated by:

\[
\text{cardiac index} = \frac{\text{cardiac output}}{\text{body weight}}
\] (1)

The total pulmonary resistance (TPR) was calculated by using the mean pulmonary arterial (PA) pressure (P):

\[
\text{TPR} = \frac{60 \times \text{mean } P_{PA} \times 1332}{\text{cardiac index} \times 1000}
\] (2)

These measurements were performed under controlled conditions and during isoproterenol infusion (1.5 \(\mu g/kg/min\)). Arterial blood gases were performed during controlled conditions to determine whether hypoxemia, which would have implicated an alveolar hypoxia vasoconstriction, was present.

Following the hemodynamic studies, pulmonary arteriograms were performed with the dogs in left lateral recumbency. Meglumine iothalamate (1 ml/kg) was power-injected while positive inspiratory pressure (15 to 20 cm H\textsubscript{2}O) was applied to the airway. Five radiographs were taken at 0.6 second intervals, with the first film taken during the injection of the final one-third of the contrast media. The PA diameters were measured for each arteriographic series with the measurements being the widest measured on each series, e.g., systole. The measurements (previously described\textsuperscript{15}) were taken of the right PA, left caudal PA, and right caudal PA. Two of the authors (CR, JK) independently and then jointly subjectively classified the peripheral dilatations and the flow obstructions in the caudal lobar arteries. Arteriograms were normal before heartworm infection and served as a reference for the 6- and 12-month arteriograms. The arteriogram with the most severe changes in the arterial shape and size and the arteriogram with the most complete obstruction of blood flow through the caudal lobar arterial branches were selected as a reference. The baseline and the most severely altered arteriograms served as references to obtain numerical ratings for peripheral dilatations and flow obstructions: 1 = normal, 2 = subtle changes, 3 = mild changes or re-
tarded flow, and 4 = severe changes or total obstruction of flow.

One week after the 12-month arteriographic and hemodynamic studies, the dogs were intravenously given 2 ml/kg of 1% Evan’s blue in Tyrode’s buffer (pH = 7.4). One hour later, we harvested the pulmonary arteries after inducing anesthesia with thiamyl sodium (20 mg/kg), approaching the heart and lungs via a left-side thoracotomy, injecting 5000 U heparin into the right ventricle, and removing the heart and lungs. The pulmonary arteries were perfused with Tyrode’s solution (pH = 7.4, 280 mOsm) until the effluent was cleared of blood. The lung was fixed by instilling Trump’s fixative in Tyrode’s buffer into the pulmonary arteries at physiologic pressures and into the trachea. After 4 hours of initial fixation, the pulmonary arteries were incised and the extent of the Evan’s blue staining, as an indication of endothelial permeability, was noted. Since most DI adults live in the right caudal PA, these arteries were studied more critically. Blue-stained and nonstained segments of the right caudal lobar PA were removed and processed for scanning electron microscopy. Sections were fixed for 1 additional day, then postfixed in 1% osmium tetroxide overnight, rinsed in distilled water, and dehydrated in a graded ethanol series. The specimens were critical-point dried, coated with gold, and examined in a Joel scanning electron microscope.20

The extent of villous proliferation on the arterial surface was evaluated by a modification of the morphometric technique of Yamaki and Wagenvoort.21 Five specimens were examined from each dog’s right caudal lobar PA, and three representative photomicrographs (original magnifications, × 100) were taken of each area. Prints (8 × 10 inches) were developed and then overlaid with a grid composed of 1-cm squares. The intersection points that occurred over villous lesions were counted as hits. The percentage of arterial surface covered by proliferative lesions was determined by dividing the number of hits by the total intersections that overlaid the print. The complexity and severity of the arteriosclerotic plaques was assessed and quantitated by a graded scale for the plaques. Each print was blindly evaluated by three observers using the following numerical scale: 0 = no lesions observed, 1 = small pinpoint lesions, 2 = small linear lesions, 3 = moderate round lesions, 4 = moderate linear lesions, and 5 = large complex lesions.

All numerical data were characterized by descriptive statistics, and the group means were compared by analysis of variance with multiple range tests. Statistical significance was defined as p being less than 0.05.

Results

Mean pulmonary arterial pressure in all three groups was higher than baseline at 6 months, but this increase was not statistically significant. From baseline to 6 months, there was a significant increase in pulmonary arterial pressure during isoproterenol infusion in each group. There was no significant difference between groups in pulmonary PA or TPR during control and isoproterenol conditions at any of the three time periods (Table 1). Since most of the heartworm silhouettes were in the two caudal pulmonary arteries, the trauma was localized there, and comparisons of the arterial changes focused on these caudal pulmonary arteries (Figures 1–3). The mean diameters of the right and left caudal pulmonary arteries increased from baseline to 6 months in Group A and then did not change significantly. In contrast, the Group B diameters increased from baseline to 6 months and then decreased from 6 to 12 months during aspirin administration. The arterial diameters in Group C did not change during the 12-month study. The shape changes of the caudal pulmonary arteries became progressively more severe in Group A, in contrast to

Table 1. Pulmonary Arterial Changes

<table>
<thead>
<tr>
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<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Aspirin</td>
<td>Aspirin</td>
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<td>Mean pulmonary arterial pressure during control conditions (mm Hg)</td>
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<tr>
<td>Base</td>
<td>11.7</td>
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<td>6 mos</td>
<td>14.7</td>
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<td>12 mos</td>
<td>17.4</td>
<td>16.5</td>
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<td>Mean pulmonary arterial pressure during isoproterenol infusion</td>
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<tr>
<td>Base</td>
<td>12.6a</td>
<td>17.8a</td>
<td>11.4a</td>
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<tr>
<td>6 mos</td>
<td>20.8b</td>
<td>26.6c</td>
<td>22.3d</td>
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<td>12 mos</td>
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<td>Diameter of right caudal pulmonary artery (mm)</td>
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<tr>
<td>Base</td>
<td>11.6a</td>
<td>11.4a</td>
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<td>6 mos</td>
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<td>Diameter of left caudal pulmonary artery (mm)</td>
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<td>12 mos</td>
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<td>Shape changes of the caudal pulmonary arteries (standardized ratings)</td>
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<tr>
<td>Base</td>
<td>1.1a</td>
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<td>6 mos</td>
<td>2.8b</td>
<td>2.4b</td>
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<tr>
<td>12 mos</td>
<td>3.2c</td>
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<td>1.8b</td>
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<td>Flow obstruction in the caudal pulmonary arteries (ratings)</td>
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<tr>
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<tr>
<td>6 mos</td>
<td>3.4a</td>
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<tr>
<td>12 mos</td>
<td>3.5a</td>
<td>2.5a</td>
<td>1.7</td>
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<td>Percentage of right caudal lobar pulmonary arterial surface exhibiting lesions</td>
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<tr>
<td>12 mos</td>
<td>48.4</td>
<td>10.3*</td>
<td>16.2*</td>
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<td>Complexity of the arteriosclerotic lesions using a &quot;blind&quot; panel with a standardized grading scale</td>
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<tr>
<td>12 mos</td>
<td>3.98</td>
<td>2.61*</td>
<td>2.69*</td>
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Means within a column for each measurement are significantly different (p < 0.05) if their letter subscripts are different. The asterisks (*) in the bottom two rows indicate a significant difference from the mean of the control group.
Groups B and C, where changes increased in severity from baseline to 6 months and then remained unchanged. The flow was more obstructed at 6 and 12 months than at baseline in Groups A and B but the flow was not significantly obstructed at any time in Group C. The group means for both shape changes and flow obstruction at 12 months were significantly different; in Group A, it was worse than in Group B, while in Group B it was worse than in Group C (Figures 4 and 5).

Scanning electron microscopy of the control Group A revealed large areas with villous proliferations (Figure 6). Most of these lesions were the large complex villous and rugose proliferative lesions characteristic of severe heartworm disease. In Group A, 48% of the right caudal lobar pulmonary arteries had arteriographic shape changes rated as 1 (normal). In each group, one dog had aortic arteriographic shape changes rated 1.5. Flow was normal in this and all baseline arteriograms. B. Note the focal dilations of the arteries (solid white arrows) and the abrupt narrowing of the small arteries (black and white arrows). The open white arrow indicates flow decrements in the right caudal artery. The shape changes were rated as 3, and the flow changes were rated as 4.

Figure 1. Arteriograms of caudal lobar pulmonary arteries in Group A before (A) and after (B) 12 months of harboring 28 heartworms and receiving no treatment. A. In this and most baseline arteriograms (18 of 21), shape changes were rated 1 (normal). In each group, one dog had arteriographic shape changes rated 1.5. Flow was normal in this and all baseline arteriograms. B. Note the focal dilations of the arteries (solid white arrows) and the abrupt narrowing of the small arteries (black and white arrows). The open white arrow indicates flow decrements in the right caudal artery. The shape changes were rated as 3, and the flow changes were rated as 4.

Figure 2. Arteriogram of Group A caudal lobar pulmonary arteries after 6 months of harboring 28 heartworms and receiving no treatment. Note areas of dilation (white arrows) before truncation (black and white arrow) into distal arteries with small diameters. The severity of shape changes was rated as 3. The reduction of arterial flow in the right caudal artery was rated as 2.5. Evaluation of flow obstruction was based upon sequential arteriograms on both ventrodorsal and lateral views.

Figure 3. Arteriogram of Group C caudal lobar pulmonary arteries after 12 months of harboring 28 heartworms and being treated with aspirin. Arteries gradually taper (1.5 rating) and peripheral perfusion is normal. The longitudinal negative contrast silhouettes within the pulmonary arteries are heartworms.
arterial surface had these proliferations. Both of the aspirin-treated groups had less surface involved; Group B had 10% and Group C had 16% involvement (Table 1, Figures 7–9). Lesion complexity scores were also significantly more severe in Group A (3.98) than in either Group B (2.61) or Group C (2.69) (Figure 10). The two aspirin groups were not significantly different in the extent or severity of the arterial lesions. Villous proliferations in all three groups were the typical myointimal proliferation previously described10–12 (Figures 11 and 12).

**Discussion**

Aspirin decreased the macroscopic and microscopic arteriosclerosis which developed in this model of chronic, low-grade vascular injury. Aspirin permitted resolution of the arteriosclerosis in Group B, in which lesions had developed over a 6-month period. This resolution was probably the result of an attenuation of myointimal proliferation while the body’s healing response reduced the severity of the 6-month lesions. In previous studies, the pulmonary arterial lesions and hypertension decreased after removal of the vascular insult by medical elimination of the DI. We also previously observed a reduced development of myointimal proliferation during aspirin administration in dogs infected for 30 days with nine DI adults. In the current study the pulmonary arteries were traumatized for 1 year by three times as many DI adults and were treated with only two-thirds the amount of aspirin.

The platelet-inhibitory effects of the aspirin were probably the most important action in reducing myointimal proliferation in this study.22–25 We feel that an anti-inflammatory effect was unlikely. Although there was a mild inflammatory response to heartworms, it seemed to be restricted to the vascular surface and most of the lesions did not have the typical inflammatory appearance. The intimal proliferations were composed primarily of smooth muscle cells and collagen, with only a few monocytes. At the doses used in this and previous studies, aspirin did not decrease leukocyte adhesion to the arterial surface.17 Although inflammatory mediators may be important in attracting platelets to participate in the vascular response, a cyclooxygenase inhibitor with almost no anti-inflammatory action26 was found to be just as effective as aspirin in reducing intimal proliferation in a study in which heartworms subsequently induced injury.27,28 A direct effect of aspirin on DI viability was also unlikely since all DI adults were live, active, and excluded Evan’s blue tagged albumin at the termination of the experiment.

Conflicting results have been reported in other studies using platelet inhibitors to reduce arteriosclerosis.29–31 The studies showing little effect have usually been short-term ones31 or have used higher dosages of aspirin.2,3,2,33 When large areas of endothelial cells were removed and trauma extended deeper into the vascular wall during the acute injuries of balloon catheter withdrawal and air infusion, antiplatelet drugs have had little effect.31,34,35 Myointimal proliferative lesions have decreased during platelet inhibition in low-grade trauma models of antigenic injury,36–38 chemical toxins (homocysteine),2 dietary manipulation (hyperlipidemia),29 parasite injury (dirofilariasis),18,19 and some forms of catheter injury.40 During heartworm infection, the trauma appears to be limited to the endothelium and produces only partial loss of the endothelium. In the current study, the hemodynamic effect of hypertension did not vary between the three groups and should not have had a preferential impact on any of the groups.
Figure 6. Scanning electron micrographs of the surface of right caudal lobar pulmonary arteries from a Group A control dog. Note extensive rugose and villous myointimal lesions. A. Large round lesions. B. Large linear lesions. × 100.
Figure 7. Scanning electron micrographs of the surface of right caudal lobar pulmonary arteries from a Group B dog that received aspirin for only the final 6 months. A. The few areas of moderate, linear lesions. B. Most areas contain a few small, linear lesions. × 100.
Figure 8. Scanning electron micrographs of the surface of right caudal lobar pulmonary arteries from a Group C dog that received aspirin throughout the 12 months of harboring heartworms. × 100.
Figure 9. Percentage of right caudal pulmonary arterial surface exhibiting lesions (mean of seven dogs in each group) as determined by the morphometric technique of Yamaki and Wagenwoort. The two aspirin groups differed significantly from the control group.

Figure 10. Complexity of the atherosclerotic lesions (mean of seven dogs in each group) in the right caudal lobar pulmonary arteries. Scores were determined by a blind panel using a standard grading (from 0 = no lesions observed to 5 = large complex lesions.) Both aspirin groups were significantly different from the control group.

Figure 11. Light micrograph from the epoxy-embedded arterial tissue in Figure 6B. This is a dog in the control Group A. Staining was done with methylene blue-azure II-basic fuchsin. Villi were produced by myointimal proliferation. × 125.

Figure 12. Light micrograph from the epoxy-embedded arterial tissue in Figure 8B. This is an aspirin-treated dog in Group C. Staining was with methylene blue-azure II-basic fuchsin. Villi were produced by myointimal proliferation, but most were smaller than in Group A. Both aspirin groups had similar villi. × 85.
We suggest that the inhibitory effect of aspirin on myointimal proliferation in this study was due to aspirin's effect on platelet adhesion. Platelet adhesion was reduced during aspirin administration in a similar study where this was mild chronic injury using DI infections (90% reduction) and also in a study by Silver et al. who used another model of mild arterial injury. Platelet adhesion to damaged arteries appeared to be less in the untreated controls subjected to mild chronic arterial injury (such as heartworm infection) than in more traumatic injury (balloon or other forms of mechanical injury). Since the interaction and accumulation of platelets on the arterial surface is an important component of the release reaction, we propose that the beneficial effects of aspirin in our model derive from the inhibition of adhesion; platelet involvement may be so great in more traumatic models that any platelet inhibition is less likely to significantly reduce the effect of the platelet release reaction on myointimal proliferation. The results of this study would suggest that antiplatelet therapy can have an important role in the mild chronic injury forms thought to produce human atherosclerosis.

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References

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