

EXPERIMENTAL STUDIES

Experimental Aortic Valve Stenosis in Rabbits

Marie-Claude Drolet, MSc, Marie Arsenault, MD, Jacques Couet, PhD

Quebec, Canada

Aortic valve stenosis (AVS) is the most common valvular heart disease in Western countries. Because AVS was always considered to be simply a degenerative disease of the valve, virtually no attention was given to its potential medical treatment other than surgical replacement of the valve in severe symptomatic cases. However, many recent observations have suggested a link between atherosclerosis and the development of AVS. The differences noted between hypercholesterolemic animals with or without vitamin D2 supplementation imply a significant role of calcium in the development of AVS, meriting further attention. (J Am Coll Cardiol 2003;41:1211–7) © 2003 by the American College of Cardiology Foundation

OBJECTIVES

We studied a known rabbit model of atherosclerosis to assess the effect of a hypercholesterolemic diet on aortic valve morphology and function. We also evaluated the effects of the combination of this diet with vitamin D supplements on the development of the disease and the occurrence of valve calcification.

BACKGROUND

Aortic valve stenosis (AVS) is the most common valvular heart disease. Recent observations have suggested a link between atherosclerosis and the development of AVS. However, until now, there has been no solid direct proof of this potential link.

METHODS

Rabbits were divided in three groups: 1) no treatment; 2) cholesterol-enriched diet (0.5% cholesterol); and 3) cholesterol-enriched diet plus vitamin D2 (50,000 IU/day). Echocardiographic assessment of the aortic valve was done at baseline and after 12 weeks of treatment. The aortic valve area (AVA) and maximal and mean transvalvular gradients were recorded and compared over time.

RESULTS

Control animals displayed no abnormalities of the aortic valve. Despite important increases in blood total cholesterol levels, animals in group 2 did not develop any significant functional aortic valve abnormality over 12 weeks. However, eight of 10 of the animals in group 3 developed a significant decrease in AVA (p = 0.004) and significant increases in transvalvular gradients (p = 0.003).

CONCLUSIONS

This study supports a potential link between atherosclerosis and the development of AVS. The differences noted between hypercholesterolemic animals with or without vitamin D2 supplementation imply a significant role of calcium in the development of AVS, meriting further attention.

METHODS

Animals. Twenty-two male New Zealand White rabbits (weight 2 to 2.5 kg) were divided in three study groups (6 to 10 animals per group): 1) controls receiving normal rabbit chow without any dietary supplement; 2) animals fed with 0.5% cholesterol-enriched chow (Harlan, Indianapolis, Indiana); and 3) animals fed with 0.5% cholesterol-enriched chow plus 50,000 IU/day vitamin D2 (Sigma; Markham, Ontario, Canada) in drinking water. Vitamin D2 has been shown by others to accelerate the atherosclerotic process in this animal model. All animals were treated in accordance with the recommendations of the Canadian Council for Animal Care. The protocol was approved by the Université Laval Animal Protection Committee.

Blood samples were taken through the marginal vein of the ear at baseline and weekly thereafter for the measurement of cholesterol as well as calcium levels in serum.

Echocardiography. Echocardiography was performed at baseline and 12 weeks after the beginning of treatment. Animals were sedated with an intramuscular injection of ketamine (30 mg/kg), midazolam (0.5 mg/kg), and butorphanol (0.5 mg/kg). Ultrasound images were obtained with a 12-MHz phased-array probe connected to a Sonos 5500 echograph (Philips Medical Imaging, Andover, Massachusetts).
The aortic valve area (AVA) was measured by the standard continuity equation, as currently recommended in the literature, in the same manner that is performed in humans with suspected AVS. A parastrnal long-axis view was used to measure the diameter of the left ventricular outflow tract and to observe the morphology and opening of the aortic valve. The aortic valve was also imaged in the short-axis view to assess leaflet morphology. An apical five-chamber view was obtained to measure the stroke volume with pulsed-wave Doppler proximal to the aortic valve, as is routinely done in humans. Continuous-wave Doppler was used to record the maximal and mean aortic transvalvular gradients and to obtain the integral of transvalvular flow to be used in the continuity equation for AVA calculation. The AVA was indexed to the animal’s body weight.

All echocardiographic imaging and analyses were performed throughout the protocol by the same investigator experienced in performing echocardiographic studies in rabbits and other small animals. Inter- and intra-observer variability was assessed blindly for half of the animal studies for AVA measurement.

**Histology.** At the end of the protocol, the ascending aortic section containing the aortic valve was dissected, rinsed in phosphate-buffered saline, embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, California), and snap-frozen in liquid nitrogen. Cross-sectional serial sections were prepared, and alizarin red S and von Kossa’s silver stains were employed to visualize calcium deposition.

**Statistics.** The results are presented as the mean value ± SEM. Repeated-measures one-way analysis of variance with the post hoc Tukey’s test or paired t test was used for intragroup comparisons to assess the evolution of Ca^{2+} serum levels over time. The unpaired t test was used for intergroup comparisons (total cholesterol levels). The paired t test was used for comparisons of the effect of diet on echocardiographic parameters. Statistical significance was set at p < 0.05.

**RESULTS**

**Cholesterol and calcium levels (Fig. 1).** Animals in group 1 displayed no modifications of those two parameters. Total cholesterol circulatory levels rose to high levels in both groups 2 and 3 (Fig. 1A). Interestingly, vitamin D2 caused an additional increase in serum cholesterol despite similar cholesterol intake. Calcium levels were slightly more elevated for the animals in group 3. This mild difference reached statistical significance at week 8, although calcium levels were elevated throughout the protocol (Fig. 1B).

**Development of atherosclerosis.** The arteries of the control group remained normal. As expected, and based on previous observations by others, the animals fed with the cholesterol-enriched diets (groups 2 and 3) developed severe atherosclerotic involvement of their arteries, which was evident on macroscopic as well as microscopic examination (not shown).

**Aortic valve (Figs. 2 to 6).** Baseline AVAs were comparable in all three groups. When corrected for body weight, no significant change was noted in the animals in the control and cholesterol groups (Fig. 2, middle column). However, the animals in the cholesterol plus vitamin D2 group behaved differently (Fig. 2, bottom row). The AVA decreased in eight of 10 animals after 12 weeks of treatment, and the transvalvular gradients also increased in eight animals. This decrease was even more pronounced when
AVA was indexed for the animal's weight. The transvalvular gradients remained normal in the control and cholesterol-fed groups, but increased significantly in the cholesterol plus vitamin D$_2$ group, as AVA decreased (Fig. 3).

Overall, AVA decreased by 36%, the maximal gradient increased by 300%, and the mean gradient increased by 107% (all $p > 0.05$). Two-dimensional imaging of those valves showed an increase in the thickness and echogenicity of the leaflets and a reduced mobility compatible with leaflet sclerosis and areas of calcification. This is confirmed by visual inspection of excised valves (Fig. 4). Histologic examination demonstrated leaflet thickening and calcium deposition both at the aortic attachment site and in the leaflet itself (Fig. 6). Calcifications were more prominent at

Figure 2. Effects of hypercholesterolemia in rabbits receiving (Chol+vit D$_2$) or not receiving (Chol) daily supplements of vitamin D$_2$ on aortic valve function, as assessed by echocardiography. The aortic valve area (AVA) (left column), indexed AVA (arbitrary units [AU]) (middle column), and maximal gradient (mm Hg) (right column) were calculated as described in the Methods section and are represented for each individual rabbit. ns = not significant.
the attachment site and the base of the leaflets. Histologic evidence of calcium deposition is displayed in Figure 6. Inter- and intra-observer variability was blindly assessed in half of the animals for the echocardiographic measurement of AVA by the continuity equation. Intra-observer variability was <5%, and inter-observer variability was 8%.

**DISCUSSION**

The theory of atherosclerotic pathogenesis of AVS in humans is becoming more and more popular. Aortic valve stenosis had been described many years ago in patients suffering from cholesterol metabolism abnormalities, such as Tangier’s disease and severe homozygous familial hypercholesterolemia (7–9). Following this lead, several recent publications have demonstrated a clear statistical link between AVS, per se, and specific cardiovascular risk factors, such as hypercholesterolemia, systemic hypertension, and diabetes mellitus (1,2,4,6,10–13). Unfortunately, most of the evidence currently available relies on observational cohort studies. Prospective studies are clearly needed to assess the effect of those risk factors, as well as the potential effect of pharmacologic treatment, on the evolution of AVS.

In this study, we have been able to induce peripheral atherosclerosis and, more importantly, AVS in animals fed with a cholesterol-rich diet supplemented with vitamin D₂. Aortic valve leaflet thickening and reduced systolic motion of those leaflets were clearly identified by echocardiography. Transvalvular gradients increased significantly compared with controls, and most importantly, the aortic valve opening was significantly reduced at echocardiographic examination (two-dimensional and calculated AVA).

To our knowledge, this is the first description of a clear prospective link between atherosclerosis and AVS in an animal model. Previous studies have demonstrated atherosclerotic involvement of the aortic valve in hypercholesterolemic animal models (14–19). Unfortunately, an in vivo evaluation for detecting a potential stenosis of the valve was never investigated in those animals.

As for atherosclerotic plaques, the mechanisms of aortic valve calcification that contribute to the progressive stenosis of the valve are still incompletely understood. In this protocol, we used vitamin D supplementation in combination with a hypercholesterolemic diet, a supplementation that has been used for decades by investigators studying
rabbit models of atherosclerosis to enhance and accelerate the development of the calcification process (20–23). Vitamin D metabolites have been shown to induce a disruption in the integrity of the arterial wall and some degree of smooth muscle necrosis of the arterial media of swine and rabbits, thereby enhancing the integration of lipid particles by the arterial wall (24,25). This mechanism may explain the accelerating effect of vitamin D on atherosclerosis development in hypercholesterolemic rabbits. Conversely, calcium channel blockers have been proven to delay the development of atherosclerosis in hypercholesterolemic rabbits (20). We hypothesized that the aortic valve leaflets would demonstrate the same behavior when subjected to hypercholesterolemia and vitamin D supplementation and that animals treated with this combination would develop more severe aortic valve abnormalities earlier than those treated with a high-cholesterol diet alone. Our results confirm this hypothesis. Indeed, animals not receiving supplements of vitamin D2 did not display any significant degree of AVS after 12 weeks of treatment, despite very high levels of blood cholesterol (>20 mmol/l) and the presence of peripheral atherosclerotic lesions. Unexpectedly, animals receiving vitamin D had significantly higher cholesterol levels than those not receiving it. The reason for this remains unclear. However, because both groups of animals received a similar amount of cholesterol supplements in their food, we suspect that cholesterol absorption was increased in the vitamin D group or that cholesterol metabolism was influenced by vitamin D. We cannot actually confirm whether vitamin D itself played a direct role in the development of aortic stenosis in our animals or whether it simply amplified the effects of hypercholesterolemia. The role of vitamin D in our model remains to be explored. In humans, vitamin D overload has been mostly associated with supra-valvular AVS, which was seen in our animals in whom the level of stenosis was at the aortic valve itself (26–28). Interestingly, a recent observation linked a vitamin D receptor gene polymorphism to calcific aortic stenosis in humans (29).

Our model offers many advantages over most of those previously published. Pressure-overload animal models with banding of the ascending aorta have been used to mimic the situation seen in AVS (30–33). However, this model has the disadvantage of producing a supra-coronary obstruction that is not reflective of the situation of valvular stenosis, in which the obstruction is proximal to the implantation of the coronary arteries. In larger animals such as sheep, AVS has also been induced by surgical procedures. Because the disease is artificially created, it is impossible to study the physiopathology of the disease on the valve and potential strategies for preventing or slowing the degenerative process. We describe a model in which the pathogenesis of AVS is probably related to the same atherogenic factors that also seem to be implicated in human disease. All animal models have their limitations, and, of course, we are aware that the animals in this protocol were submitted to extreme conditions of hypercholesterolemia combined with vitamin D supplementation, a combination that will never be encountered in humans. Multiple risk factors that are clearly absent from our model interact in humans to induce AVS. The long-term evolution of our animal model has not been assessed, and we still do not know if the disease will continue to progress over time. Neither the reversibility nor the effect of withdrawing the offending factors has been assessed.

Conclusions. We report an experimental model of acquired AVS in rabbits fed with a high-cholesterol diet and supplements of vitamin D2. These results support the hypothesis of the link between atherosclerosis and AVS in humans. This experimental model may prove useful in studying the early evolution of the disease, so the effects of various atherosclerotic risk factors on this evolution and the use of pharmacologic treatments to delay or even stop the evolution.
REFERENCES


Figure 6. Calcium deposits as assessed by alizarin red S (left column) and von Kossa’s (right column) staining in the attachment site (B, F), aortic valve leaflet (C, G), and aortic annulus (D, H) in rabbits treated with cholesterol enriched diet plus vitamin D3. (A and E) Sections from a normal aortic valve.


