Original Article

Mast Cells Might Have a Protective Role against the Development of Calcification and Hyalinisation in Severe Aortic Valve Stenosis

(mast cells / remodelling / inflammation / aortic valve stenosis)

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Abstract. Aortic valve stenosis is characterized by inflammation and extracellular matrix remodelling. The aim of this study was to analyse the impact of mast cells on the occurrence of histopathological changes of aortic valves in patients with severe grade, non-rheumatic degenerative aortic valve stenosis. Valve specimens were obtained from 38 patients undergoing valve replacement. The role of mast cells was analysed by dividing the specimens into two groups, characterized by the presence (group A, N = 13) or absence of mast cells (group B, N = 25). There were no significant differences in clinical data between the two groups. In group A, T cells and macrophages were present in all aortic valves, as compared to a significantly lower proportion of valves with T cells and macrophages in group B. Valves in group A were less often calcified and hyaline-degenerated than valves in group B. There were no changes in fibrosis between the two groups. We found a positive correlation between the presence of mast cells and macrophages/T cells, a negative correlation between the presence of mast cells and calcification/ hyaline degeneration, and no correlation between the presence of mast cells and fibrosis. There was also a negative correlation between the presence of macro-
phages/T cells and calcification. The linear regression model identified only the presence of mast cells as an independent negative prediction value for calcification. In conclusion, mast cells might have a protective role against the development of calcification and hyaline degeneration in severe grade, non-rheumatic aortic valve stenosis.

Introduction

Aortic valve disease is the main cause of valvular surgery in Europe and North America. It represents up to 43 % of all valvular heart diseases (Thom et al., 2006). The prevalence of calcific aortic stenosis increases with age, being present in 2 % to 4 % of adults over the age of 65. Unless treated, aortic stenosis patients have a poor prognosis (Mazzone et al., 2004).

In the early nineties, T-cell infiltration was reported in degenerative aortic valve stenosis, indicating the importance of inflammatory component in its development (Ollson et al., 1994; Mohler et al., 2001; Wallby et al., 2002). Inflammatory cells were the predominant cell type reported already in early aortic valve lesions (Otto et al., 1994; Mazzone et al., 2004; Freeman and Otto, 2005). Contemporary studies have confirmed that degenerative aortic valve disease is a chronic inflammatory process with infiltrates comprising lymphocytes, macrophages and mast cells (Steiner et al., 2012). Inflammatory cells play an important role in the initiation and progression of degenerative aortic valve disease (Helske et al., 2006, 2007; Parolari et al., 2009).

In the pathogenesis of degenerative aortic valve disease, several processes, such as endothelial dysfunction, activation of interstitial cells, lipid accumulation, infiltrations of inflammatory cells (such as macrophages, T, B cells and mast cells) and calcification, are involved (Olsson et al., 1994; Otto et al., 1994; Mohler et al., 2001).

The dysfunctional endothelial cells display increased permeability and up-regulated adhesion molecule expression. Monocytes attach to adhesion molecules, migrate to subendothelial space, and differentiate into macrophages. Macrophages and T cells that are present in the aortic valve lesion secrete a number of inflammatory effector molecules and cytokines. This biochemical environment promotes differentiation of valve interstitial cells, matrix remodelling, fibrosis, and calcification of leaflet tissue. The result of extracellular matrix remodelling is a stiff aortic valve that is prone to restricted movement and stenosis (Mahler and Butcher, 2011). Macrophages capture and present antigens to effector T cells, which mature into a form capable of actively carrying out immune defences (Galli and Nakae, 2003; Urb and Shepard, 2012).

Mast cells and macrophages produce a similar type of mediators, such as inflammatory cytokines, growth factors, proteases, and reactive oxygen species. Both are antigen-presenting cells and can mediate T-cell proliferation. Macrophages and lymphocytes may activate each other and cause further activation and increase of adhesion molecules, scavenger receptors and extracellular matrix-degrading protease. Mast cells are engaged in the migration and accumulation of the macrophages at the site of inflamed valve tissue. They can contribute to remodelling in tissues at the sites of persistent mast cell activation. These activities of mast cells may also affect recruitment of other inflammatory cells. Mast cells also co-localize with macrophages, suggesting their interaction either by direct cell-cell contact or via inflammatory mediators (Xu and Shi, 2012).

The aim of this study was to analyse the impact of mast cells in relation to other types of inflammatory cells, such as T cells and macrophages, on the morphological features of symptomatic non-rheumatic severe grade aortic valve stenosis.

We used haematoxylin and eosin (HE) and toluidine blue staining to identify mast cells. HE staining was also used to visualize the presence of inflammatory infiltrate, fibrosis, calcifications and hyaline degeneration, whereas to detect T cells and macrophages, the imunohistochemical method (anti-CD68 and anti-CD3) was used.

Material and Methods

Patients and tissue sampling

Valve specimens were obtained from 38 patients (12 women, 26 men, mean age 57.8 years, range 29–81 years) referred to the hospital for aortic valve replacement because of non-rheumatic symptomatic severe grade of aortic valve stenosis (mean pressure gradient across the valve > 40 mm Hg, aortic valve area < 1.0 cm^2 and maximum velocity > 4.0 m/sec) (Novaro and Griffin, 2003). The diagnosis was made by preoperative Doppler echocardiography. The clinical characteristics of the patients are given in Table 1. There were no significant differences in clinical data between the two groups.

Tissues were collected at the time of surgery. The study was approved by the National Medical Ethics Committee (trial registration 170/07/13). All participants provided their written informed consent to participate in this study. The study was conducted according to the Declaration of Helsinki.

Upon dissection, tissues were immersed in 10% buffered formalin, embedded in paraffin and cut in a microtome into 4 μm sections. The sections were mounted on silane-coated slides (Dako North America, Inc., Carpinteria, CA) and stored at room temperature until histological staining.

Staining of tissue sections for mast cell, macrophage and T-cell identification and analysis

After deparaffinization, the sections were stained with HE, with toluidine blue solution (Pleskovič et al., 2011), and Movat pentachrome method to identify mast cells, or they were used for immunohistochemistry methods to identify T cells and macrophages. Histological analysis was performed by a trained pathologist blind to the treatment protocols.

The degenerative aortic valve leaflets of 38 patients were divided into two groups with regard to the presence or absence of mast cells: 13 valves with mast cells (group A) and 25 valves without mast cells (group B). Between the two groups of patients, there were no statistically significant differences in clinical data (Table 1, Student's t- test, $P < 0.05$).

The two groups of degenerative aortic valve leaflets were then compared. The inflammatory mononuclear infiltrate was analysed in sections stained with HE. To identify mononuclear T cells, the valve tissue sections were stained with antibodies for CD3 (pan-T-cell antigen, Dako 1 : 400). Macrophages were stained with an-

Table 1. Clinical characteristics of patients

	Group A (13 cases) Mean (± SD)	Group B (25 cases) Mean (± SD)
Age (years)	54 ± 14	60 ± 14
RR1 (mm Hg)	134 ± 22	134 ± 21
EF (%)	62 ± 6	57 ± 13
Heart rate	67 ± 8	74 ± 15
Glucose (mmol/l)	5.8 ± 1.2	6.8 ± 1.9
HDL (mmol/l)	1.2 ± 0.2	1.1 ± 0.3
LDL (mmol/l)	3.3 ± 0.8	3.0 ± 0.8
Triglycerides (mmol/l)	1.9 ± 2.4	1.6 ± 0.7
Cholesterol (mmol/l)	4.6 ± 0.9	4.8 ± 1.0

Clinical characteristics of patients undergoing valve replacement due to non-rheumatic severe aortic valve stenosis with regard to the presence (group A) or absence of mast cells (group B). Note that there were no significant differences in clinical data (Student's t-test, $P < 0.05$) between the two groups.
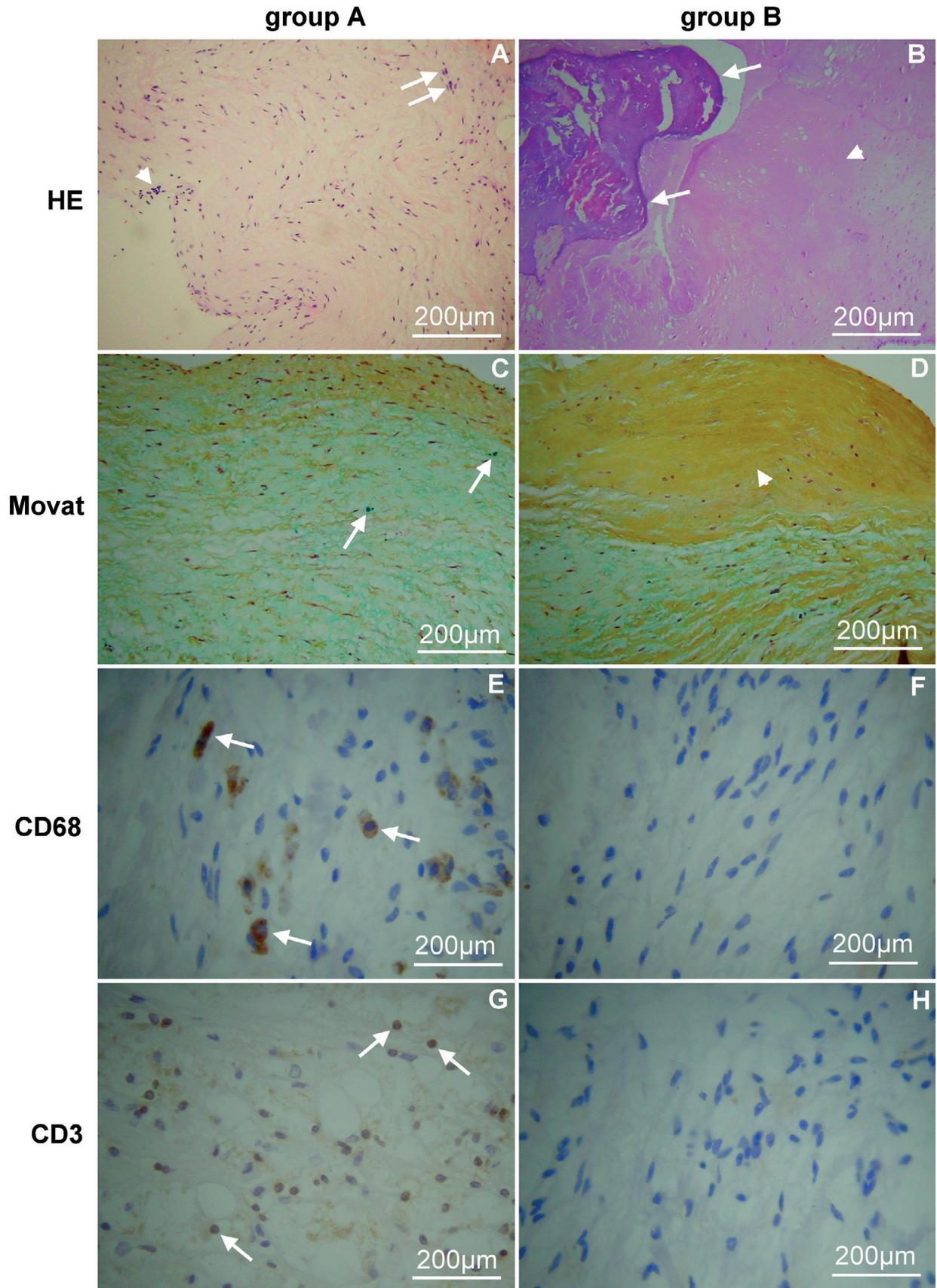
Fig. 1. Valve sections in the group A (**A**, **C**, **E**, **G**) and B (**B**, **D**, **F**, **H**) stained for inflammatory cells, hyaline degeneration and calcification. Valve sections stained with HE (**A**, **B**), Movat pentachrome method (**C**, **D**), anti-CD68 (**E**, **F**) and anti-CD3 (**G**, **H**) for inflammatory mononuclear infiltrates – HE (**A** arrowhead), mast cells – HE (**A**, arrows) and Movat pentachrome method (**C**, arrows), macrophages – anti-CD68 (**E**, arrows), T cells – anti-CD3 (**G** arrows), hyaline degeneration – HE (**B**, arrowhead), Movat (**D** arrowhead), and calcification – HE (**B**, arrows).

tibodies for CD68 (macrophage antigen, Dako 1 : 100) (Wallby et al., 2013). Cells in tissue sections were analysed using high-power view optical microscopy (400×). The inflammatory cells were evaluated as: 0 = absence of inflammatory cells, 1 = presence of inflammatory cells.

Histopathological analysis

Fibrosis, calcification and hyaline degeneration were estimated in sections stained with HE. Calcifications, fibrosis and hyaline degeneration were categorized as: 0 = absence/not visible deposits in low-power view optical microscopy (25×), 1 = visible in low-power view optical microscopy (25×) (Wallby et al., 2013).

Statistics

The percent ratio of leaflets with or without macrophages and T cells, with the presence or absence of fibrosis, calcifications and hyaline degeneration was calculated for each group. The comparison between groups with mast cells (group A) and without mast cells (group B) was performed using the Mann-Whitney test. Statistical significance was set at P < 0.05.

Pearson coefficient of correlation was calculated between the presence of mast cells, macrophages and T cells and calcification, hyaline degeneration and fibrosis. Results with correlation r < 0.03 and P values < 0.05 were considered as significant.

Linear regression analysis (r^2) was performed to evaluate the potential contribution of mast cells, T cells and macrophages to calcification. Statistical significance was set at P < 0.05.

Results

Histological analysis of valve sections regarding the presence or absence of mast cells

Histological sections of leaflets were obtained from 38 patients with symptomatic severe degenerative aortic valve stenosis. The leaflets were divided into two groups according to the presence (group A, N = 13) or absence (group B, N = 25) of mast cells. All valves from group A contained mononuclear infiltrate (100 %) as compared to only about half of the valves pertaining to group B (52 %, P < 0.01) (Fig. 1 A; Fig. 2).

Immunohistological staining of the valves showed that macrophages (Fig. 1 E, F) and T cells (Fig. 1 G, H) were present in all valves of group A (100 %), but just in 32 % and 28 % of valves, respectively, of group B (P < 0.01) (Fig. 2). In both groups the calcified deposits (Fig. 1 B) were mainly nodular and only rarely diffusely distributed throughout the body of analysed valve leaflets. Hyaline degenerative changes (Fig. 1 B, D) and fibrosis were confined to the central and subendothelial part of the leaflets. All leaflets from group B were calcified (100 %), as opposed to only 62 % from group A (P < 0.01) (Fig. 2). The hyaline degenerative changes were also found significantly more often in group B (72 %) than in group A (38 %, P < 0.05) (Fig. 2). There were, however, no statistical differences between both groups in the occurrence of fibrosis (group A = 77 %, group B = 76 %) (Fig. 2).
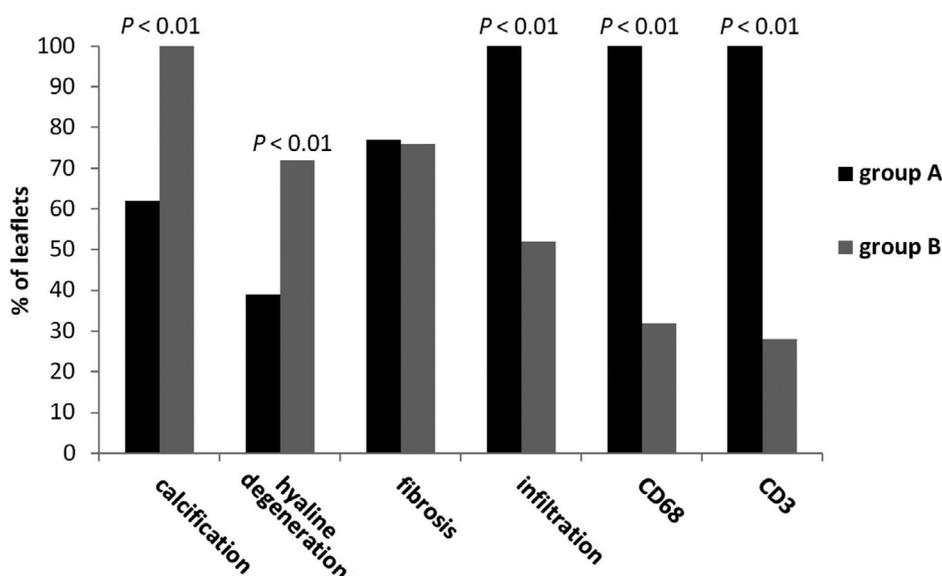

Fig. 2. The percentage of leaflets with inflammatory infiltrate and extracellular matrix remodelling with regard to the presence or absence of mast cells. The percentage of leaflets with the presence of calcification, hyaline degeneration, fibrosis, mononuclear infiltration, macrophages (CD68) and T cells (CD3) in the group with (group A; N = 13) and without mast cells (group B; N = 25); Mann-Whitney, P < 0.05, P < 0.01.

Analysis of the relationship between mast cells, macrophages and T cells and matrix remodelling of valves (fibrosis, hyaline degeneration and calcification)

Mast cells, stained with HE and Movat pentachrome method (Fig.1 A, C), were present mostly in the subendothelial part of the valves. We found a positive correlation between the presence of mast cells and macrophages (r = 0.65; P < 0.01), T cells (r = 0.68; P < 0.01), a negative correlation between the presence of mast cells and calcification (r = –0.54; P < 0.01)/hyaline degeneration (r = –0.33; P < 0.01) and no correlation between the presence of mast cells and fibrosis (r = 0.01). There was also a significant negative correlation between the presence of macrophages and calcification (r = –0.35, P < 0.05) and T cells and calcification (r = –0.37, P < 0.05). The linear regression model identified the presence of mast cells, but not T cells and macrophages as an independent negative predictive value of calcification (r^2 = 0.29, P < 0.05). There were no significant correlations between the presence of macrophages and hyaline degeneration (r = –0.29) and the presence of macrophages and fibrosis (r = 0.12), and no significant correlation between the presence of T cells and hyaline degeneration (r = –0.12) and the presence of T cells and fibrosis (r = 0.22).

Discussion

In this study we analysed the influence of mast cells and other inflammatory cells (T cells and macrophages) on the morphological features of the symptomatic severe aortic valve stenosis. We found that in leaflets with mast cells, the mononuclear infiltrate containing T cells and macrophages was always present as opposed to the valves without mast cells, where an infiltrate was present in only about half of the valves. It is known that mast cells release chemotactic factors such as osteopontin (Bulfone-Paus and Paus, 2008) for monocytes (Foris et al., 1983; Chen et al., 1998) and T cells that are involved in the sustenance of inflammation (Hieb et al., 2008). So far, several reports have tried to explain the role of inflammation in the pathogenesis of severe degenerative aortic valve stenosis (Wallby et al., 2013). In the early nineties, T-cell infiltration was demonstrated in tricuspid degenerative aortic valve tissue (Olsson et al., 1994; Otto et al., 1994; Wallby et al., 2002). The quantity, quality and architecture of valvular extracellular matrix are speculated to be the major determinants of long-term durability of native valves (Shoen, 2008).

It is known that the early phase of aortic valve remodelling is an active inflammatory process with subendothelial accumulation of oxidized lipoproteins and calcification (Jian et al., 2003). Only a few data, however, are available about the influence of mast cells and other potential defence cells on the remodelling of extracellular matrix in valve leaflets in degenerative aortic valve stenosis (Leopold, 2012). A recent study showed that the increased number of mast cells within human stenotic aortic valves was associated with the severity of aortic stenosis (Wypasek et al., 2013). Mast cells were noted in all stenotic valves (Wypasek et al., 2013) in calcified areas and in the subendothelial layer on the aortic side of the stenotic leaflets (Helske et al., 2004, 2006; Wypasek et al., 2013). These authors found a strong positive correlation between the number of mast cells and macrophages (Wypasek et al., 2013). In our experiment, we also found a positive correlation between the presence of mast cells and macrophages as well as with the presence of T cells.

Mast cells, macrophages and T cells are involved in sustained inflammation (Hieb et al., 2008). It was shown that macrophage infiltration had an impact on the degree of valve calcification (Aikawa et al., 2007; Hjortnaes et al., 2010). It has also been shown that macrophages release matrix metalloproteases and cysteine endoproteases that cause degradation of collagen and elastin linked with degenerative remodelling (Rabkin et al., 2001; Wylie-Sears et al., 2011). Calcified aortic valves have been shown to contain expanded populations of T cells (Leopold, 2012).

However, in stark contrast to the above-mentioned report by Wypasek et al. (2013), our results demonstrate that degenerated aortic valve leaflets that contained mast cells were less often calcified and hyaline degenerated. This is in agreement with the studies showing that an optimal dose of granules, isolated from mast cells, have cardioprotective roles in myocardial infarction via the decreased apoptosis of cardiomyocytes, increased infiltration of macrophages, decreased fibrosis, and preserved the left ventricular thickness and function (Kwon et al., 2011). It is also known that osteopontin, a secretory product of mast cells and a potent inhibitor of ectopic calcification in intercellular valvular tissue, could prevent valvular tissue remodelling (Giachelli and Steitz, 2000; Steitz et al., 2002). Other studies suggested that diminished numerical density of impaired mast cells might be the reason for more extensive inflammatory and immunologic atherosclerotic changes in the vessel wall of coronary arteries in patients with coronary artery disease (Pleskovič et al., 2011), implicating that mast cells may play an important role in the protection of the integrity of endothelial cell layers and the vessel wall, probably via their paracrine activity, especially through the release of heparin (Pleskovič et al., 2011). The release of heparin as an anticoagulant substance, which leads to higher endogenous heparin levels and higher levels of IgE, may have the primary role in the protective function of vessel wall endothelial cells (Sinkiewicz, 2002).

It may also be speculated that the presence of mast cells indicates an ongoing inflammatory stage that precedes the terminal stage of extensive hyaline degeneration and calcification where mast cells are no longer present.

Further investigations should try to determine the background of events in which mast cells might have a

protective role against the development of calcification and hyaline degeneration in severe grade, non-rheumatic aortic valve stenosis.

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