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Original Article

A comparative analysis of mitral valve changes in different age groups by histochemical, immunohistochemical and ultrastructural study

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ABSTRACT

Introduction: Mitral valve prolapse (MVP) is an increasingly prevalent clinical problem posing a surgical and diagnostic challenge for cardiac surgeons and cardiologists respectively. The commonest MVP etiologies are age-related degenerative (60%), rheumatic (12%) and functional (25%). The age related changes in valve can be collagen and elastic fiber disruption, calcification and mucopolysaccharides (MPS) accumulation.
Methods: Tissues from 60 formalin fixed human hearts were taken for histomorphology and immunohistochemistry and for ultrastructural analysis in 15 of them.
Results: Among 60 mitral valves, mild fibrosis was present in 4 cases (40%) in 2nd decade, 8 cases (80%) in 3rd decade, 4 cases (40%) in 4th and 5th decades, and 6 cases (6%) in 6th decade and only 1 case (10%) in 7th decade. Majority of the mitral valves (90%) in 7th decade showed moderate fibrosis. The MPS material deposition was mild in 60%, 20% and 10% cases in 2nd, 3rd and 5th decades respectively whereas it was moderate to severe in 4th to 5th decades. Immunohistochemistry showed moderate and severe decorin positivity in 18/60 (30%) and 1/60 (1.66%) cases respectively whereas mild and moderate biglycan positivity was seen in 34/60 (56.66%) and 8/60 (13.33%) cases respectively. Ultrastructurally, collagen fibers were fragmented with numerous inclusions of MPS material, and fibroblasts with increasing age.
Discussion: The present study highlighted that fibrosis and MPS accumulation in MV leaflets increases with advancing age along with the increased expression of decorin and biglycan.
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1. Introduction

Mitral valve prolapse (MVP) is an increasingly prevalent clinical problem which poses a surgical and diagnostic challenge for cardiac surgeons and cardiologists respectively. It has been reported to occur in 5–15% of the general population[1] and is often associated with serious complications like infective endocarditis, mitral insufficiency, embolic stroke, embolic systemic infarct and ventricular arrhythmias.[2–5] The commonest MVP etiologies are age-related degenerative (60%), rheumatic (12%) and functional (25%).[6] It is proposed that the underlying pathophysiological mechanism of MVP is a continuing process of repeated minor injury and repair occurring during the cardiac cycle with minor congenital anatomical variations in the valve apparatus.[7–9] The age related degenerative processes are usually progressive and physiologically irreversible.[10] Analysis of necropsy findings discloses commonly observed cardiac changes in very elderly hearts that may be considered "normal aging changes" and do not produce cardiac dysfunction during life.[11] Thus knowledge of age related degenerative changes in mitral valve is becoming important. The adult mitral valve is made up of distinct atrialis containing aligned elastic and collagen fibers covered with overlying endothelium, spongiosa layer of proteoglycans and glycosaminoglycans along with mesenchymal cells, collagen and elastic fibers.[6,12] The fibrosa is the major load bearing layer, comprising of central collagenous core of the leaflet lined by endothelial cells on the ventricular aspect.[6] The age-related changes in different layers of mitral valve can be collagen disruption, elastic fibers fragmentation, calcification and accumulation of mucopolysaccharides (MPS), matrix metalloproteinases, glycosaminoglycans and proteoglycans thus making it prone for prolapse.[6,13–17]

Previous studies have described the degenerative changes in various pathological conditions of mitral valve such as rheumatic heart disease, mitral regurgitation and mitral valve prolapse. However there is paucity of literature regarding histomorphological details of age-related degenerative changes in normal human mitral valve leaflets. Thus we planned to analyze the histomorphological features and ultrastructural changes in mitral valve along with the distribution of proteoglycans in mitral valve leaflets with aging.

2. Material and methods

The study material comprised of 60 formalin fixed human hearts including 30 males and 30 females for histomorphology and immunohistochemistry. In addition and 15 of these were subjected for ultrastructural analysis. The cases were selected without any macroscopic abnormality or pathological change at gross examination retrospectively from Department of Histopathology, Postgraduate Institute of Medical Education and Research, Chandigarh, India. All the hearts belonged to patients dying of non-cardiac disease conditions in the hospital, undergoing routine medical autopsies in the department of Histopathology with duly informed consent from patient's relatives. The patients dying of inflammatory conditions including rheumatic heart disease, myocarditis, infective endocarditis and auto-immune diseases involving heart and cases with history of coronary artery disease or ischemic heart disease were excluded from the study.

2.1. Histomorphology

In each heart, mitral valve apparatus were excised at anterior and posterior leaflets along with their chordate tendine and a part of papillary muscle. They were processed routinely for paraffin embedding and stained with Hematoxylin and Eosin (H & E) for histomorphology. Histochemical stains including Masson trichrome (MT) was done to determine the distribution of collagen and extent of fibrosis, along with Elastic Van Gieson stain (EVG) for elastic tissues. To assess the accumulation of mucopolysaccharide material, Periodic Acid Schiff Alcian blue stain (PAS-AB) was done.

2.2. Immunohistochemistry

The antibodies used immunohistochemistry (IHC) included:

a) Primary antibodies:
   - Biglycan: a goat polyclonal antibody raised against a peptide in an internal region of biglycan of human origin (Santa Cruz Biotechnology, Inc., Europe)
   - Decorin: a mouse monoclonal antibody raised against full length recombinant decorin of human origin ((Santa Cruz Biotechnology, Inc., Europe)

b) Secondary antibody: biotin-conjugated secondary antibody (Novacastra Laboratories Ltd, United Kingdom)

Formalin fixed paraffin embedded (FFPE) tissue sections used for IHC. The sections were incubated with primary and secondary antibodies using standard IHC protocol.[18]

2.3. Criteria of positivity

The tissue sections were considered positive when >25% of the section showed brown colored staining by immunohistochemistry. The antibody positivity was described as mild, moderate and strong according to the percentage & intensity of positive field.

Negative: <25% staining
Mild (+): 26–50% positive
Moderate (++): 51–75% positive
Strong (+++): >75% positive

2.4. Positive control

A case of MVP with surgical excision of mitral valve was used as positive control for decorin and placental decidual tissue as positive control for biglycan.

2.5. Negative control

2.5.1. By omitting the primary body

Each section was examined under light microscope by two observer's unbiasely (Saikia UN & Saini N). The morphometry

Table 1 – Age and sex distribution of study group.

Hearts	Group 1 (1st decade)	Group 2 (2nd decade)	Group 3 (3rd decade)	Group 4 (4th decade)	Group 5 (5th decade)	Group 6 (6th decade)
Males						
Numbers	5	5	5	5	5	5
Mean age ± SD	14 ± 2.82	26.6 ± 3.2	38 ± 3.08	47 ± 2.91	53.2 ± 1.78	64 ± 1.87
Age range	10–20	21–30	31–40	41–50	51–60	61–70
Females						
Numbers	5	5	5	5	5	5
Mean age ± SD	18.2 ± 2.04	23.2 ± 1.92	36.6 ± 2.3	46.8 ± 2.94	56.2 ± 2.48	64.8 ± 2.28
Age range	10–20	21–30	31–40	41–50	51–60	61–70

was done with the help of V-Test software. The selected sections were the selected sections were photographed by using Nikon CCD camera. The various age-related changes were reported as mild (+), moderate (++) and severe (+++). Qualitative data is expressed as numbers and percentages and quantitative data as range and mean ± SD.

2.6. *Ultrastructural study*

Small tissue piece (1–4 mm) fragments of valve leaflets were fixed in 3% glutaraldehyde in Sorensen's phosphate buffer (pH 7.2–7.4) on a non-absorbent surface. Semi-thin sections (0.5 μm) were cut using glass knife on Ultracut-E ultramicrotome. The specimens were processed for conventional electron microscopy and embedded in Epoxy resin. Sections of 60–90 mm thickness were double stained with uranyl acetate and lead citrate[19,20] and examined under Transmission electron microscope equipped with photo documentation to study the age related changes in mitral valve leaflets.

2.7. *Results*

To assess the age related histomorphologic alterations, 60 hearts (30 males and 30 females) were divided into 6 groups according different age decades (Table 1).

2.8. *Light microscopy*

In all the samples, as the age advanced, the histologic alterations included myxomatous degeneration, fibroelastic deficiency, collagen alterations, and mucopolysaccharide accumulation in the valve leaflets. In addition fibrosis and collagen deposition were noted in the chordae tendinae and papillary muscles also. Myxomatous degeneration was characterized by expansion of the spongiosa with accumulation of myxomatous substance which appeared as pale areas extending into the fibrosa having less dense collagen. Collagen alterations appeared as fragmentation of collagenous bundles within the fibrosa. The fibrosa layer was seen pale and pale eosinophilic with disintegration, fraying collagen fibers. Elastic fiber alterations were characterized by disrupted, fragmented, and granular elastic fibers that form an amorphous clump. The number of elastic fibers was also increased in the area of fibrosis of late stage.

We observed mild fibrosis (+) and MPS deposition in younger age groups whereas the degree of fibrosis and MPS deposition became moderate to severe (++ to +++) in older age groups (Fig 1A, B, C). Among 60 mitral valves sampled, mild fibrosis was present in 4 cases (40%) of 2nd decade, 8 cases (80%) in 3rd decade, 4 cases (40%) in 4th and 5th decades, and 6 cases (6%) in 6th decade and one case (10%) in 7th

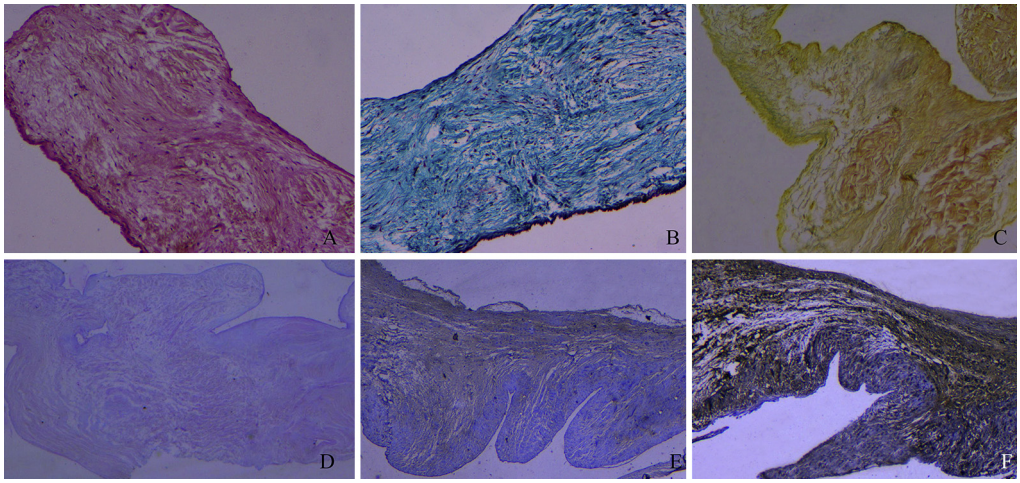

Fig. 1 – Microphotograph of mitral valve leaflets showing (A) thickened fibrosa and spongiosa (H&EX10X). (B) Masson's Trichrome stain highlighting fibrosis with collagen deposition (10X). (C) Elastin Van Gieson stain showing elastic fiber degeneration (10X). (D) Alcian blue – Periodic Acid Schiff stain showing mild accumulation of degenerated mucopolysaccharide material (4X). (E) IHC showing mild biglycan positivity (4X). (F) IHC showing moderate decorin positivity (4X).

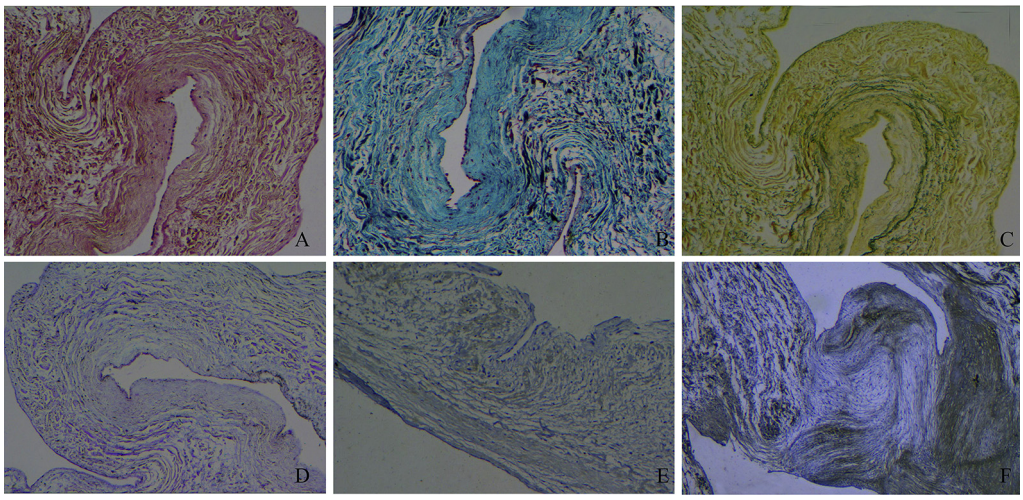

Fig. 2 – Microphotograph of mitral valve leaflets showing (A) thinning fibrosa and expansion of spongiosa (H&EX10X). (B) Masson's Trichrome stain highlighting reduced amount of collagen (10X). (C) Elastin Van Gieson stain showing elastic fiber fragmentation (10X). (D) Alcian blue – Periodic Acid Schiff stain showing moderate accumulation of degenerated mucopolysaccharide material (10X). (E) IHC showing mild biglycan positivity (10X). (F) IHC showing moderate decorin positivity (4X).

decade. Majority of the mitral valves (90%) in 7th decade showed moderate fibrosis, however severe fibrosis was found only in 10% cases in 6th decade (Fig 2B). The incidence of fibrosis was comparatively higher in females as compared to males. The most striking change with age was the accumulation of MPS material which was mild in 60%, 20% and 10% cases in 2nd, 3rd and 5th decades respectively (Fig 2C) whereas it was moderate to severe in 4th to 7th decades and maximum in 7th decade (90%) (Table 2). Inflammation without neovascularistaion within the leaflets was observed only in one case each in 6th and 7th age decades respectively.

The chordae tendinae appeared hyalinized and fibrosed with increasing age. Mild fibrosis of chordae tendiane was found in 20% cases in 3rd decade, 60%, 40% and 30% cases in 4th, 5th and 6th decades respectively, whereas moderate fibrosis was present in 30% and 100% cases in 4th and 7th decades respectively (Fig 3A, B). Mild MPS accumulation was observed in chordae tendinae in 60% and 20% cases in 4th and 6th decades respectively, while moderate MPS accumulation was reported in 20% cases in 7th decade (Fig 3C). No calcification and inflammation was reported in any of the chordae tendiane.

The papillary muscles exhibited mild to moderate degree of fibrosis with advancing age. Mild fibrosis was observed in all samples with a minimum (10%) and maximum (50%) incidence in 2nd and 6th decade respectively. Also moderate and severe fibrosis was found in 40% and 10% cases 7th decade respectively. Focal calcification was seen in 10% of papillary muscles in both males and females in 7th decade. Scattered inflammatory cells were observed within the papillary muscles in 10% cases of 2nd and 3rd decades.

To confirm MPS deposition, Periodic Acid Schiff Alcian blue stain (PAS-AB) showed increased positivity progressive with age. The valve leaflets and chordae tendinae with moderate to severe MPS deposition also showed strong AB positivity (Figs. 1D and 2D). Mild AB positivity was observed in 2 (20%) and 1(10%) cases in 2nd and 3rd decades respectively whereas moderate positivity was found in 1 (10%), 3(30%), 5 (50%) and 1 (10%) cases in 4th, 5th, 6th and 7th decades respectively. AB positivity was markedly increased and became severe in 5th, 6th and 7th decades in 7 (70%), 5 (50%) and 9 (90%) cases.

2.9. *Immunohistochemistry*

The distribution of proteoglycans (PGs) i.e decorin and biglycan was observed in different layers of valve leaflet by IHC (Figs. 1E, F and 2E, F). Thirty one (51.66%) of 60 cases had mild

Table 2 – Prevalence of fibrosis and mucopolysaccharide accumulation in mitral valve leaflets.

Age range	Number of cases ($n = 60$)	Fibrosis			Mucopolysaccharide accumulation		
		Mild (+)	Moderate (++)	Severe (+++)	Mild (+)	Moderate (++)	Severe (+++)
10–20	10	40%	–	–	60%	–	–
21–30	10	80%	–	–	20%	–	–
31–40	10	40%	60%	–	–	80%	20%
41–50	10	40%	60%	–	10%	20%	70%
51–60	10	60%	20%	10%	–	30%	70%
61–70	10	10%	90%	–	–	10%	90%

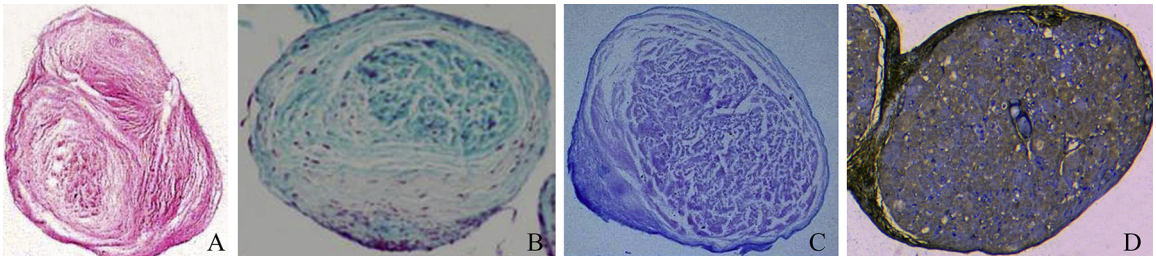

Fig. 3 – Microphotograph of chordae tendinae showing (A) hyalinization and fibrosis with MPS deposition (H&EX4X). (B) Masson's Trichrome stain highlighting fibrosis (10X). (C) Alcian blue – Periodic Acid Schiff stain showing moderate accumulation of degenerated mucopolysaccharide material (10X). (D) IHC showing moderate decorin positivity (10X).

decorin positivity, out of which maximum positivity (15%) was seen in 7th decade. Moderate to severe decorin positivity was found in 18/60 (30%) and 1/60 (1.66%) cases respectively. Biglycan positivity was observed in 34/60 (56.66%) and 8/60 (13.33%) cases respectively ranging from mild to moderate. Strong biglycan positivity was not seen in any of the sample. The decorin expression was observed more in the areas of thick fibrous tissue along the atrialis and ventricularis where the collagen fibers were densely arranged whereas the biglycan expression was more in pale areas where more MPS material and less dense collagen fibers were present.

2.10. *Ultrastructural study*

To assess the age related ultrastructural alterations, 15 hearts were divided into 3 groups: Group 1 (age 10–20 years, mean age: 14.8 ± 3.89 years), group 2 (age 30–45 years, mean age: 36.6 ± 3.20 years) and group 3 (age above 55 years, mean age: 62.6 ± 4.14 years).

In group 1, all the samples showed abundant collagen fibers which were closely packed having thin and parallel strands with stacks along with interspersed elastic fibers (Fig 4A). In the advancing age i.e group 2, the collagen seemed to be more loosely arranged in stacks. However no significant changes were noted. With further age advancement, in group 3, collagen fibers were fragmented and haphazardly arranged in many areas. In between the collagen fibers, certain inclusions of MPS material surrounded by electron dense granules were observed (Fig 4B). In addition, there was granular degeneration of collagen fibers in this group. The numerous glycogen granules were found in the areas of extensive collagen damage. Also numerous fibroblasts were observed along with the dense collagen fibers containing osmiophilic dense bodies and vacuoles (Fig 4C). We did not observe any myelin figures or any other mitochondrial inclusions.

3. Discussion

Degenerative mitral valve disease is responsible for the syndromes of billowing of mitral leaflet, mitral valve prolapse (MVP), floppy mitral valve, and flail leaflet.[21–24] The pathology of these is mainly caused by age related degenerative changes like myxomatous infiltration and fibroelastic deficiency.[17] The underlying process in the pathogenesis of mitral value prolapse is a continuing cellular proliferative response to repeated minor injury occurring in the prolapsing valve which results in increased production of collagen and mucopolysaccharide.[25] We analyzed the histomorphological and ultrastructural changes in mitral valve apparatus with advancing age in various age groups.

We observed that the major age related degenerative changes in valve leaflets were fibrosis and MPS deposition which serve as the probable underlying causes of MVP. We found mild degree of fibrosis and MPS accumulation in younger age groups (6%) compared to older age groups where degree of fibrosis and MPS deposition was moderate or severe (38.33%). In 2nd decade (age 10–20 years), fibrosa layer was

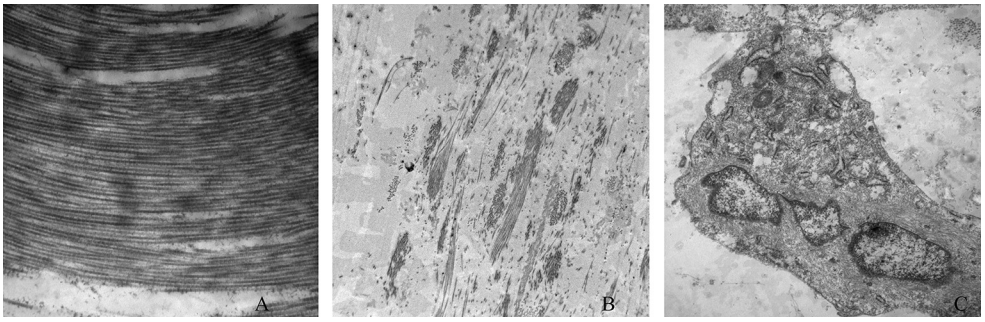

Fig. 4 – Ultrastructural photographs of mitral valve leaflets showing (A) compact stacks of collagen fibers (8400X). (B) Fragmentation of collagen fibers with clump formation (3900X). (C) Fibroblast with the dense collagen fibers containing osmiophilic dense bodies, vacuoles and rough endoplasmic reticulum (3900X).

composed of closely packed, parallel, thin collagen and elastic fibers. Among 10 mitral valves of first decade, mild fibrosis was present in 4/60 cases (40%) whereas mild MPS accumulation was observed in 6/60 (60%) cases. Previous study on mitral valves of age below 10 years revealed the similar findings where the fibrosa layer was composed of parallel, evenly stained collagen and elastic fibers with moderate PAS-positivity in the fibrous tissue. However we did not find any significant PAS positivity in any of our cases in this group.

Majority of the mitral valves (90%) in 7th decade showed moderate fibrosis whereas severe fibrosis was found only in 10% cases in 6th decade. Moderate to severe MPS accumulation was observed in 4th to 7th decades, maximum being in 7th decade with an incidence of 90%. Alcian blue (AB) staining positivity was also expressed as moderate to severe in these age groups. These results are in accordance with a previous study in which collagen fibers became dense and fibrosed after losing their parallel orientation from 3rd to 6th decades and showed progressive increase in PAS-positivity.[13] The explanation to age-dependent accumulation of collagen in valves is the increased number of cross-links between the collagen fibers, particularly between their amino acids (histidinoalanine and pentosidine) and accumulation of advanced glycation end-products in collagen fibers with age.[26] Another study highlights that the degradation of collagen fibers by specific matrix metalloproteinases reduced with advancing age which results in excess accumulation of collagen fibers within valve leaflets.[27]

We also observed that with increased MPS material, spongiosa was seen expanding into the fibrosa, causing loosening of the valve leaflet and making it prone to prolapse. This myxomatous transformation of the mitral valve leaflets due to proliferation of spongiosa is thought to be the pathologic hallmark of the primary (or idiopathic) variety of MVP syndrome.[28]

The ultrastructural features in various age groups also supported the histologic findings. In younger age group, abundant collagen fibers were closely arranged having thin and parallel strands with stacks along with interspersed elastic fibers. As the age advances, the collagen appeared more loosely arranged in stacks. Collagen fibers were fragmented and frayed in many areas with abundant glycogen granules in older age group. Numerous inclusions of MPS material surrounded by electron dense granules were observed in between the collagen fibers. Fibroblasts were observed along with the dense collagen fibers containing osmiophilic dense bodies and vacuoles. However to support our observation there is no such study available in the English literature to the best of our knowledge.

It is suggested that with increasing age, the regular wavy pattern of collagen changes and they eventually become randomized.[29] Further, the dense collagenous core reduces in cross-sectional area and MPS starts accumulating which may lead to stretching of chordae tendinae and become the underlying cause of chordae tendinae rupture (CTR).[30] In our study, the chordae tendinae appeared hyalinized and fibrosed after losing their normal wavy arrangement in older age groups. Mild degree of fibrosis was found in 4th, 5th and 6th decades respectively, whereas moderate fibrosis was seen in 4th and 7th decades. Mild MPS accumulation was also observed in chordae tendinae in 60% and 20% cases in 4th and 6th decades respectively, while moderate MPS accumulation was seen in 20% cases in 7th decade. Previous study explaining structure of chordae tendinae suggested that the collagen fibers in young subjects were arranged at regular periodicity and with age, they become elongated with irregular broad striped pattern.[29] Our observations are in accordance with this study suggesting that the process of gradual disorganization of collagenous core of chordae tendinae is related to aging.

Mild to moderate degree of fibrosis was found in papillary muscles with advancing age. Majority of the papillary muscles (31.66%) showed mild fibrosis, however moderate and severe fibrosis was found in 10% and 1.66% cases respectively. Similarly to our findings diffuse type of papillary muscle fibrosis; probably an aging change was present in almost half of the autopsies along with focal fibrosis in a previous study.[31] Fibrosis within the papillary muscles could limit the ability of the muscles to contract or to sustain contraction, thus compromising the tautness of chordae tendineae which in turn could cause eversion of the mitral leaflet into the left atrium and cause reguritation.[28]

IHC moderate to severe decorin positivity was found in 18 (30%) and 1 (1.66%) cases respectively whereas mild and moderate biglycan positivity was seen in 34 (56.66%) and 8 (13.33%) cases respectively. Decorin expression was observed in the areas of thick fibrous tissue along the atrialis and ventricularis where the collagen fibers were densely arranged whereas the biglycan expression was more in pale areas where more MPS material and less dense collagen fibers were present. Thus our results suggest a negative association between the expression of decorin and biglycan proteoglycans. In a previous study of localization of decroin and biglycan, authors have found decorin was strongly colocalized with collagen fibers and is in accordance with our findings whereas biglycan was found in both collagen and elastin rich regions.[32] However our results did not show increased iglycan expression in the fibrous areas. This may be associated with the presence of other matrix components i.e versican and hyaluronic acid in mitral valves which were not done in our study.

In conclusion we suggest that degenerative mitral valve changes is an age dependent condition caused by collagen disruption, elastic fibers fragmentation, calcification and accumulation of mucopolysaccharides, and proteoglycans within the valve leaflet which predisposes it to MVP. This is undetected most of the times in the clinical settings until the symptoms become severe. Hence it is important to screen patients even with the mildest symptoms to rule out an overt MVP disease. This may help in early detection of MVP with timely surgical correction if required.

Limitations of our study

This study is limited by less number of cases and non-inclusion of surgical tissue. Other types of proteoglycans and MPS material also need to be studied.

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Conflicts of interest

All authors have none to declare.

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