CONTINUING EDUCATION IN TOXICOLOGIC PATHOLOGY
RESPIRATORY AND CARDIOVASCULAR SYSTEM

Fourth Conference

ORGANIZED BY

SOCIETY OF TOXICOLOGIC PATHOLOGY - INDIA (STP-I)

NOVEMBER 1-3, 2012

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Drug-induced Valvulopathy

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Fourth STP-I Conference, 1-3 November 2012

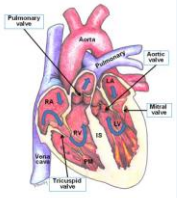
Points to cover

- Heart valve outlook
  - Comparative functional histology
- Drugs known to cause valvulopathy in humans & pathogenesis
- Animal models to screen drug-induced valvulopathy?

Heart valves

- Four heart valves
  - Semilunar valves = aortic & pulmonary valves
  - Atrioventricular valves = mitral & tricuspid valves
- Play a critical role in ***unidirectional hemodynamic flow***

Defects → Serious health consequences

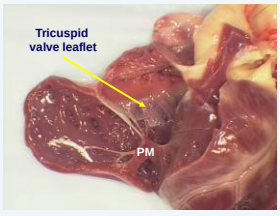


Heart valves

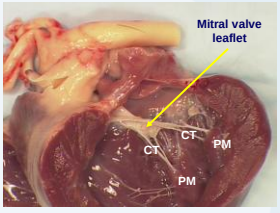
- Screening of drug-induced valvular effects – Preclinical toxicity studies
- Commonly used preclinical species – Gross evaluation of heart valves

☑ **Dogs, non-human primates**
☒ **Not routinely in rodents ➲ small heart size**

Atrioventricular (AV) valves: Mitral & Tricuspid valves



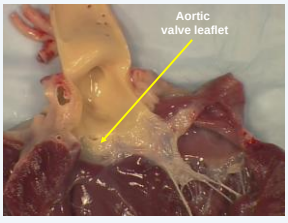
Tricuspid valve, dog



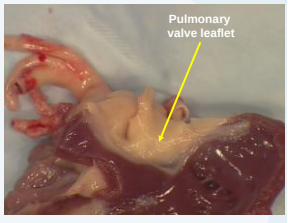
Mitral valve, dog

Valvular apparatus – annulus, leaflets, chordae tendineae (CT) & papillary muscle (PM)

Semilunar valves: Aortic & Pulmonary valves



Aortic valve, Dog



Pulmonary valve, Dog

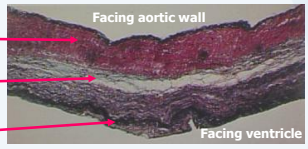
Histology of heart valves

- Described inconsistently
  - Veterinary medicine
- Deficiencies – Rodents
- Valvular cell-types & functional updates

Heart valves (Human)

- Three layers – Atrioventricular & Semilunar valves*

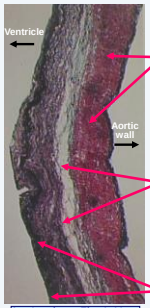
1. Fibrosa
2. Spongiosa
3. Ventricularis



Aortic valve – Elastic Van Giesen, (collagen – red; elastic fibers – black)

*Liu et al. (2007). Am J Pathol; Schoen FJ (2005). Cardiovascular Pathol; Schoen FJ (2005). Robbins and Cotran Pathological Basis of Disease, 7th ed.

Functional Histology of Heart Valves - Human



Aortic valve – Elastic Van Giesen, Collagen – red; Elastic fibers – black

Layer	Predominant component	Function
Fibrosa	Dense collagen fibers, ~ Valvular Interstitial Cells (VICs) & very fine elastic fibers	Provide strength, stiffness & maintain coaptation during diastole, prevents regurgitation (**mechanical integrity**)
Spongiosa	Glycosamino-glycans, ~ Loose collagen fibers & VICs	Absorb shear forces & cushion shock between layers during cyclic valve motion (**shock absorber**)
Ventricularis	Elastic fibers & ~ VICs	Extend in diastole & contract in systole

Atrioventricular & Semilunar valves (Human)

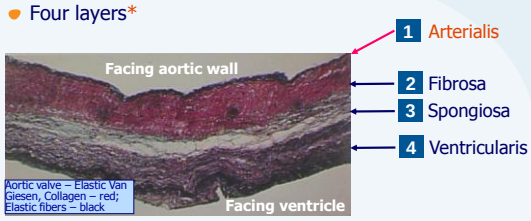
- ~~Three layers: Fibrosa, Spongiosa & Ventricularis – Most recent publications & textbooks~~
- **Four layers –** Publications & Textbook*

1. ***Arterialis*** – facing arterial wall (semilunar)
   Auricularis/atrialis – facing atrium (atrioventricular)
2. Fibrosa
3. Spongiosa
4. Ventricularis

* Pellerin et at., 2002, Schoen & Edwards, Cardiovascular Pathology, 2001

Aortic valve – Human

- Four layers*



Aortic valve: Arterialis (facing arterial wall) – elastic fibers

*Pellerin et at., 2002, Schoen & Edwards, Cardiovascular Pathology, 2001

Heart valves (Veterinary Histology)

Heart valves:

- **Four** layers to
- **No mention** of specific layers

Examples: Atrioventricular valves

- **Two layers**©
  - **(1)** Spongiosa (Stratum spongiosum)
  - **(2)** Fibrosa (Stratum fibrosum)

©Dellmann's Textbook of Veterinary Histology, ***2006***
©Jubb, Kennedy & Palmer's Pathology of domestic animals, ***2007***

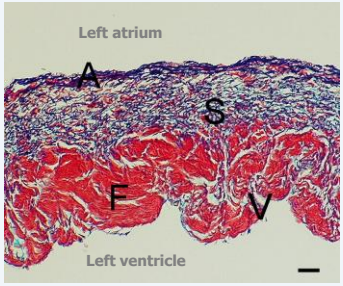
- **No specific mention of layers**© **–** Fibrous core, also known as fibrosa

©Handbook of Toxicologic Pathology, 2002

- ***2009*** **Publications*** **– Four layers** (atrialis, spongiosa, fibrosa & ventricularis)

*Aupperle et al. (2009). J Comp Pathol
Aupperle et al. (2009). The Veterinary Journal

Mitral valve – Dog



Atrialis (A)

Spongiosa (S)

Fibrosa (F)

Ventricularis (V)

Atrialis (A) violet; Spongiosa (S) torquoise; Fibrosa (F) red & Ventricularis (V), Picrosirius red stain (×100, bar = 100 µm).

*Aupperle et al. (2009). J Comp Pathol; Aupperle et al. (2009). The Veterinary Journal

Valves = Cells & Extracellular matrix

- **Valvular cells** = I. Valvular Endothelial Cells & II. Valvular Interstitial Cells
- **I. Valvular Endothelial Cells[1] (VECs)**
  - Lining valvular surface; highly responsive to chemicals & mechanical forces
  - Display heterogeneity[1] – Differences in gene expression (~ 400 genes) profiles & phenotype
    - VECs ≠ Aortic endothelial cells
    - VECs lining aortic side ≠ ventricular side
    - Implications of these differences – not yet known

[1]Schoen (2006). Cardiovascular Pathology; Butcher et al. (2006) Arterioscler Throm Vasc Biol

Cellular updates in valves

- **II. Valvular Interstitial Cells (VICs) –** Most prevalent cells & found in all layers (i.e. fibrosa, spongiosa & ventricularis)
  - Described as ~~***valve fibroblasts, myofibroblasts or smooth muscle cells***~~ (≈ morphologic & functional similarities)
  - **[1]Recommended to abandon these terminologies & should be replaced by VICs**
    - Specific features at the time of embryonic endothelial-to-mesenchymal transformation
    - VICs are not smooth muscle cells (SMCs)
      - SMCs – Intact basement membrane
      - VICs – Incomplete basement membrane

[1]Liu et al. (2007). Am J Pathol

Valvular Interstitial Cells (VICs)

- **Currently five identifiable phenotypes**
  1. Embryonic progenitor endothelial/mesenchymal (EPE/M) cells
  2. Progenitor VICs (pVICs)
  3. Quiescent VICs (qVICs)
  4. Activated VICs (aVICs)
  5. Osteoblastic VICs (obVICs)
- **Transitions – among various phenotypes**
  - e.g. qVICs → aVICs; pVICs → qVICs & so on

Valvular Interstitial Cells (VICs)[1]

Cell type	Location	Functions & Markers
EPE/M cells[2]	Embryonic cardiac cushions	**Give rise to resident qVICs** during the process of valve formation in embryo; Detected by the loss of endothelial & the gain of mesenchymal markers
Progenitor (pVICs)	Bone marrow, circulation, &/or heart valve leaflet	**To provide aVICs – Valve repair/remodeling (i.e. another source of aVICs),** may be CD34-, CD133-, &/or S100-positive
Quiescent (qVICs)	Heart valve leaflet	Maintain **physiologic valve structure & function** (inhibition of angiogenesis in the valve leaflets)
Activated (aVICs)	Heart valve leaflet	Valvular injury/disease conditions, qVICs → aVICs; **aVICs – features of myofibroblasts, express α-SMA & other contractile proteins (e.g. striated muscle isoform of myosin heavy chain)**; repair (proliferation, migration & matrix remodeling)
Osteoblastic (obVICs)	Heart valve leaflet	Regulate – valvular calcification, chondrogenesis & osteogenesis; secrete alkaline phosphatase, osteocalcin, osteopontin & bone sialoprotein

[2]EPE/M cells = Embryonic progenitor endothelial/mesenchymal cells

[1]Fayet et al. (2007). Cardiovascular Pathology; Liu et al. (2007). Am J Pathol

Valvular Extracellular Matrix (ECM)

- Valvular ECM changes – Injury & disease conditions
- Three primary components: Collagens (I, III & V), Elastin & Proteoglycans (PGs – decorin, biglycan, versican & hyaluronan)
- All glycosaminoglycans (GAGs) – components of PGs except for Hyaluronan (unsulfated form)
  - Secreted by aVICs
  - Decorin & biglycan (4-sulfated) – abundant in tensile loading regions (e.g. chordae tendineae)
  - Hyaluronan & versican (6-sulfated) – regions experiencing compression (i.e. free edges – leaflet)
  - ***Distinctive GAGs changes in drug-induced valvulopathy & other conditions***

Rodent heart valves

Rodent heart valves

- **Histological assessment of *all four heart* valves – Not routinely done in toxicity studies**

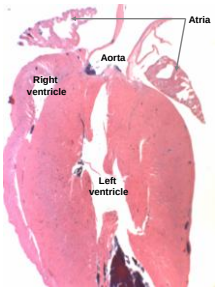
  1. Small size and often missed

  2. No consistency – Trimming/Sectioning
  - Longitudinal section (most labs): >1 or no valves
  - Combination of longitudinal & transverse sections

  Advantage – to diagnose myocardial hypertrophy

  3. Hardly request for re-cut of missing valves

Rodent Heart Trimming – Books



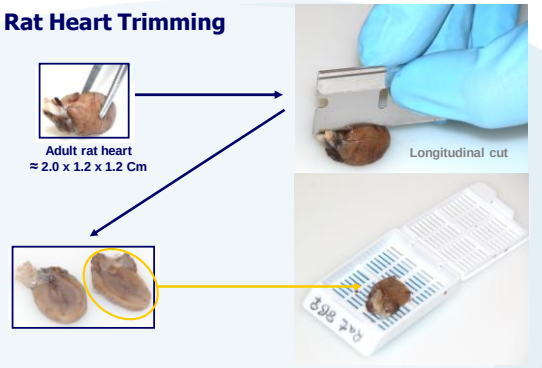
"For most studies, the heart is sectioned through its *longitudinal axis to include one or ideally both the right and left ventricle, interventricular septum, portions of both atria, and the major vessels* at the base of the heart."

Pathology of the Mouse, Cache River Press, 1999

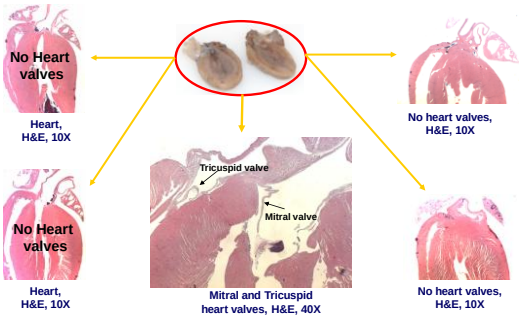
In small laboratory animals, a *longitudinal section through the heart taken perpendicular to the ventricular septum is often sufficient."*

Handbook of Toxicologic Pathology, Academic Press, 2002

Rat Heart Trimming



Inconsistent Rodent Heart sections



Rodent heart valves (contd.)

- **Generally under-evaluated and under-represented tissue**
- **Concentrated in myocardial changes**
  > Often ignore the examination of valves

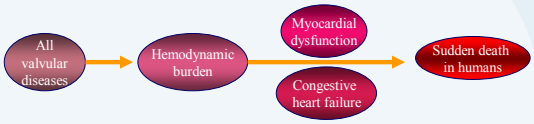
Citation from the book chapter…

"Whereas histopathological examination of the ***myocardium is the principle component of cardiovascular assessment in toxicity studies***, histological examination of blood vessels, measurements of heart weight, blood pressure and heart rate as well as electrocardiography are important."

Histopathology of Preclinical Toxicity Studies, Elsevier, ***2007***

Heart valves

- **Very relevant tissue**



- **Drug-induced valvulopathy in humans**
- **Drug-induced valvulopathy in animals**

Drug-induced valvulopathy in humans

- **Anorexigens (anti-obesity drugs)**
  Fenfluramine (Pondimin®),
  Dexfenfluramine (Redux®)
- **Anti-migraine**
  Ergot alkaloids – Ergotamine,
  Dihydroergotamine, Methysergide
- **For Parkinson disease & Hyperprolactinemia**
  Ergot derivatives – Pergolide (Permax®),
  Cabergoline (Dosinex®)
- **For metabolic syndrome & Type 2 diabetes**
  Benfluorex (fenfluramine derivative, Mediator®)
- **Recreational – Ecstasy**
  3, 4-Methylenedioxymethamphetamine (MDMA)

Anorexigen-induced Valvulopathy

- 1997: First report of valvulopathy by ***Fen-Phen*** (Fenfluramine + Phentermine) in 24 patients – Connolly et al., Mayo Clinic
- Fenfluramine – 5HT (5-Hydroxytryptamine) reuptake inhibitor
  Phentermine – Norepinephrine/dopamine reuptake inhibitor
- Incidence of valvulopathy:
  - 38% (US department of Health & Human services)
  - Other studies: 5-15%
  - Most recent population-based study: 32% (mild aortic regurgitation) (Palmieri et al., AJM 2002)
- Fen-Phen medication for > 6 months increases the risk

Anorexigen-induced Valvulopathy

- September 1997: Fenfluramine (Pondimin®) and Dexfenfluramine (Redux®) were withdrawn from the US market
- One of the costliest drug recalls in US history > 21 Billion dollars

2012: Benfluorex (Mediator®, Servier)

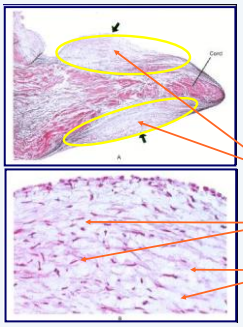
- Fenfluramine derivative, licensed for use by diabetics but widely prescribed in France as a ***slimming aid***
- In 2009, Benfluorex – pulled from the European market
  ⛔ Valvulopathy & Pulmonary hypertension
- **2012 reports –**
  - Around 3,100 people required hospitalization
  - At least 1,300 deaths (estimated death toll between 1,000 & 2,000)
  - Deaths from faulty heart valves among major users
- Its French manufacturer, Servier is being probed on suspicion of dishonest practices & deception

Anorexigen-induced Valvulopathy

- Distribution:
  - Aortic & mitral valves
  - Little or no effect on tricuspid & pulmonary valves
- Thickening of affected valves due to
  - Proliferation of myofibroblasts*/**aVICs**
  - Increased extracellular matrix, primarily GAGs (glycosaminoglycans)
- Microscopically similar to:
  1. Valvulopathy induced by ergot alkaloids (ergotamine & methysergide)
  2. Valvulopathy – Carcinoid syndrome

*Connolly et at., 1997; Steffe et al., 1999

Fen-Phen associated valvulopathy in humans



- 44-Year old woman*
  - Treated with Fenfluramine (20mg, t.i.d.) & Phentermine (30mg o.d.) for 1 year
- Dyspnea & heart murmur
- Thickened mitral valve

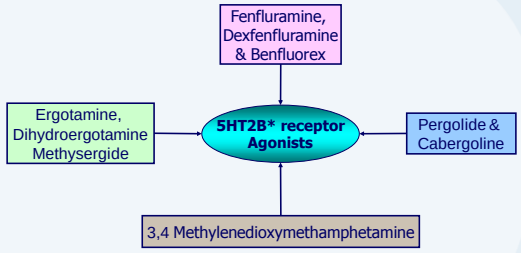
Subendocardial fibromyxoid tissue

Myofibroblasts (**aVICs**)

Extracellular matrix (clear spaces)

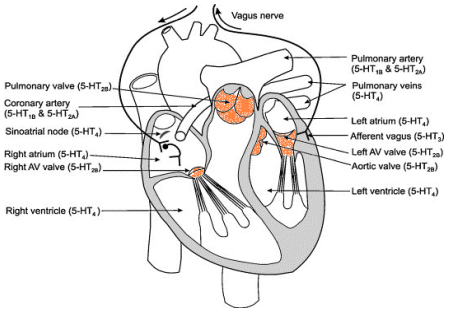
*Connolly et al., Mayo Clinic, NEJM 1997

Mechanism of valvulopathy



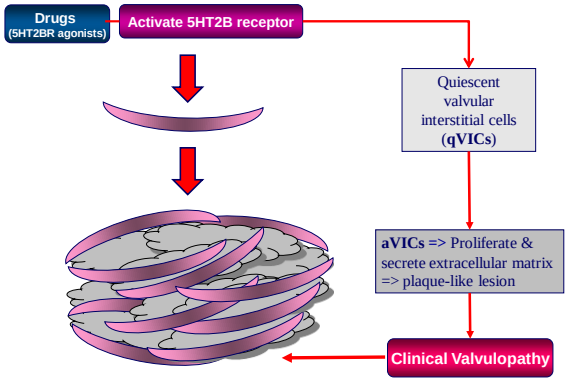
***5HT2B = 5-Hydroxytryptamine 2B**

Location of 5HT2BR (humans) – All four valves



Kaumann & Levy. Pharmacol Therap, 2006

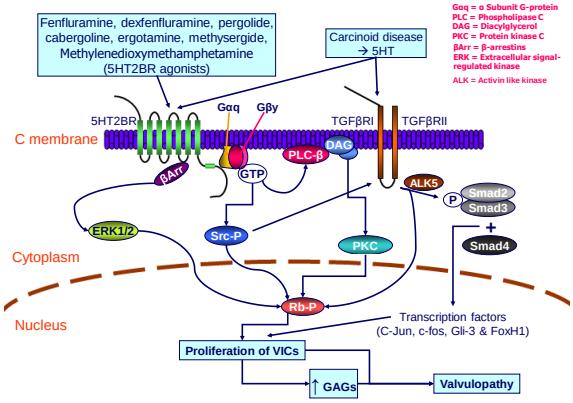
5HT2BR-related mechanism



5HT2BR stimulation

Valvulopathy

- ❑ Complex
- ❑ Not fully understood



*Elangbam, 2010

Does anorexigen exposure produce a distinctive morphological lesion?

- An interesting finding from Dr. McManus's group (St. Paul's Hospital, Canada), 2002
- Quantitative analysis of human heart valves from various disease conditions:
  1. **Anorexigen-exposed mitral valve**
  2. **Rheumatic** – Immunologically mediated (group A streptococci?)
  3. **Carcinoid** – Tumor secreting 5HT
  4. **Floppy/Prolapse** – Mutations in gene encoding fibrilin (Marfan Syndrome)?

Dr. McManus's group concluded……

Anorexigen-exposed heart valves:

Distinctive microscopic features that separate from normal valves and valve lesions with other pathologies –

Degree of GAGs (glycosaminoglycans) deposition
Infiltration of leukocytes
Presence of vessels (neovascularization)

PRECLINICAL TOXICOLOGY

No validated preclinical models exist for the anorexigen-induced heart valve lesions

In-vivo models to screen drug-induced valvulopathy ?

Two Reasons

- **Valvulopathy liability**
  - Lessons from Fenfluramine & Dexfenfluramine
  - Particularly serotonergic compounds (5HT2B agonists) & for prolonged indication (e.g. Anti-obesity)

- **GSK compound 'X': 2-Year Oncogenicity Study**
  - Significant increase in the incidence/severity of mitral valvulopathy in high-dose rats
  - Exacerbation?

Compound 'X' - Background

- Sympathomimetic compound – treatment of obesity & depression
- Significant increase in the incidence/severity of mitral valvulopathy (endocardial myxomatous change) in high-dose rats
- **Microscopically similar to Fen-Phen associated valvulopathy in humans**

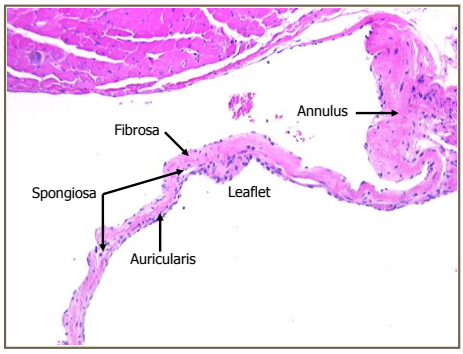


Figure 1. Normal mitral valve leaflet from a 730-day old rat. H&E, 100X

Elangbam et al., Toxicol Pathol 2002

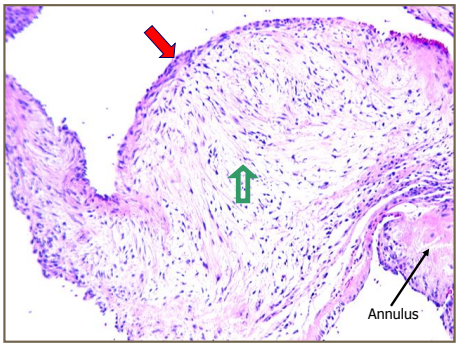


Figure 2. Mitral valve leaflet with valvulopathy from a 684-day old rat. Note marked thickening (solid arrow) of valve leaflet due to fibromyxoid tissue (open arrow) in the subendocardium H&E, 100X.

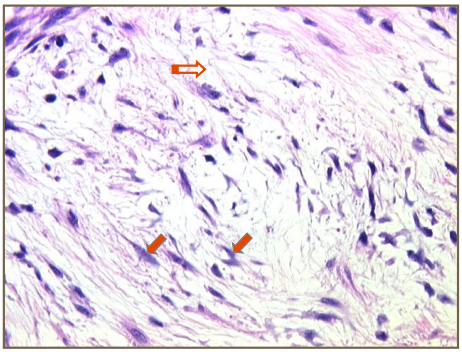


Figure 3. High power of figure 2. Note fibroblast like cells (solid arrows) and adjacent clear extracellular matrix (open arrow). H&E, 400X.

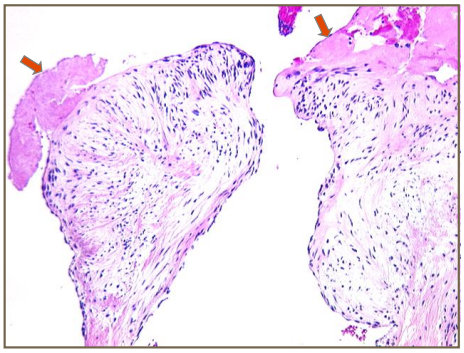


Figure 4. Mitral valve leaflet with valvulopathy from a 643-day old rat. Note Presence of thrombi (solid arrows) on the valve leaflet. H&E, 100X.

Compound 'X': Mitral valvulopathy (MV) in S-D rats

Decided to explore

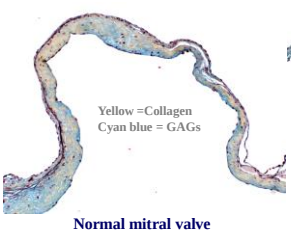
- Role of 5HT receptors in rat MV?
- Compositional changes (GAGs & Collagen) in rat MV?
- In-vivo models to screen drug-induced valvulopathy?

Compound 'X' study

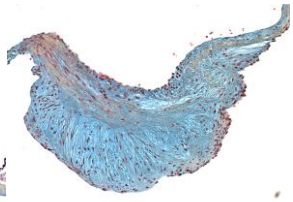
RESULTS:

Compositional analysis
Mitral valve

Movat's Pentachrome Stain, 200X
Black = Elastin; Red = Muscle-like cells
Cyan blue = GAGs; Yellow = Collagen



Normal mitral valve from a 730-day old SD-Rat

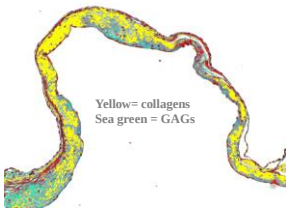


Mitral valve with valvulopathy from a 642-day old SD-Rat

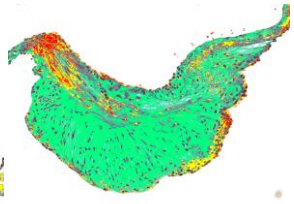
1. **GAGs (blue) = Increased**
2. **Collagen (yellow) = Decreased & disorganized**

Elangbam et al., Exp Toxicol Pathol 58:89-99, 2006

ImagePro color segmentation, 200X



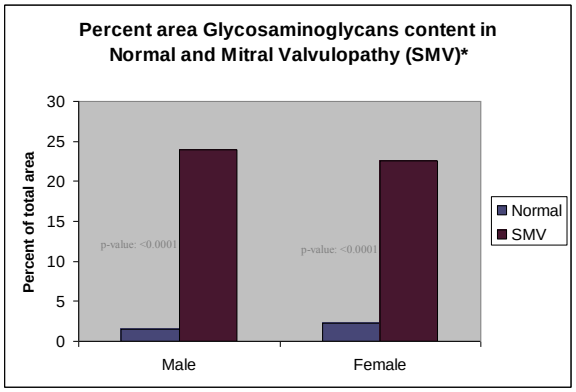
Normal mitral valve



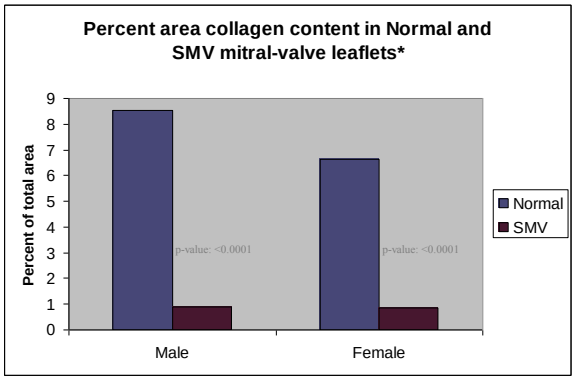
Mitral valve with valvulopathy

1. **GAGs (green) = Increased**
2. **Collagen (yellow) = Decreased & disorganized**

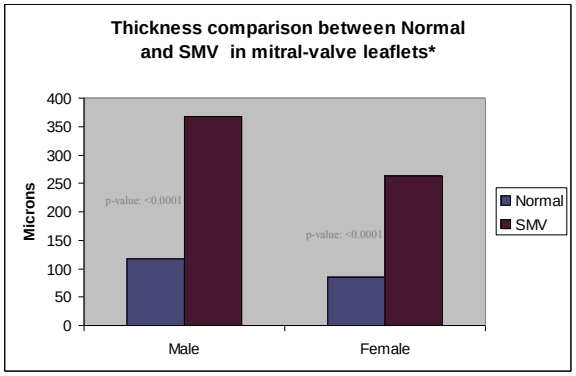
Elangbam et al., Exp Toxicol Pathol 58:89-99, 2006



*Elangbam et al., Exp Toxicol Pathol 58:89-99, 2006



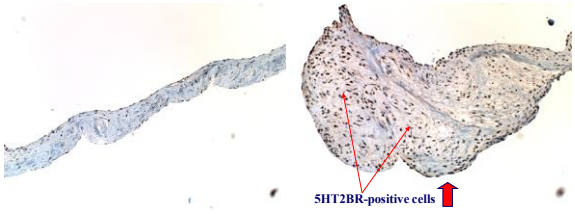
*Elangbam et al., Exp Toxicol Pathol 58:89-99, 2006



*Elangbam et al., Exp Toxicol Pathol 58:89-99, 2006

Immunohistochemistry 5HT2B receptor – Compound X study

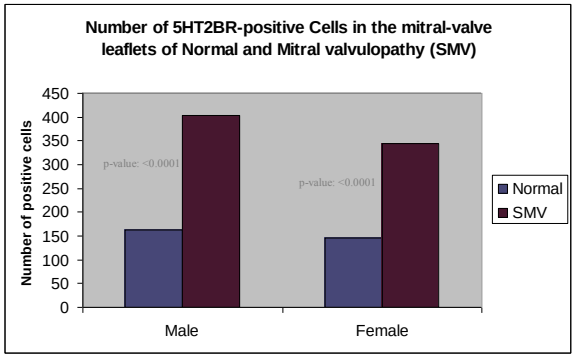
5HT2B receptor Immunohistochemistry



Normal mitral valve, 200X

Mitral valve with valvulopathy, 200X

Elangbam et al., Exp Toxicol Pathol 58:89-99, 2006



Elangbam et al., Exp Toxicol Pathol 58:89-99, 2006

Compound 'X' study – Conclusions

Mitral Valvulopathy in S-D rats

- **Morphology (H&E)**
  - Nodular or segmental thickening of affected heart-valves due to subendocardial fibromyxoid tissue
- **Compositional analysis:**
  - Significant increase in the GAGs content (quantitative analysis)
  - Significant loss/disorganization of collagen
- **These changes – similar to anorexigen associated valvulopathy in humans**

Compound 'X' study – Conclusions

- **Immunohistochemistry:**
  - Significant increase in the number of 5HT2BR-positive cells
  - 5HT2BR may be involved (?)

An investigative study in rats

Animal model: 5HT's role in valvulopathy

7-DAY INVESTIGATIVE STUDY

Experimental design

- 24 CD™ IGS virus antibody free male SD rats, 11 to 12 weeks old, two groups, 12/group

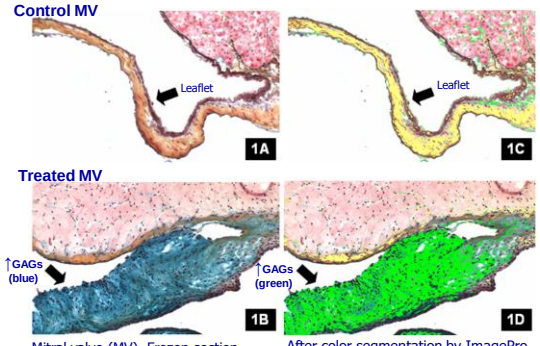
Groups	Treatment & dose
Control	Sterile water, daily s/c injections for 7 days
Treated	5HT, daily s/c injections (75 mg/kg for the first 3 days and 60 mg/kg thereafter) for 7 days

- Mitral & aortic valves – compositional analysis & RT-PCR (5HT2BR & 5HTT)

Results

Mitral & Aortic valves:

- Compositional changes
- Transcriptional 5HT2BR and 5HTT changes

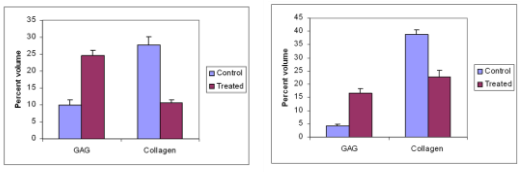


Mitral valve (MV). Frozen section, Movat's Pentachrome, 200X

After color segmentation by ImagePro plus; GAGs (green), collagen (yellow)

Elangbam et al., Exp Toxicol Pathol 60:253-262, 2008

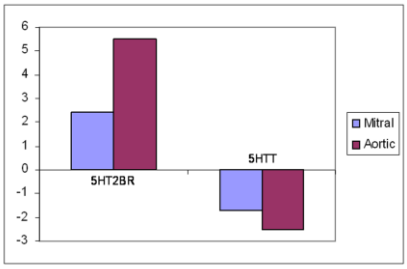
Compositional Morphometry – Percent volume occupied by GAGs and collagen. Bars represent mean ± SE. Note increased percent volume of GAGs ($p \leq 0.0001$) and decreased collagen ($p \leq 0.0001$)



Mitral valve

Aortic valve

Elangbam et al., Exp Toxicol Pathol 60:253-262, 2008



TaqMan data – Log 2 relative fold expression. Note up-regulation and down-regulation of 5HT2BR and 5HTT genes, respectively in mitral and aortic valves of 5HT treated SD rats.

Investigative study - Conclusions

- 7-Day 5HT treatment
  - Compositional alterations in GAGs & collagen
  - Correlated with up-regulation of 5HT2BR & down-regulation of 5HTT genes
- 5HT2BR & 5HTT – Pathogenesis of 5HT-induced vavulopathy
- Animal model for 5HT-induced valvulopathy
  - In vivo-screening for serotonergic drugs with 5HT2BR agonism

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  - Compositional analysis
- Dr. Leah Zadrozny, Former summer GSK Intern (2006) – 7-Day Investigative study
- Dr. Lauren Job, Former summer GSK intern (2007)
  - Compositional analysis