CONTINUING EDUCATION IN TOXICOLOGIC PATHOLOGY
RESPIRATORY AND CARDIOVASCULAR SYSTEM

ORGANIZED BY
SOCIETY OF TOXICOLOGIC PATHOLOGY - INDIA (STP-I)

NOVEMBER 1-3, 2012
The Atria Hotel, # 1, Palace Road, Bangalore - 560 001
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

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

Semilunar valves: Aortic & Pulmonary valves

Atrioventricular (AV) valves: Mitral & Tricuspid valves

- Tricuspid valve, dog
- Mitral valve, dog

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**
   - Facing aortic wall

2. **Spongiosa**
   - Facing ventricle

3. **Ventricularis**
   - Aortic valve – Elastic Van Giesen, collagen – red, elastic fibers – black


**Functional Histology of Heart Valves - Human**

<table>
<thead>
<tr>
<th>Layer</th>
<th>Predominant component</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fibrosa</strong></td>
<td>Dense collagen fibers, ~ Valvular Intersitial Cells (VICs) &amp; very fine elastic fibers</td>
<td>Provide strength, stiffness &amp; maintain coaptation during diastole, prevents regurgitation (mechanical integrity)</td>
</tr>
<tr>
<td><strong>Spongiosa</strong></td>
<td>Glycosaminoglycans, ~ Loose collagen fibers &amp; VICs</td>
<td>Absorb shear forces &amp; cushion shock between layers during cyclic valve motion (shock absorber)</td>
</tr>
<tr>
<td><strong>Ventricularis</strong></td>
<td>Elastic fibers &amp; ~ VICs</td>
<td>Extend in diastole &amp; contract in systole</td>
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</tbody>
</table>
Atrioventricular & Semilunar valves (Human)

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

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

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

Aortic valve – Human

- Four layers*

1. Arterialis
2. Fibrosa
3. Spongiosa
4. Ventricularis

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

*Pellerin et al., 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)

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

- **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) turquoise; 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** (VECs)
  - Lining valvular surface; highly responsive to chemicals & mechanical forces
  - Display heterogeneity – 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

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


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 \(\rightarrow\) aVICs; pVICs \(\rightarrow\) qVICs & so on


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

\(^1\) Fayet et al. (2007). Cardiovascular Pathology; Liu et al. (2007). Am J Pathol

\(^2\) EPE/M cells = Embryonic progenitor endothelial/mesenchymal cells
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

- 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

Adult rat heart ≈ 2.0 x 1.2 x 1.2 Cm

No heart valves, H&E, 10X

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
  - All valvular diseases
  - Hemodynamic burden
  - Myocardial dysfunction
  - Congestive heart failure
  - Sudden death in humans

- 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\(^*\) \(\text{aVICs}\)
- Increased extracellular matrix, primarily GAGs (glycosaminoglycans)

Microscopically similar to:
1. Valvulopathy induced by ergot alkaloids (ergotamine & methysergide)
2. Valvulopathy – Carcinoid syndrome

\(^*\)Connolly et al., 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 (\(\text{aVICs}\))
  - Extracellular matrix (clear spaces)

\(^*\)Connolly et al., Mayo Clinic, NEJM 1997

Mechanism of valvulopathy

- Fenfluramine, Dexfenfluramine & Benfluorex
- Ergotamine, Dihydroergotamine & Methysergide
- 5HT2B* receptor agonists
- Pergolide & Cabergoline
- 3,4 Methylenedioxymethamphetamine

\(^*\)5HT2B = 5-Hydroxytryptamine 2B
Location of 5HT2BR (humans) – All four valves

5HT2BR-related mechanism

Drugs (5HT2BR agonists) → Activate 5HT2B receptor → Quiescent valvular interstitial cells (qVICs) → aVICs ⇒ Proliferate & secrete extracellular matrix ⇒ plaque-like lesion → Clinical Valvulopathy

Kaumann & Levy. Pharmacol Therap, 2006
5HT2BR stimulation

Valvulopathy

- Complex
- Not fully understood

Carcinoid disease - 5HT

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*

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**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.**
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?
**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
**Percent area Glycosaminoglycans content in Normal and Mitral Valvulopathy (SMV)**

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

**Percent area collagen content in Normal and SMV mitral-valve leaflets**

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

**Thickness comparison between Normal and SMV in mitral-valve leaflets**

*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

Number of 5HT2BR-positive Cells in the mitral-valve leaflets of Normal and Mitral valvulopathy (SMV)

Elangbam et al., Exp Toxicol Pathol 58:89-99, 2006
Compositional analysis:
- Significant increase in the GAGs content (quantitative analysis)
- Significant loss/disorganization of collagen

These changes – similar to anorexigen associated valvulopathy in humans

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

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment &amp; dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Sterile water, daily s/c injections for 7 days</td>
</tr>
<tr>
<td>Treated</td>
<td>5HT, daily s/c injections (75 mg/kg for the first 3 days and 60 mg/kg thereafter) for 7 days</td>
</tr>
</tbody>
</table>

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

Morphology (H&E)
- Nodular or segmental thickening of affected heart-valves due to subendocardial fibromyxoid tissue

Immunohistochemistry:
- Significant increase in the number of 5HT2BR-positive cells
- 5HT2BR may be involved (?)
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 ($p \leq 0.0001$)

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 SHT treated SD rats.

Investigative study - Conclusions

- 7-Day SHT treatment
  - Compositional alterations in GAGs & collagen
  - Correlated with up-regulation of 5HT2BR & down-regulation of 5HTT genes

- 5HT2BR & 5HTT – Pathogenesis of SHT-induced vavulopathy

- Animal model for SHT-induced valvulopathy
  - In vivo-screening for serotonergic drugs with 5HT2BR agonism

Acknowledgements

GSK
- David Krull
  - Immunohistochemistry
- Joanna Barton
  - Histology, Movat’s pentachrome
- Lawrence Yoon
  - TaqMan, LCM/TaqMan
- Don Creech
  - TaqMan, LCM/TaqMan
- Lisa Gates
  - Histology, Histochemistry
- Robert Geske
  - Immunohistochemistry
- Kerry Crabb

NTP archives
- Dr. Melvin Hamlin
- Mary Ellen Sutphin
- Keith Connelly

NIHES
- Dr. Abraham Nyska
  - Peer review
- Dr. Grace Kissing
  - Statistical analysis
- Drs. David E. Malarkey & Robert Maronpot – Reviews

College of Veterinary Medicine, NCSU
- Dr. Torrie Crabb, Former summer GSK Intern (2005)
  - NTP study review
- Dr. John Weber, Former summer GSK Intern (2006)
  - Compositional analysis
- Dr. Leah Zadrozny, Former summer GSK Intern (2006)
  - 7-Day Investigative study
- Dr. Lauren Job, Former summer GSK Intern (2007)
  - Compositional analysis