Pathological Indicators for Reflux-Related Changes in Oral (Gavage) Studies

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Gastroesophageal Reflux: Human

- **In human: a long term condition in which stomach contents rise up into the esophagus (acid reflux)**
- **Resulting in either symptoms or compli-cations**
- **Causing reflux esophagitis, esophageal strictures, intestinal metaplasia in esopha-gus (Barretts), esophageal carcinoma**

Kahrilas PJ, Shaheen NJ, Vaezi MF. American Gastroenterological Association, Institute; Clinical Practice and Quality Management, Committee (October 2008). "American Gastroenterological Association Institute technical review on the management of gastroesophageal reflux disease". *Gastroenterology*. **135** (4): 1392–1413,

Gastroesophageal Reflux: Laboratory Animals

- **'…After oral gavage dosing of rats, reflux may occur, resulting in serious respiratory effects and mortality…'**
- **'…Increased incidences of such "gavage accidents," however, might be an alert that the underlying pathogenesis is more complex than just a result of technical error, in particular if encountered only in treated animals…'**

Damsch S, Eichenbaum G, Tonelli A, Lammens L, Van den Bulck K, Feyen B, Vandenberghe J, Megens A, Knight E, Kelley M (2011). Gavage-related reflux in rats: identification, pathogenesis, and toxicological implications (review). Toxicol Pathol. 2011 Feb;39(2):348-60.

Aspiration or Reflux?

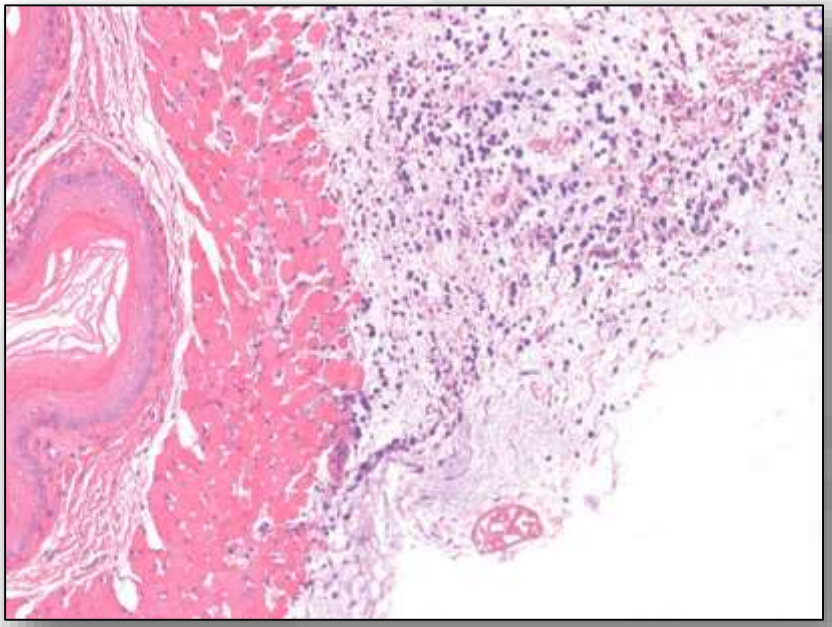
‘….even in the cases of correct oral dosing a small amount of the compound may be inadvertently deposited at the laryngeal orifice and would be inhaled during inspiration…’, …many compounds …., although well tolerated orally, may be highly toxic to pulmonary tissue..’

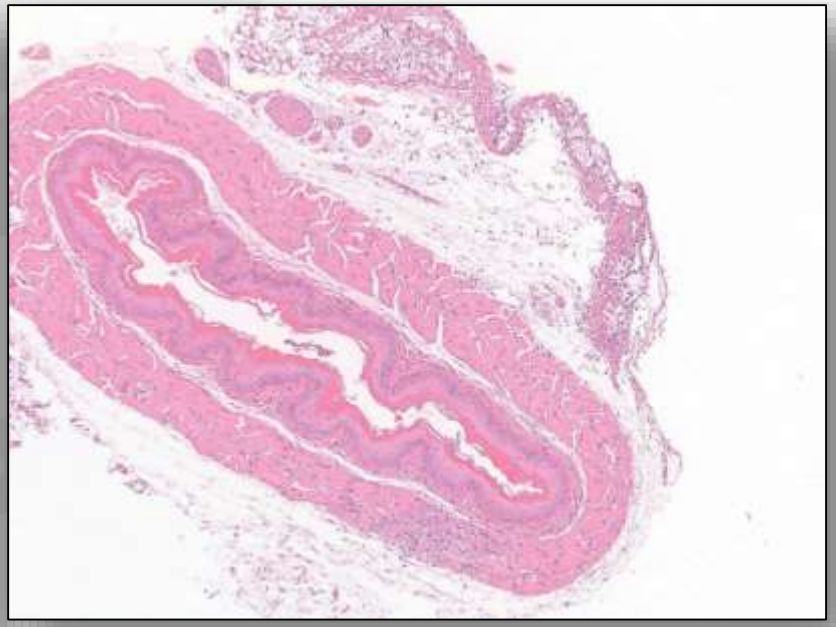
Gopinath C, Prentice DE, Lewis DJ (1987):
Atlas of Experimental Toxicological Pathology, MTP Press Limited, Lancaster, England: 41

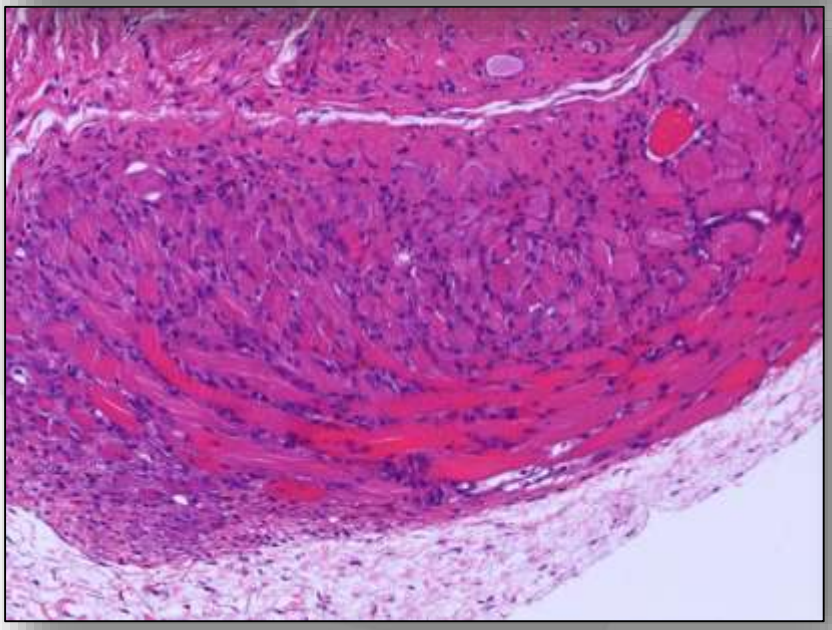
Misgavage

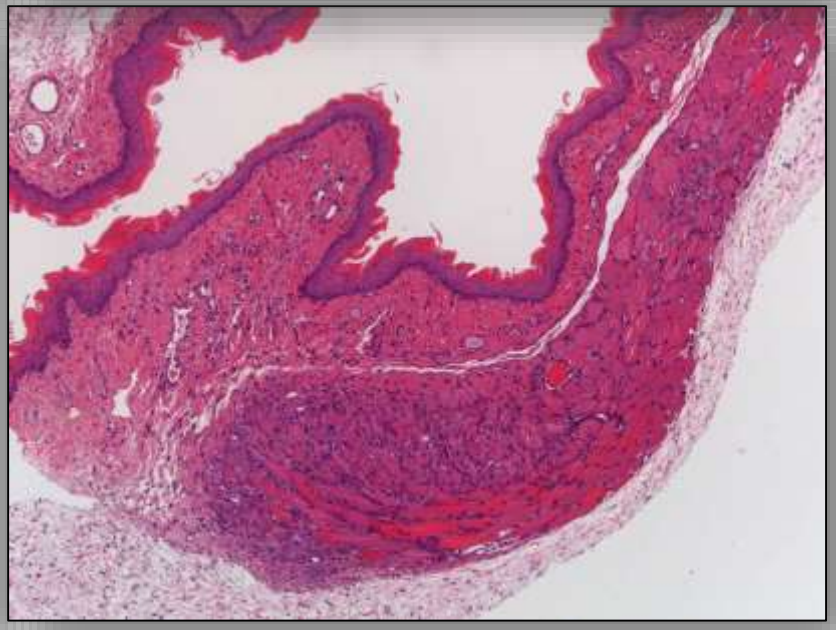
- **Accidental instillation into trachea or lungs**
- **Perforation of esophagus**
- **Traumatic insult on esophagus or stomach due to inadequate gavage probes**
- **Irritating effects to parasympaticus (N.vagus)**

Indicators of Misgavage: Esophagus

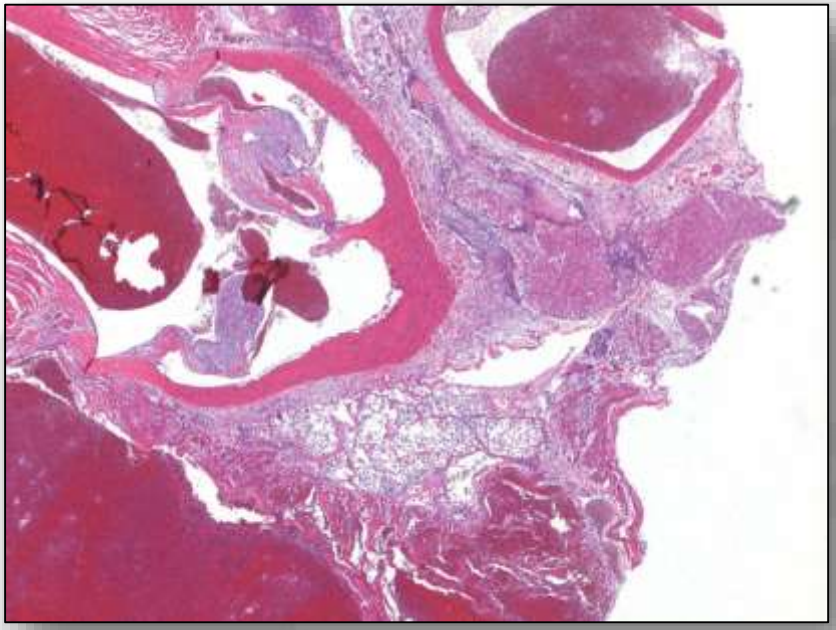


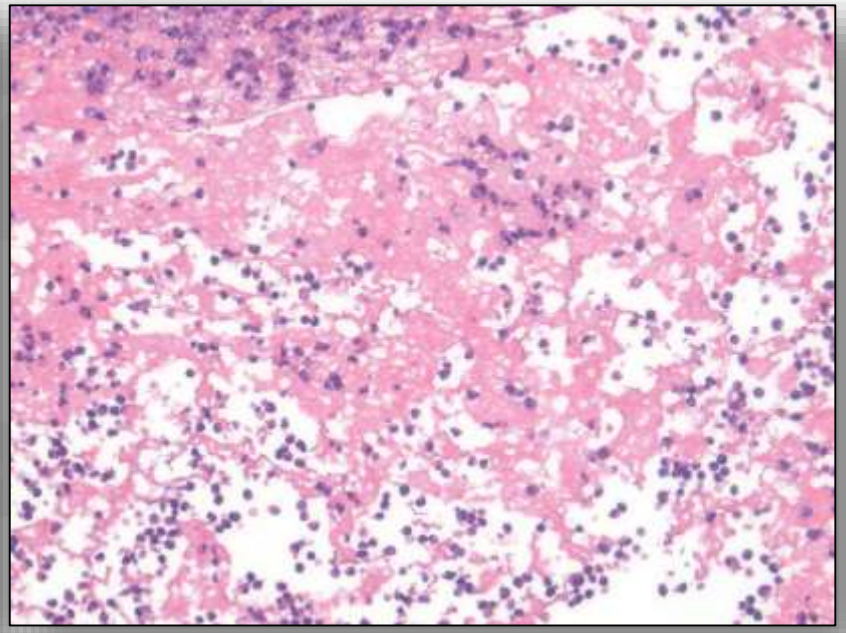


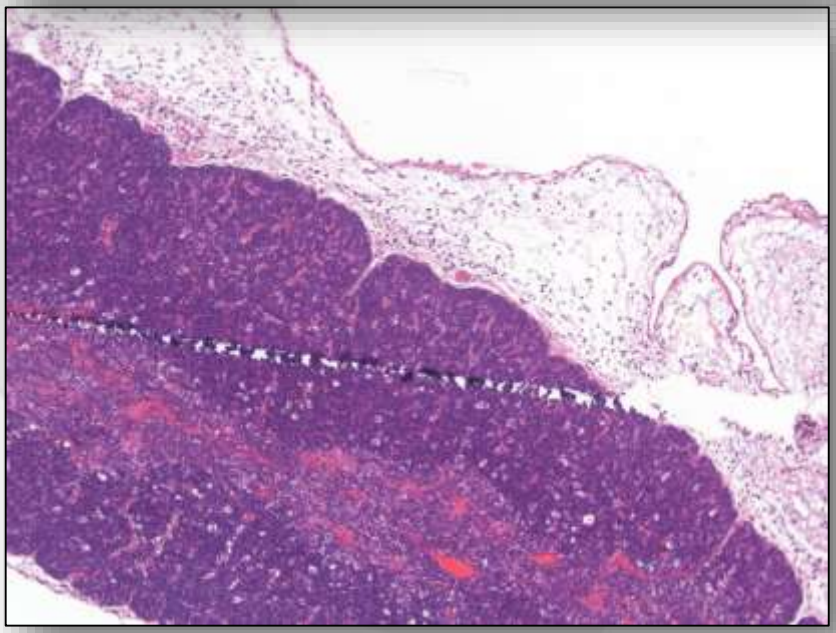


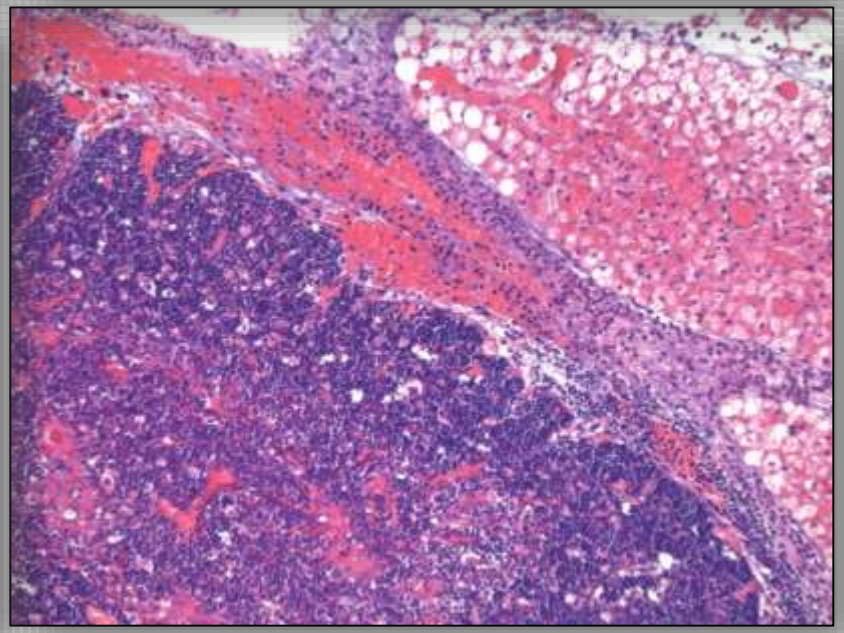


Indicators of Misgavage: Heart and Thymus

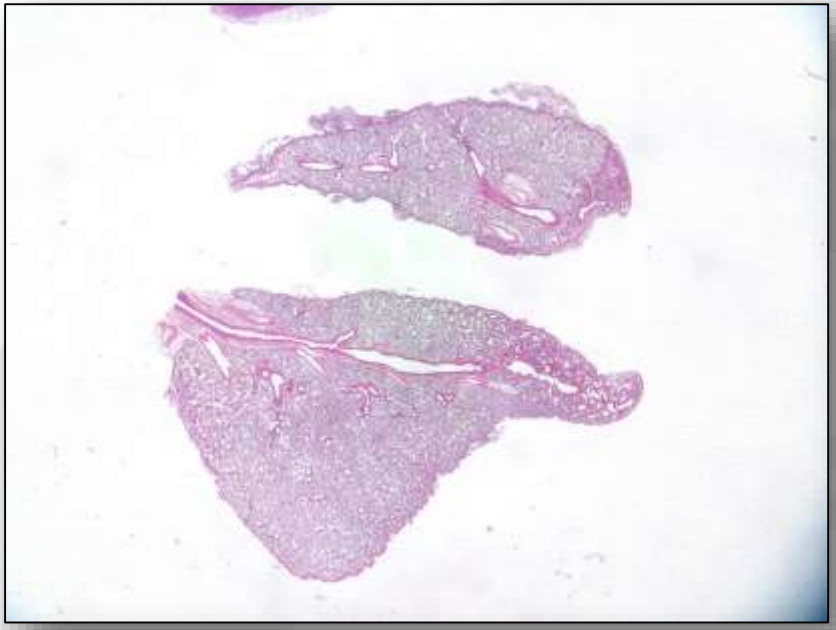


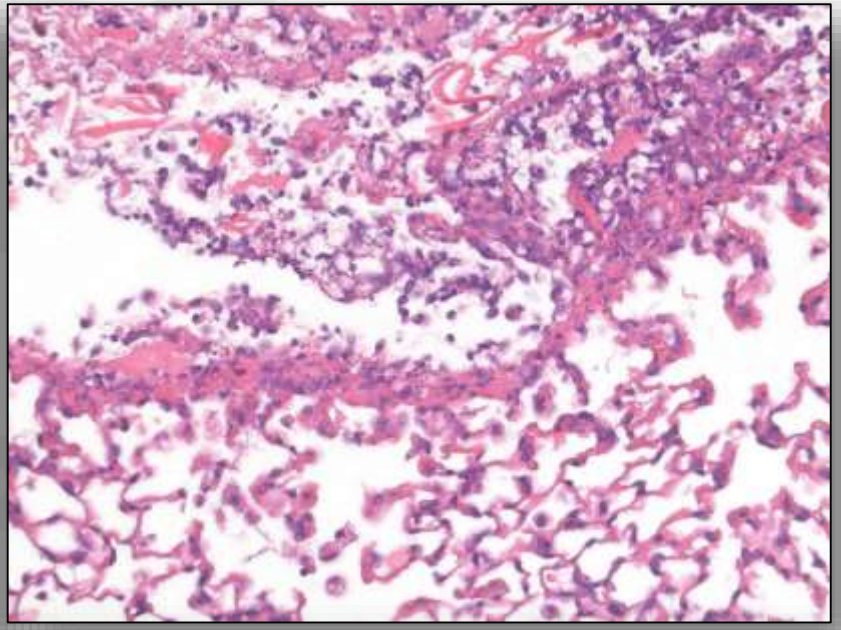


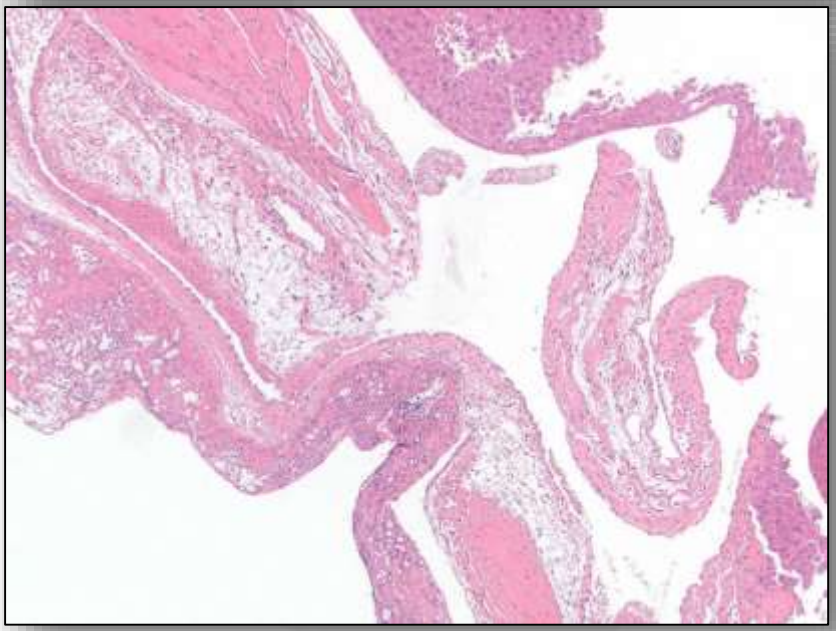


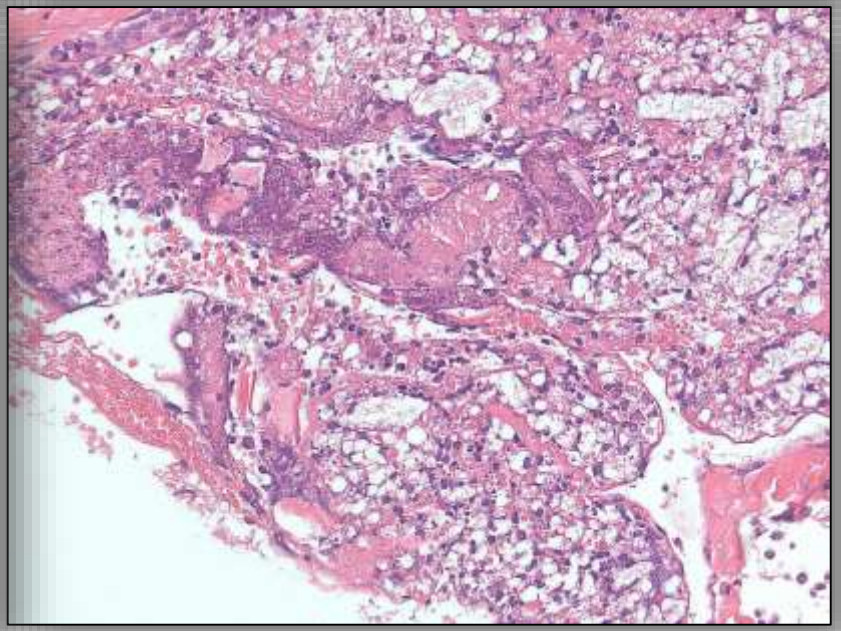


Indicators of Misgavage: Lung and Diaphragm



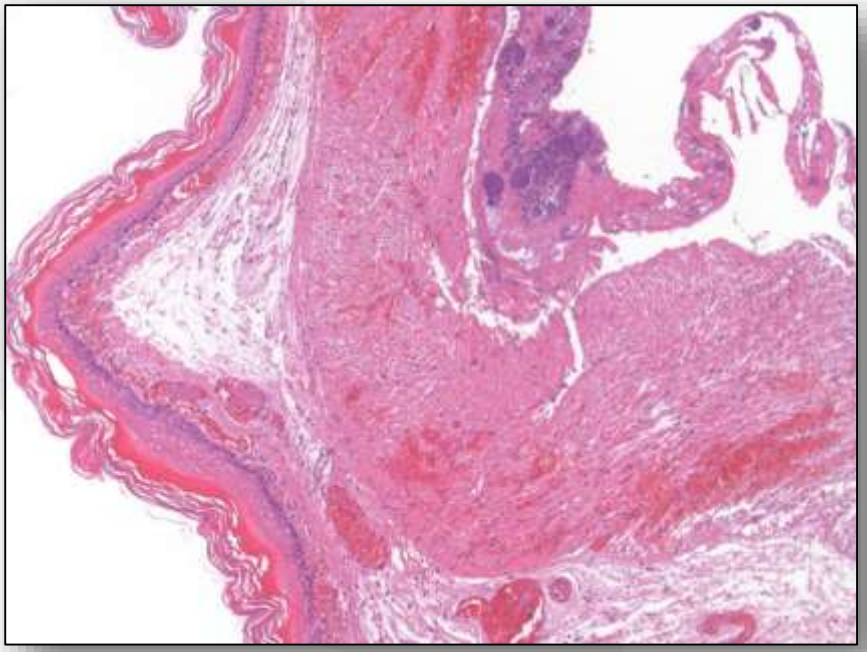


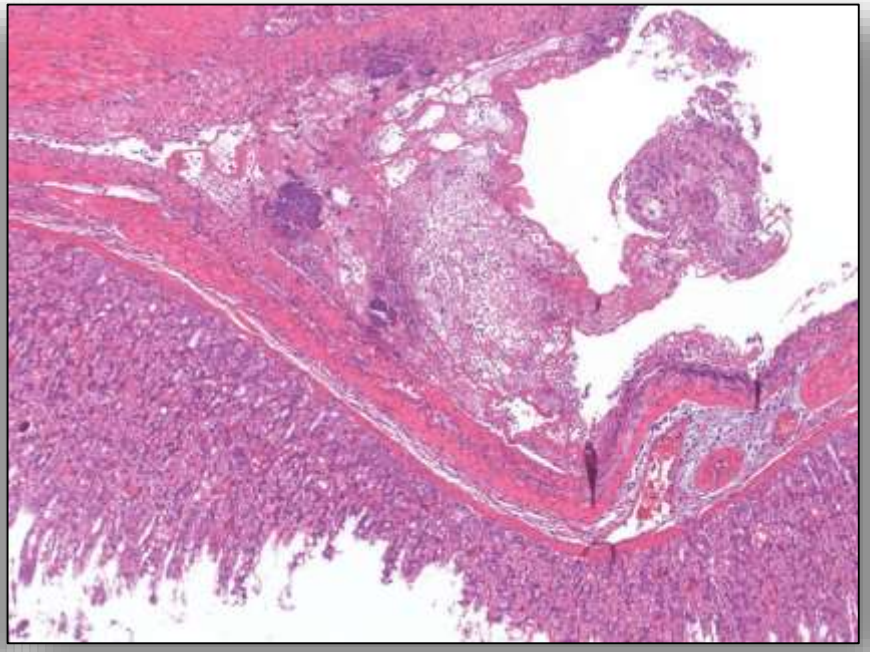




Misgavage and Reflux: Irritation, Stomach

- **Possible technical issues due to inadequate gavage probe material**
- **Possible technical issues due to too long gavage probes**
- **Example: Oily Test Item in Hamster, Reflux and Misgavage by Stress**

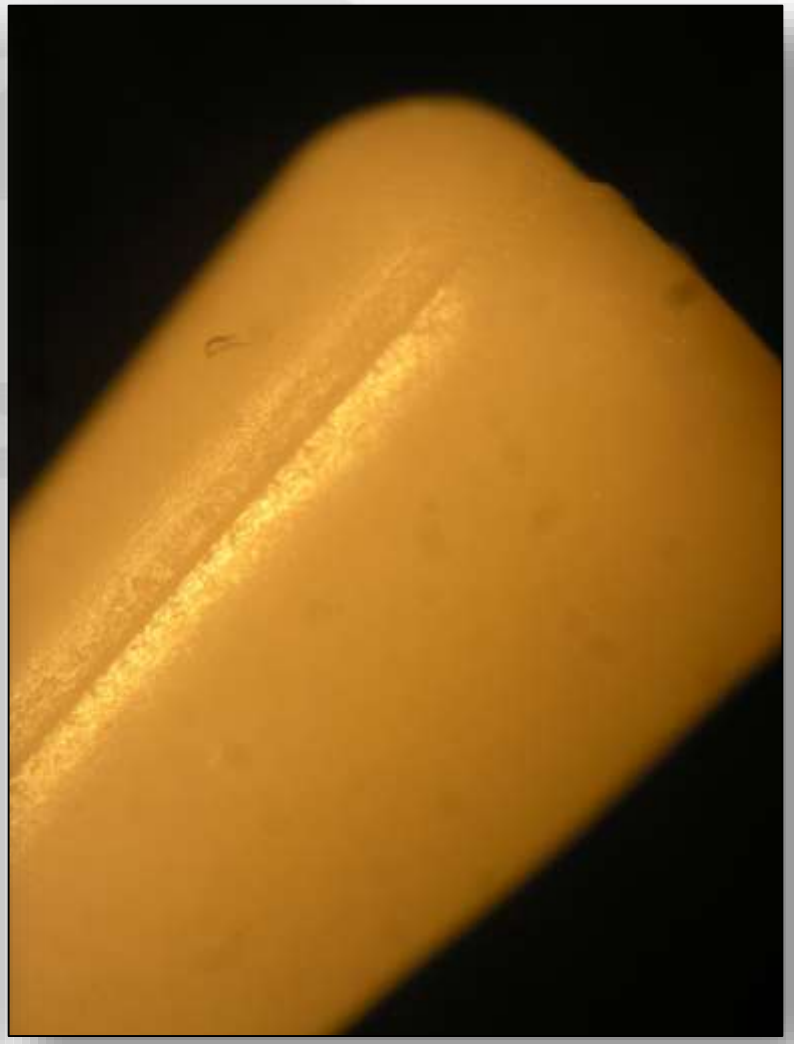




Silicone Probe-Related Irritation and Reflux

Changing Commercially Available Probes

- Silicone Ring
- Killer probe



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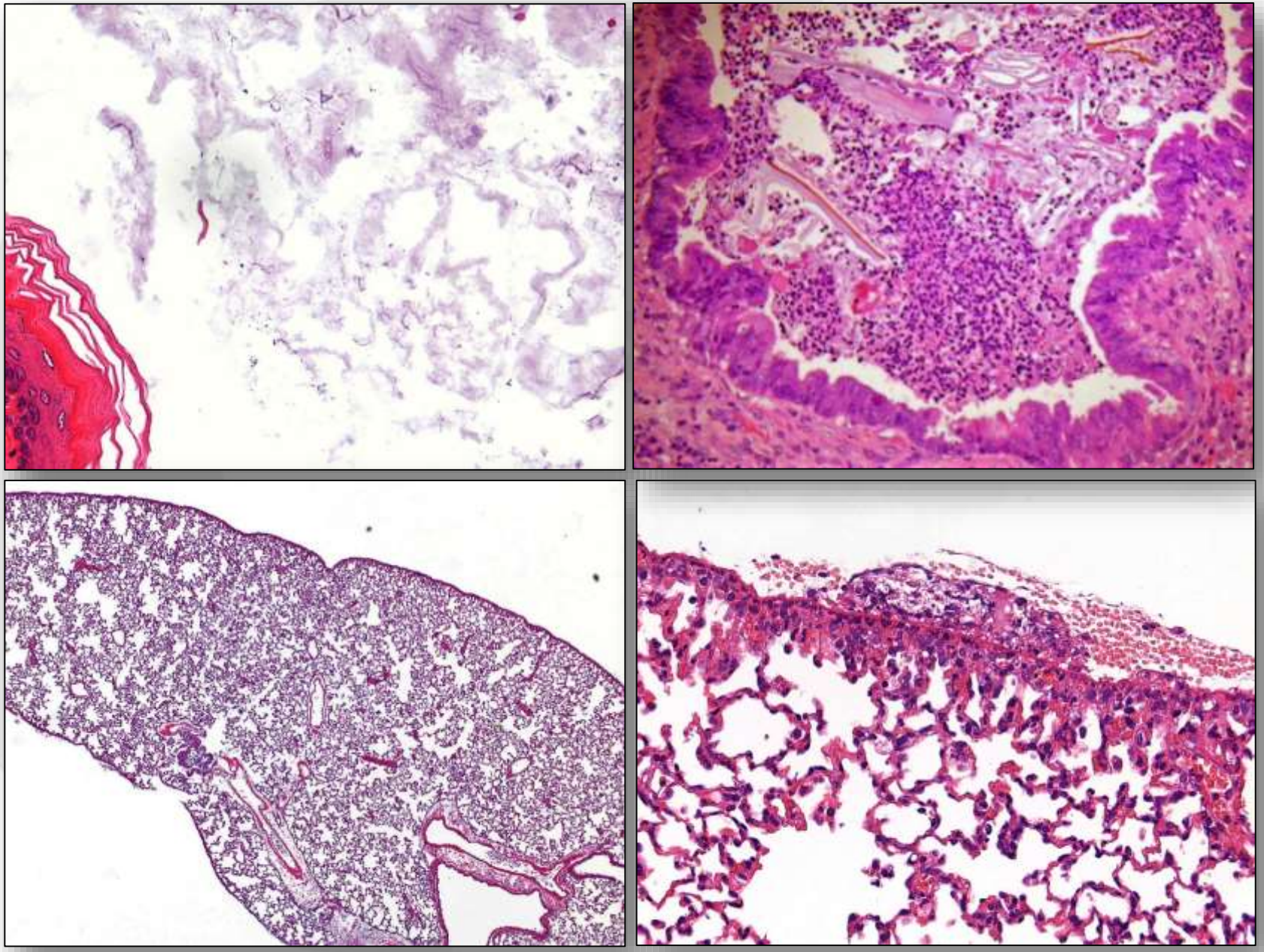
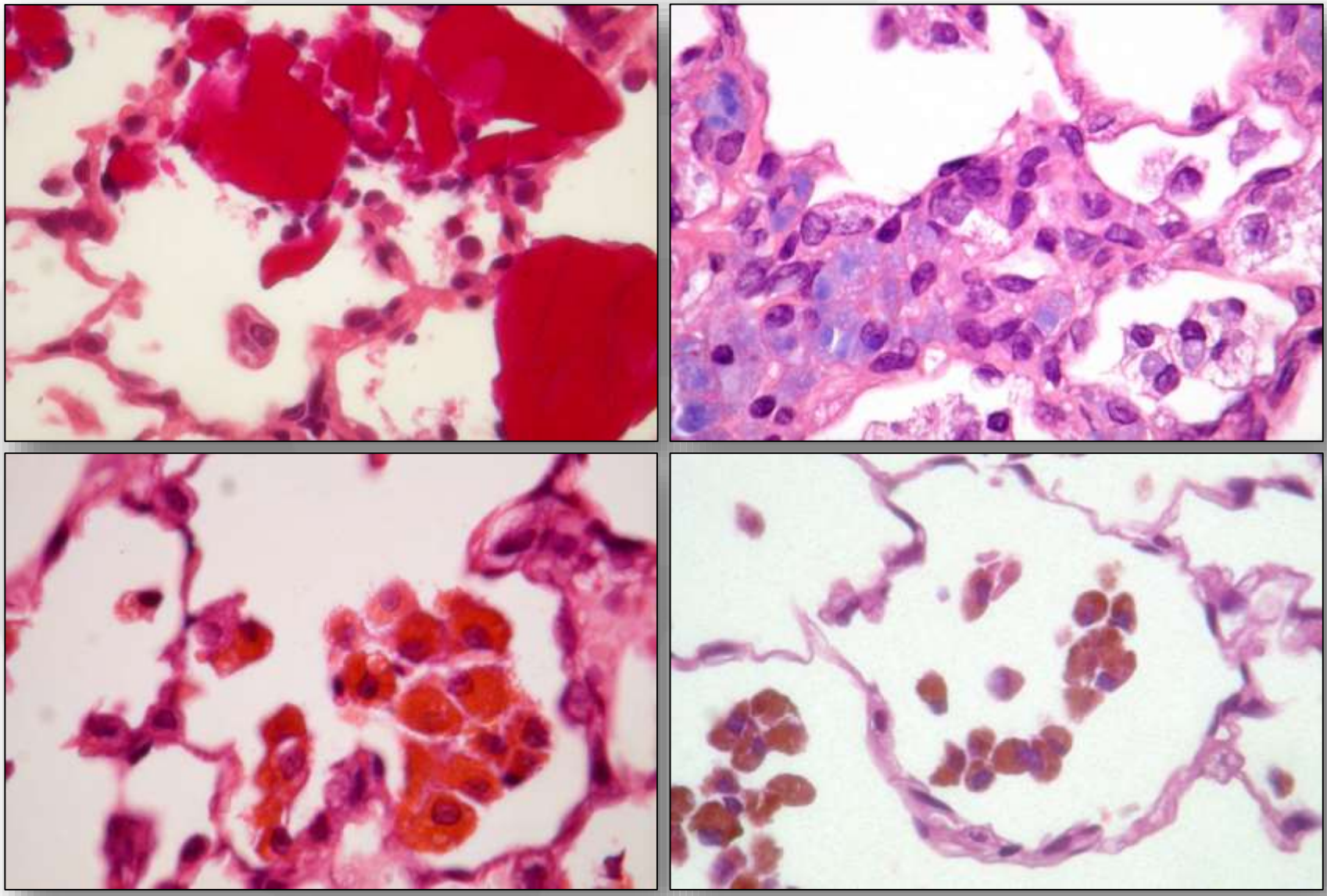
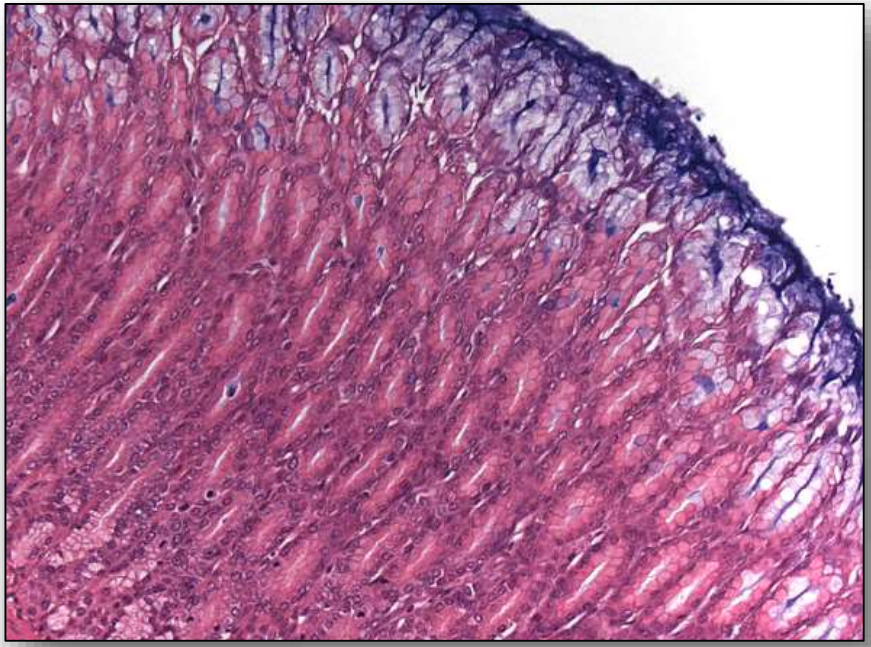
Stomach Irritation and Reflux: Hamster, Oily TI

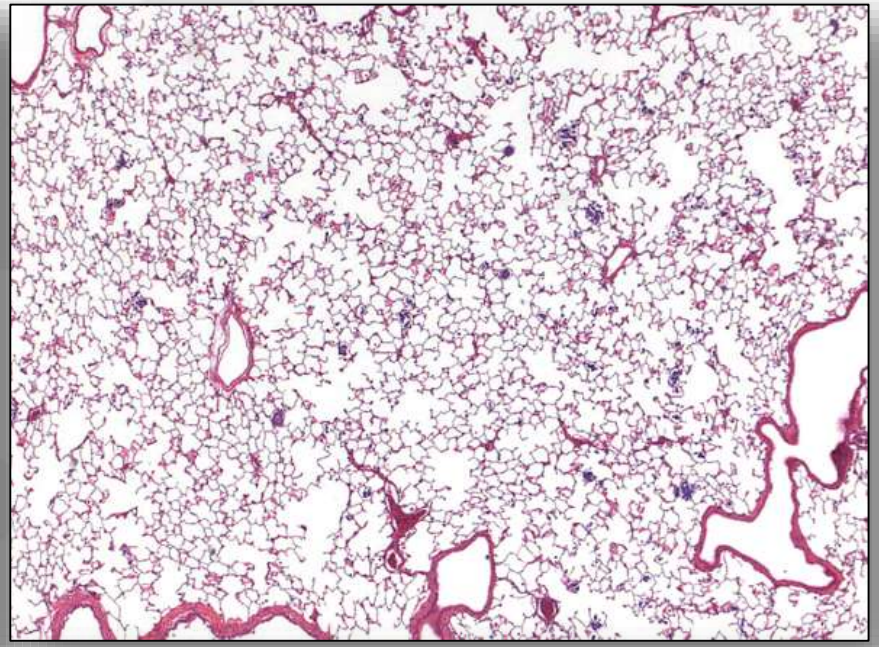


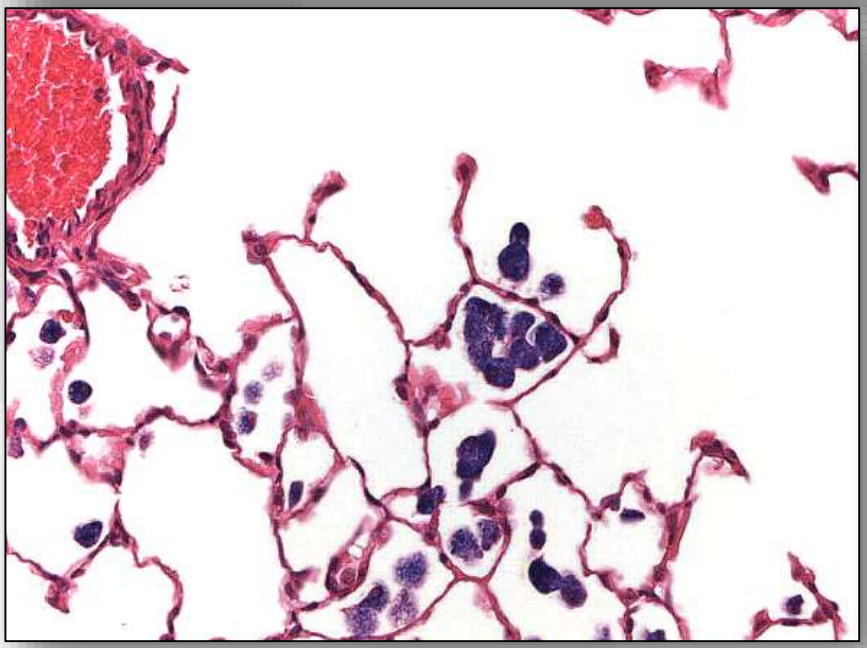
Simple Aspiration Must Not Reflect Reflux (Dye Stuff)

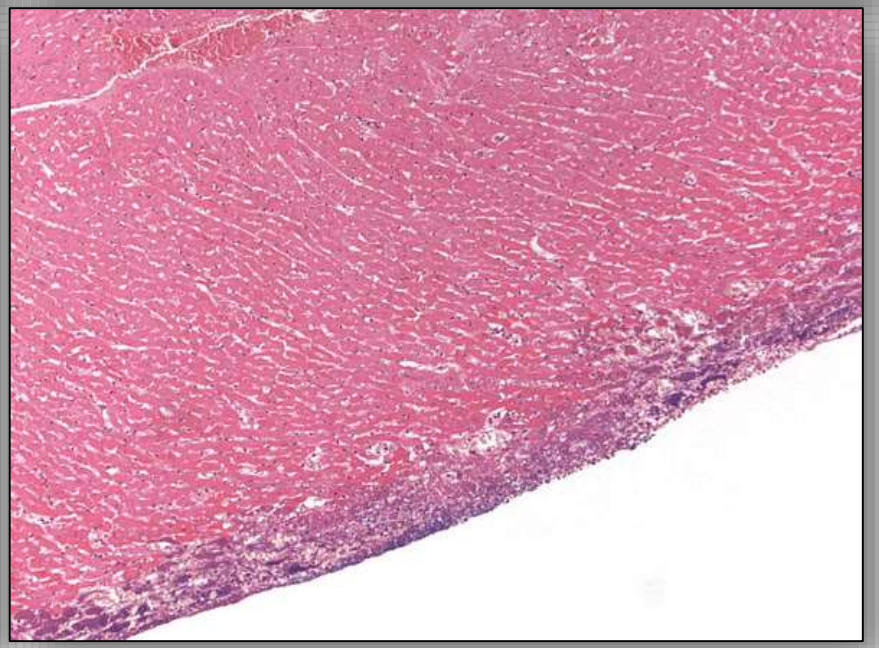


But it could be Reflux…..or even, Misgavage….





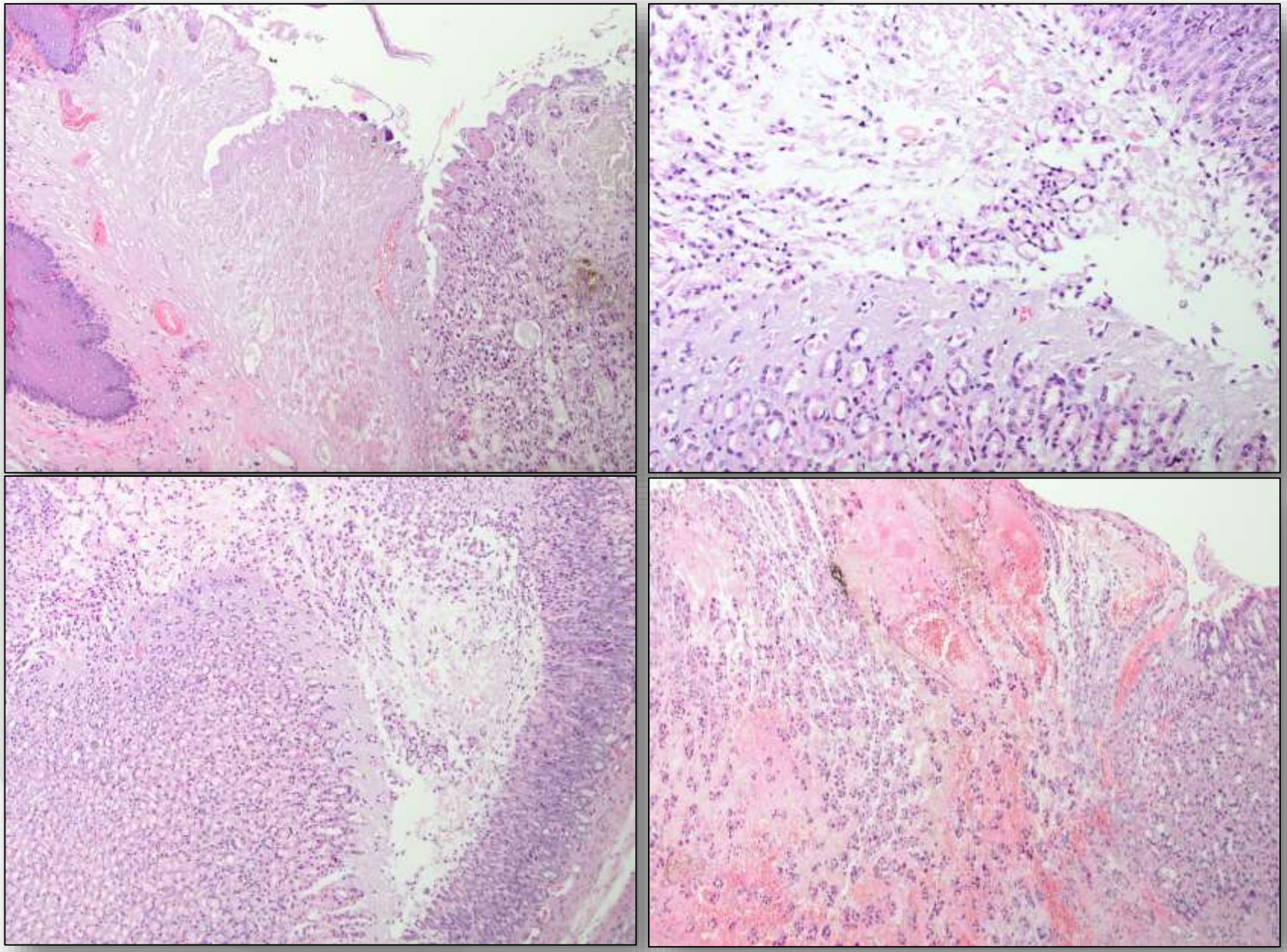




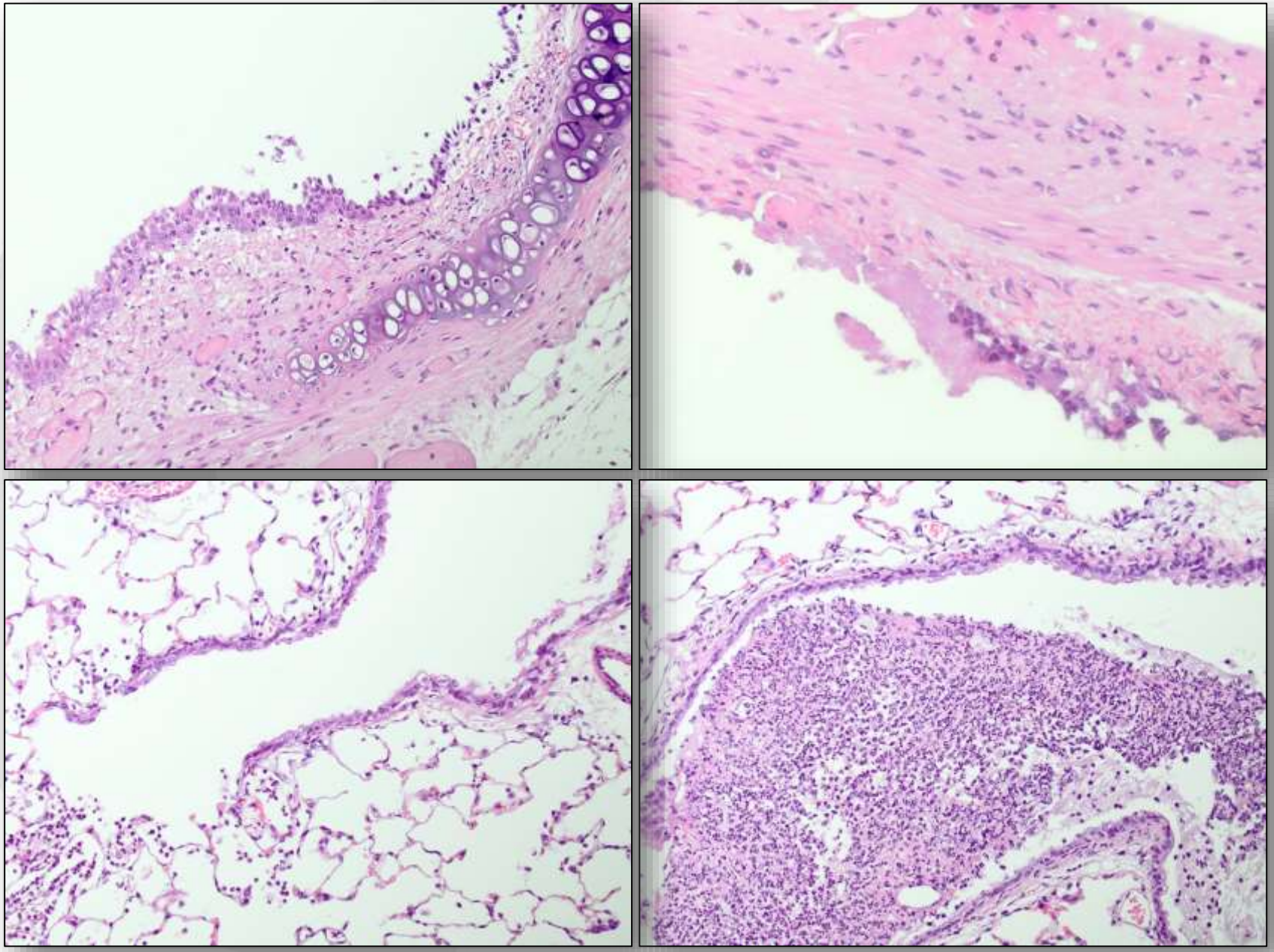
Reflux by Changing the pH in Stomach

- **Caustic test item**
- **Causes pH 0.6 bei release of HCl**

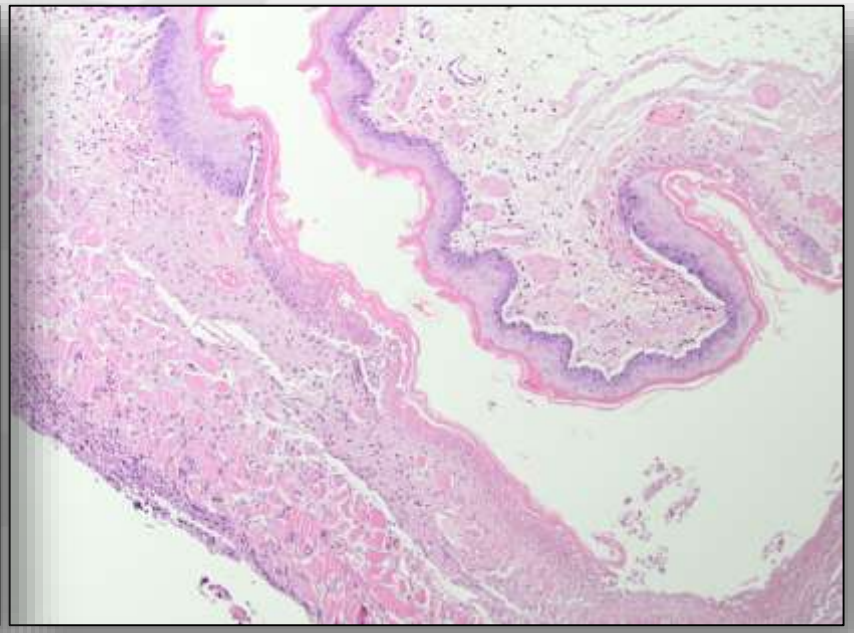
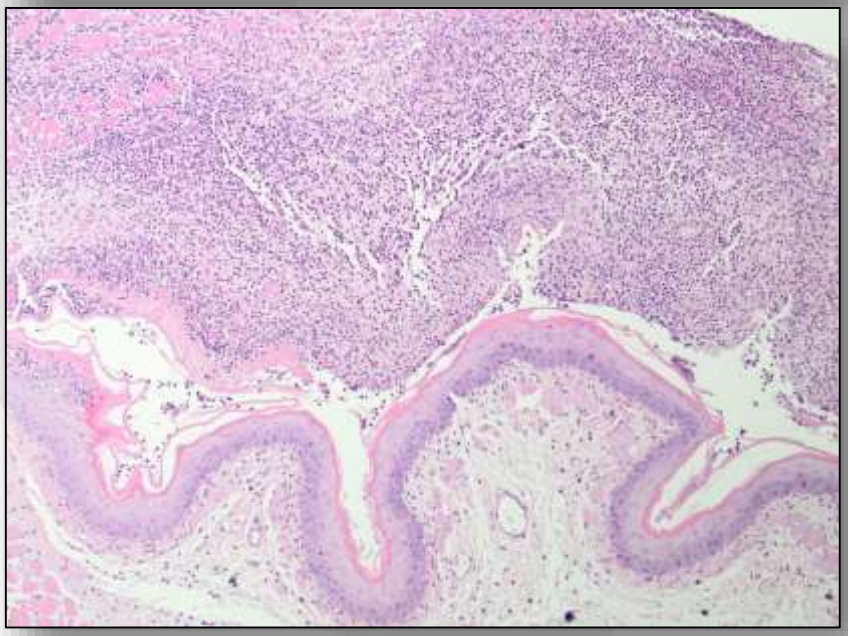
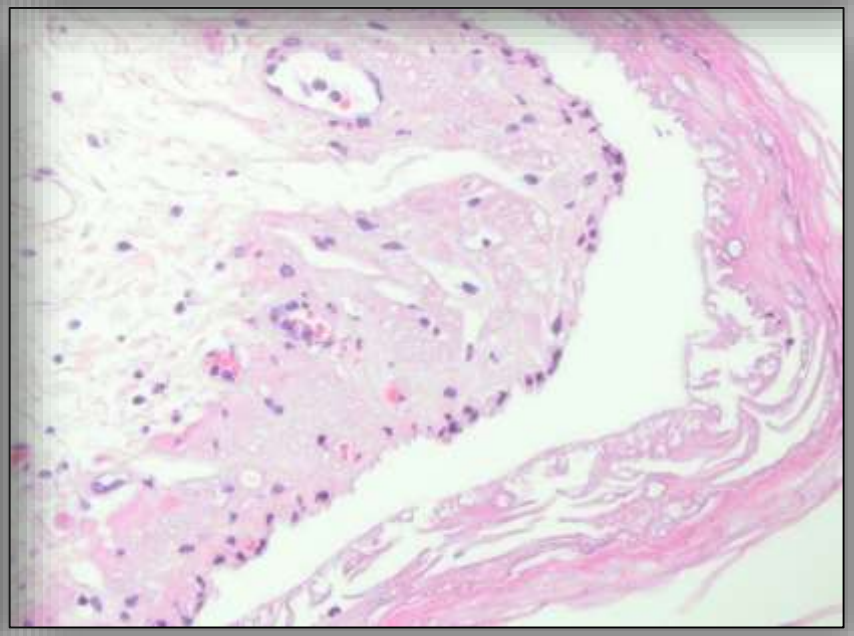
Reflux by Changing the pH in Stomach



Consequences: Trachea, Lung (Reflux)



Consequences: Esophagus (Reflux)

Reflux by Changing Viscosity: CMC

- **Vehicle for pharmaceutical**
- **Vehicle in toxicity studies**
- **Usually used as sodium salt in concentrations of 1-2% w/v to maintain good suspending properties**
- **Sodium carboxymethylcellulose solution usually at approximately pH 10**
- **Incompatible with some acidic drugs**

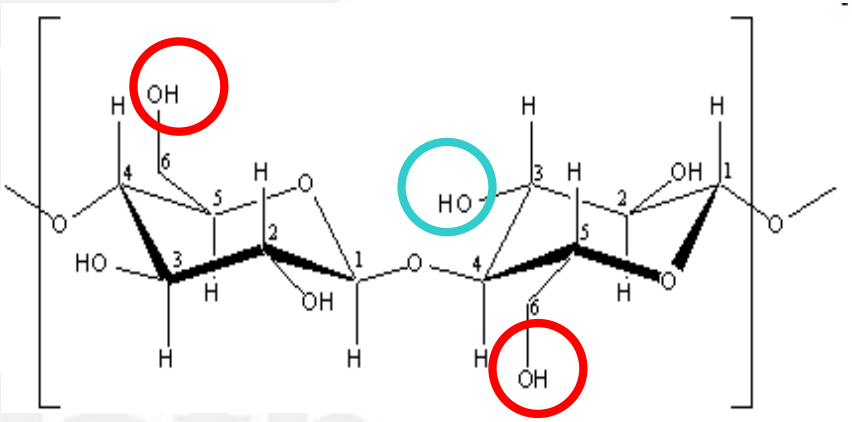
Weber K, Thio T, Iwata H (2009). Compounds causing local intolerability: Carboxymethylcellulose. Classic Examples in Toxicologic Pathology, 3rd Edition 2009, Eds: Drommer W, Karbe E, Germann PG. ISBN: 978-3-00-028588-2.

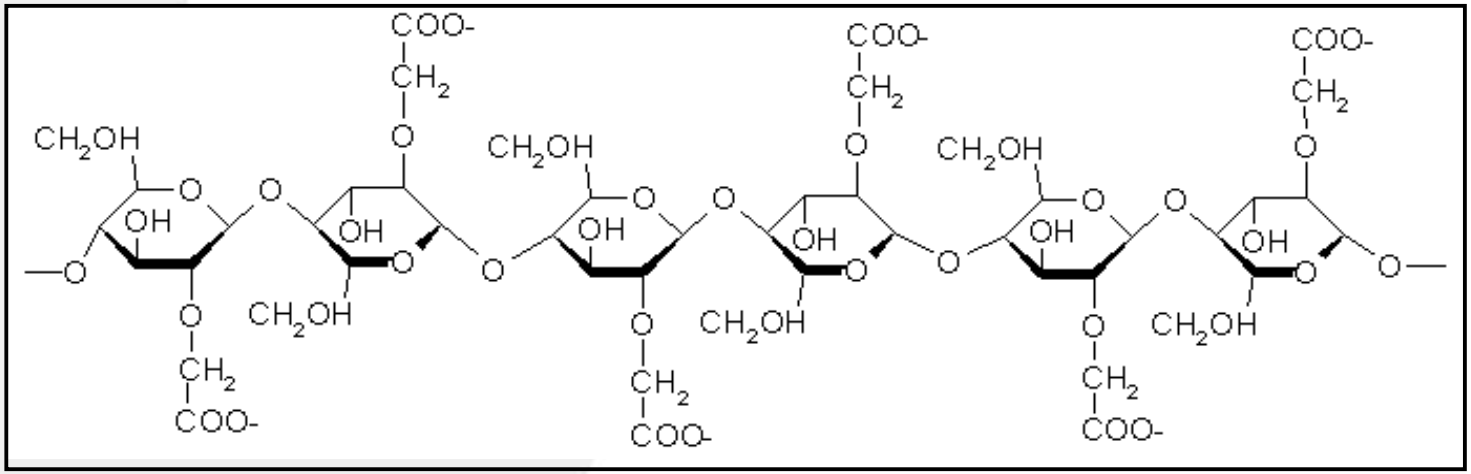
Reflux by Changing Viscosity: CMC

β-1,4-glycosidic ligation of 2 β(1,4)-D-glucopyranose molecules that are turned for 180° at the β-1,4-glycol-sidisic ligation

(2,6-di-O-compounds)

(3-O-compounds)



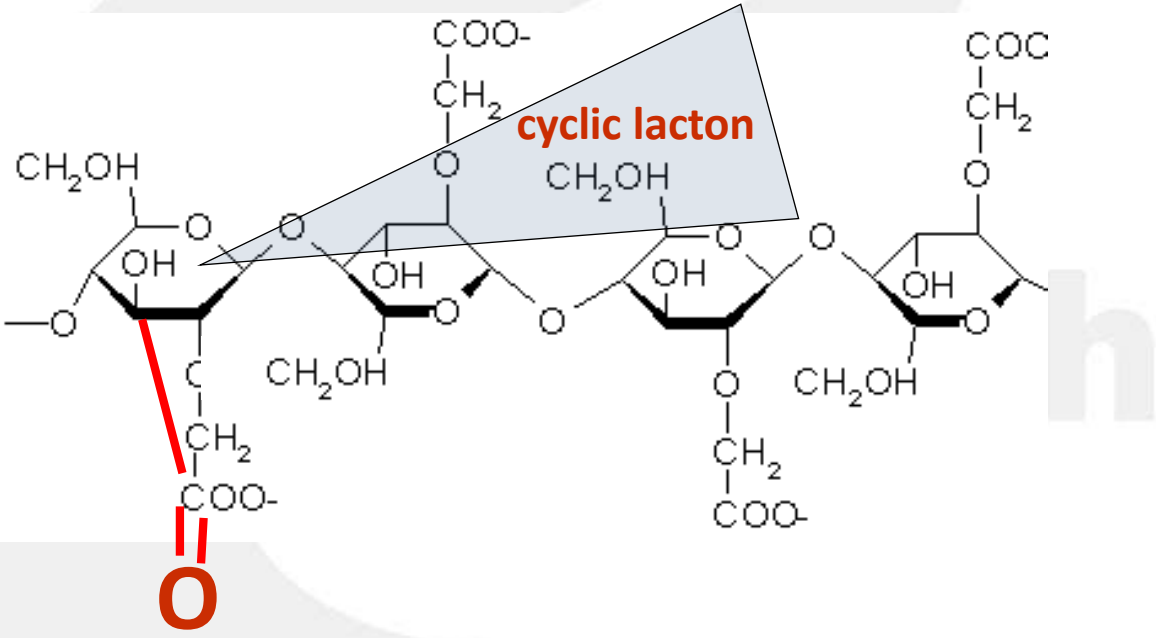


Substitution mainly by linkage to 2-O- and 6-O, resulting in 2,6-di-O-compounds (followed by 3-O-, 3,6-di-O-, 2,3-di-O- and 2,3,6-tri-O-linked)

Reflux by Changing Viscosity: CMC, Structure

- Slightly shorter than native cellulose
- Different preparations with different degrees of substitution
- Generally 0.6 - 0.95 derivatives per monomer unit
- Best extension at low concentration
- Overlap and coil up leading to a gel (thermoreversible) at higher concentrations
- Viscosity increases with decreasing pH values or increasing ionic strength (both cause increased coiling)
- At low pH, CMC may form cross-links through lactonization between carboxylic acid and free hydroxyl groups.

Reflux by Changing Viscosity: CMC, Changes in Structure



- **Pseudoplastic when hydrophoby of CMC increases = increased viscosity**
- **Generally with concentration**
- **Lower substitution causes tixotrophic behaviour**

Reflux by Changing Viscosity: CMC, Examples

- **26-week oral gavage study**
- **Vehicle: 1% CMC solution: 0,01 N HCl solution (pH 2)**
- **Viscosity of 500m Pa.s, 50:50 v/v**

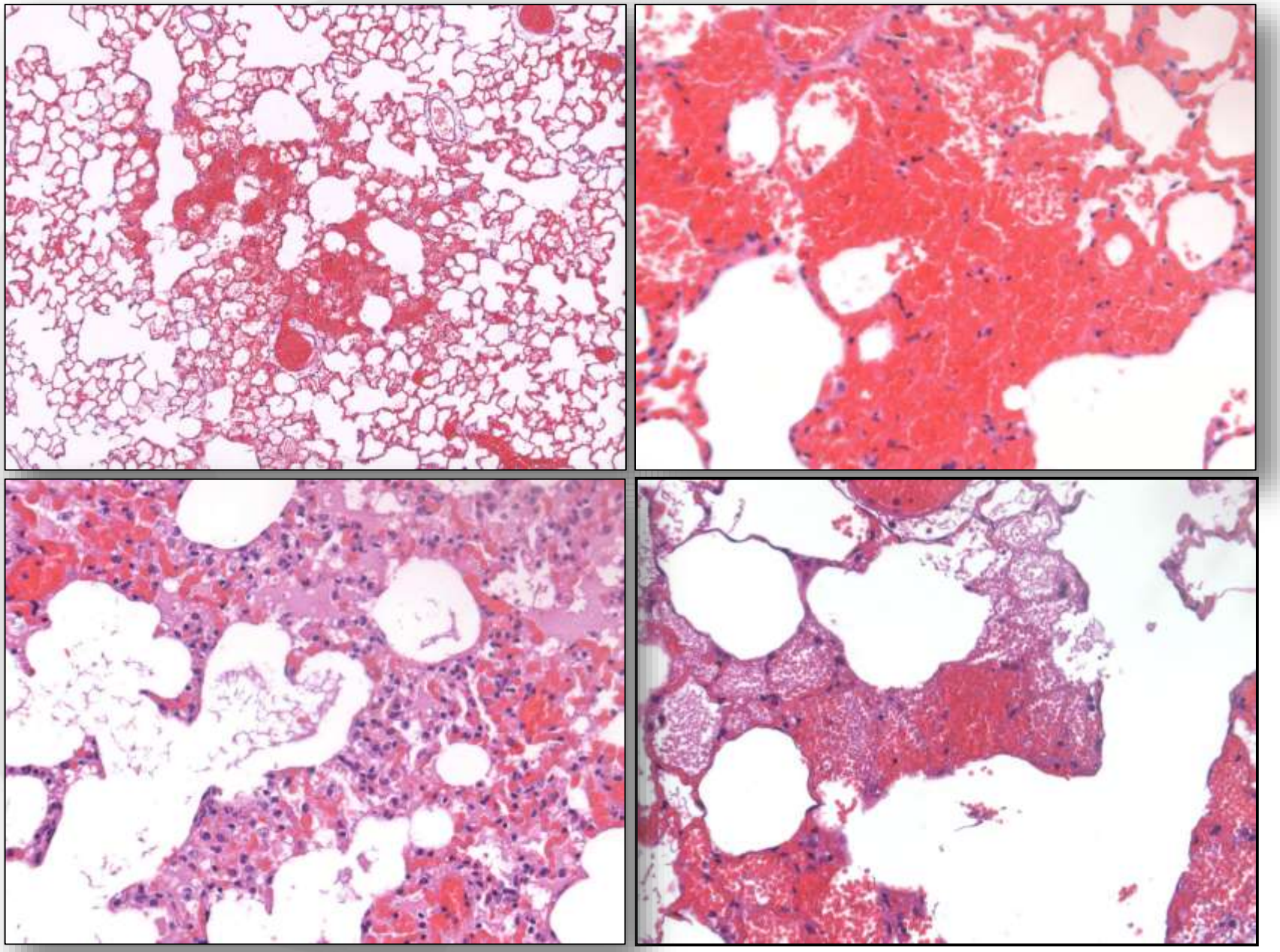
- **104-week oral gavage studies in rats and mice**
  Vehicle: 1% CMC solution: viscosity of 15-50mPa.s thereafter
  (pH 2)

$1\ Pa = 1\ kg/m \cdot s^2 = 1\ N/m^2$

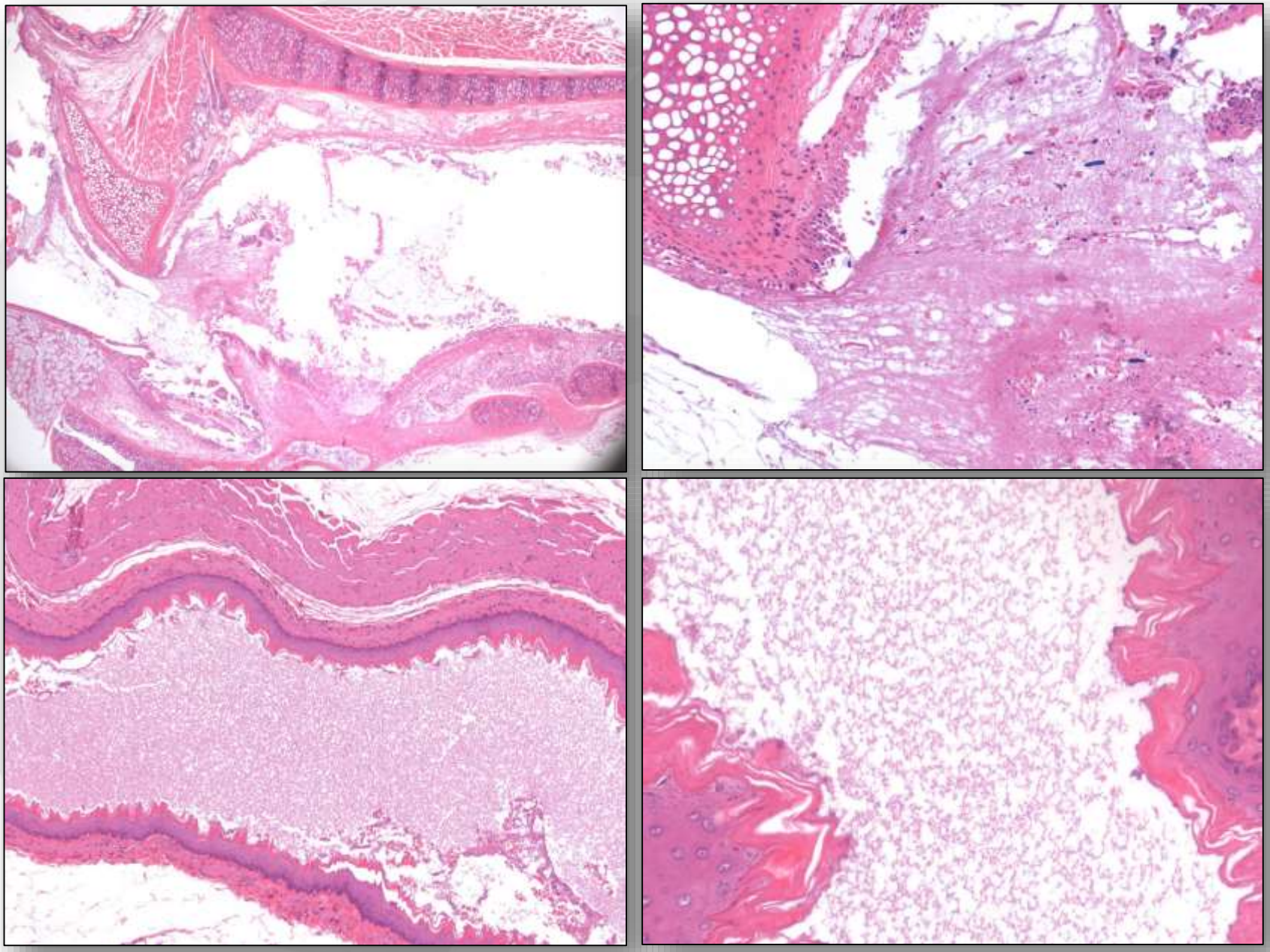
Reflux by Changing Viscosity: CMC, Examples

- Laboured respiration and breathing noises
- Lungs of some decedents were not or incompletely collapsed
- In some animals: lungs with gray white foci and dark red discoloration
- Inflammation, necrosis and perforation of the esophagus

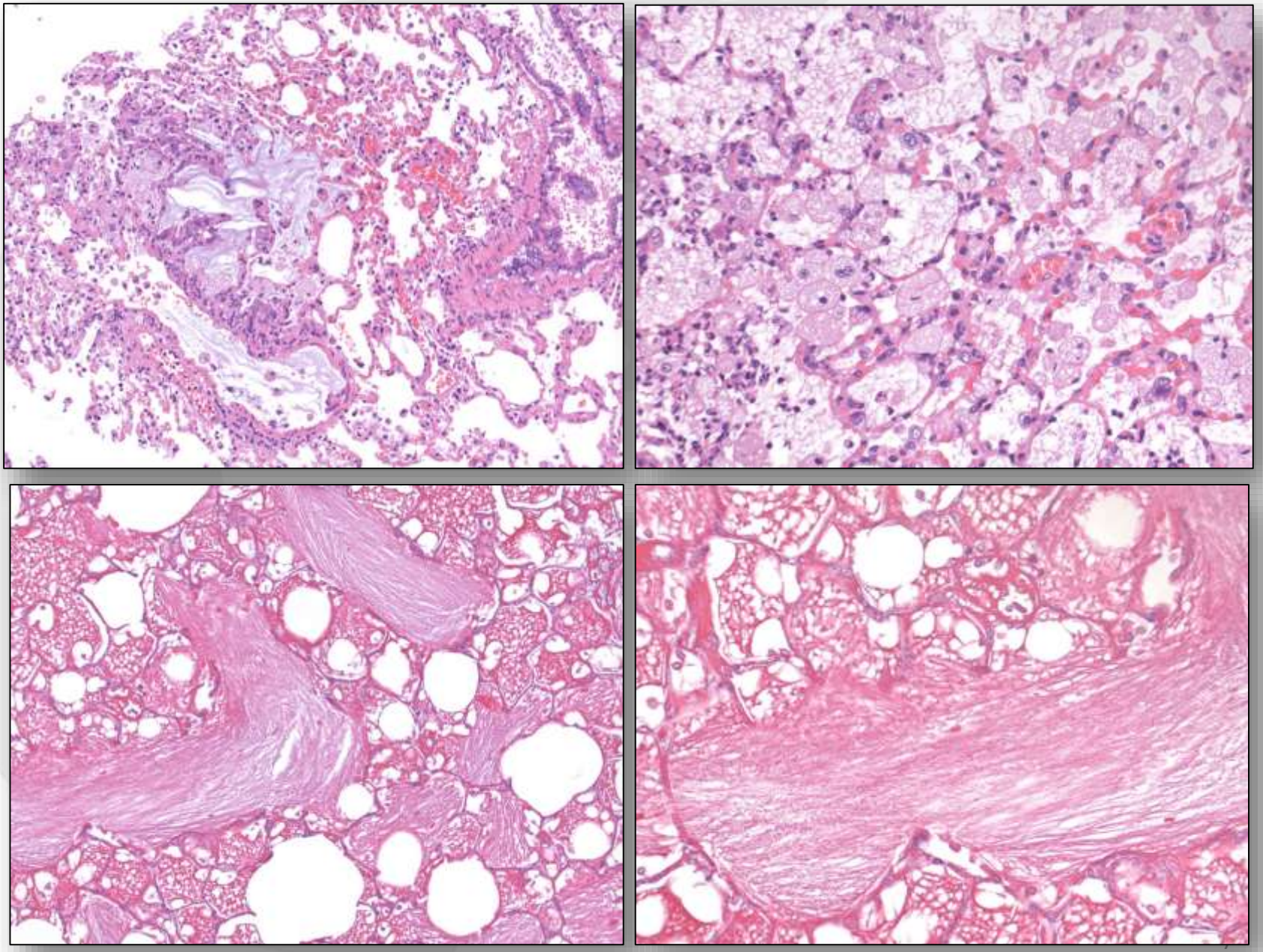
Reflux by Changing Viscosity: CMC, 26-W, Rat



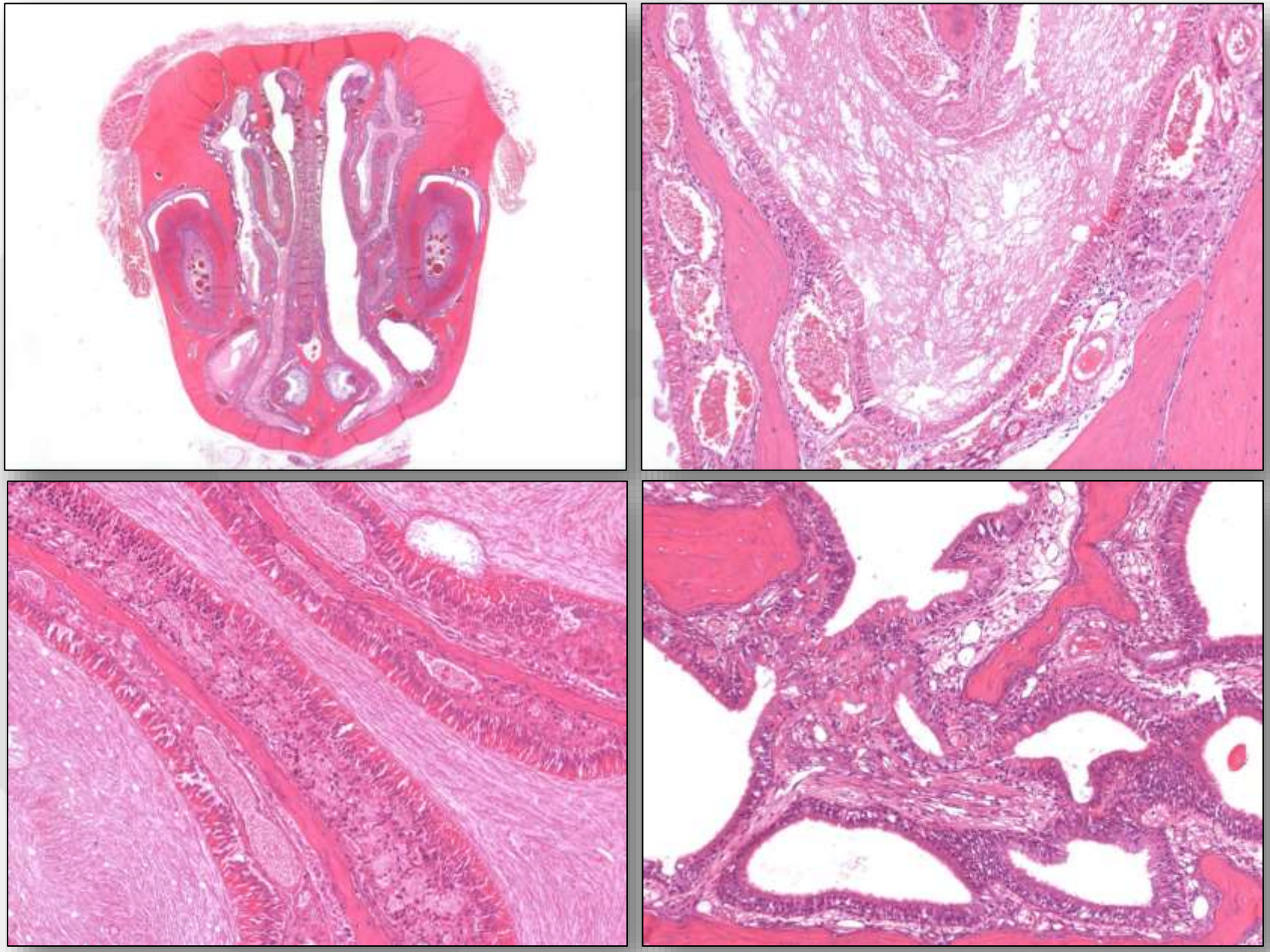
Reflux by Changing Viscosity: CMC, 26-W, Rat



Reflux by Changing Viscosity: CMC, 104-W, Rat



Reflux by Changing Viscosity: CMC, 104-W, Rat



Reflux: Consequences in Other Organs

Reflux-Related Otitis Media and Meningitis in Rats after Gavage Dosing

Hetty van den Brink-Knol[1], Nils Krueger[2], Klaus Weber[3]
[1]WIL Research Europe B.V., 's-Hertogenbosch, The Netherlands, [2]Evonik Resource Efficiency GmbH, Hanau-Wolfgang, Germany,
[3]AnaPath GmbH, Liestal, Switzerland

charles river
WIL RESEARCH

1 Introduction

In a 28-day toxicity study three rats that were euthanized moribund showed a granulocytic inflammation of the meninges at microscopic examination. Meningitis of the brain and spinal cord is a very uncommon finding in rats in toxicity studies and has, to our knowledge, never been reported as test item or treatment induced (WDD intrathecal dosing). To explore the possible pathogenesis histopathologic examination of possibly associated regions (i.e. different levels of the nasal cavity/ skull) was performed.

2 Material and methods

30 male and 30 female rats (CR:WI(Han)) were allocated to four dose groups including a vehicle control group (propylene glycol) and were treated once daily by oral gavage (dose volume of 5 ml/kg) for 28 days. The animals were group housed with 5 animals per sex under SPF conditions. Histopathological examination was performed on routinely prepared H&E slides of an extensive list of organs and tissues of all control and high dose animals and premature decedents. From the premature decedents additional sections were prepared of level 3, 7 and 8 of the nasal cavity and posterior levels of the skull (**Drawing 3**) and an additional Warthin-Starry stain for bacteria was performed on the brain sections.

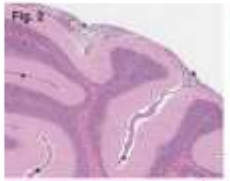
3 Results

Clinical signs and mortality
Four animals (mid and high dose Groups) showed lethargy, hunched posture, labored respiration, rales and/or salivation and were euthanized for ethical reasons between Day 6 and Day 14 of treatment.

Microscopic examination
Animals surviving their scheduled study periods:
- No microscopic findings in the brain and spinal cord.

Premature decedents: Animals A (male mid dose), B (male high dose) and C (female mid dose):
- Brain: A diffuse, granulocytic (suppurative) inflammation of the meninges of cerebrum and cerebellum; inflammation diffusely around the brain and extending into the ventricles (**Fig. 1, 2, 3**). The Warthin-Starry stain of animal C showed small bacterial colonies (**Inset Fig. 1**).
- Spinal cord: Granulocytic inflammation of the meninges (**Fig. 4**), with highest severity in the cervical part. In animal C mixed inflammatory cells at the central canal of the spinal cord (**Fig. 5**) and extending into the vertebral bone (**Fig. 6**).
- Level 8 nasal cavity/skull: Moderate to marked pyogranulomatous inflammation in the sinus of the tympanic bullae (middle ear) (otitis media) (**Fig. 7**). A granulocytic cell infiltrate in the inner ear (**Fig. 8**) and around the trigeminal (Gasserian) ganglion in the cranial cavity (**Fig. 9**).
- Nasopharynx: Lymphogranulocytic inflammatory infiltrate and/or squamous metaplasia.

Premature decedent: Animal D (male high dose):
- Nasal cavity level 3: mucopurulent exudate and multifocal erosion/ulceration/ fusion of the nasal turbinates (**Fig. 10**), within the mucopurulent exudate eosinophilic amorphous material (**Fig. 11**). Early inflammatory lesions in the middle ear (**Fig. 12**).



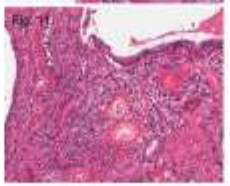
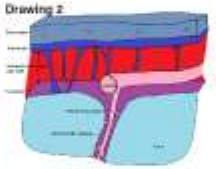
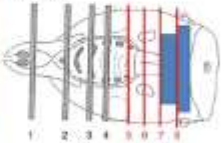
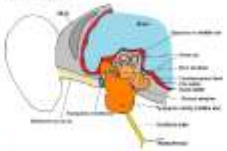


Figure legends
*Refers to granulocytic inflammation (all figures)
Brain (animal C: Fig. 1, 2) and 8 (Fig. 3)
Figure 1. Overview cerebellum, midbrain (H&E). Extent of the granulocytic inflammation of the meninges and lateral ventricles. Inset: Warthin-Starry stain: Bacterial colonies (arrow).
Figure 2. Detail cerebellum (H&E): Granulocytic inflammation meninges.
Figure 3. Detail midbrain (H&E): Granulocytic inflammation in lateral ventricle and formation of perivascular cuffs.
Spinal cord-Cervical part (H&E, Animal C)
Figure 4. Extent of the granulocytic inflammation surrounding the spinal cord and nerve root.
Figure 5. Granulocytic cells in central canal.
Figure 6. Inflammation progressing to the surrounding vertebral bone.

Drawing 1


Drawing 2


Drawing 3


Drawing 4


Level 8 of the Nasal cavity (H&E, Animal C) (Fig. 7), Animal A (Fig 8, 9)
Figure 7. Granulocytic inflammation sinus at tympanic bulla (middle ear).
Figure 8. Inflammatory cells enter part of the inner ear.
Figure 9. Granulocytic inflammatory infiltrate surrounding the Trigeminal ganglion.
Nasal cavity (level 3) and middle ear of animal D (H&E)
Figure 10. Multifocal erosion/ulceration/fusion of the nasal turbinates and mucopurulent exudate.
Figure 11. Detail of mucopurulent exudate with eosinophilic material.
Figure 12. Granulocytic inflammatory infiltrate in middle ear.
Drawing 1. Schematic overview of anatomy brain and spinal cord.
Drawing 2. Schematic overview of histology meninges.
Drawing 3. Nasal cavity, rat. Trimming levels. Levels 1-4 following RITA trimming guides for inhalation studies. Additional levels further posterior to evaluate the extension of lesions caudalward (Dernisch et al., 2011).
Drawing 4. Schematic overview of the anatomical structures of level 8 of the nasal cavity/skull.

4 Discussion

Gavage-related reflux and retrograde aspiration of gastric contents can be associated with unexpected mortality and has been described for rodents to cause changes in nasal cavity/nasal sinuses. A high dosing volume, a high viscosity and irritating properties of the test item can play a role (Dernisch et al., 2011, De Jonghe et al., 2008).

Meningitis of the brain and spinal cord is a very uncommon finding in rats in toxicity studies. The findings in the current study resemble the suppurative meningitis caused by infectious agents. Inflammation of the inner and/or middle ear with progression via neuronal pathways to the meninges has been described as a possible route of infection in rodents (Marra and Brigham, 2001, Schachern et al., 1992).

In the current study the meningitis probably resulted from reflux/regurgitation of gastric contents with stepwise inflammatory changes in nasopharynx/nasal cavity, with progression via the auditory tube to the sinus of the tympanic bullae (middle ear) and subsequently (via the inner ear or directly) to the meninges in the cranial cavity. When stomach contents end up in nasal cavities conditions are generated for ubiquitary bacteria to cause an infection. The inflammation of the meninges of the brain probably progressed via the cerebrospinal fluid (CSF) to the spinal cord and into the ventricles of the brain. Schematic drawings of the anatomical structures involved further explain the distribution and extend of the inflammatory lesions (**Drawing 1, 2, 4**).

5 Conclusion

Toxicologic pathologists should be aware that the well-known changes in nasopharynx and nasal cavity caused by gavage-related reflux/regurgitation in rodents, can incidentally progress from the nasopharynx via the middle ear to the brain and spinal cord resulting in otitis media and meningitis. It is of high importance for a balanced interpretation of study results to differentiate between effects secondary to local exposure and true systemic toxicity.

6 References

Dernisch, S., Eichenbaum, G., Torelli, A., Lammens, L., Feyen, B., van den Bulck, K., Knight, E., Kelley, M. and Torelli, A. (2011). Unexpected nasal changes in rats related to reflux after gavage dosing. *Toxicol Pathol* **39**, 337-347.

De Jonghe, S., Lammens, L., Rasoet, A., Steemans, K., Broeksaert, F., Verbeeck, J., van Goethem, F. and Hanton, G. (2008). Lethal rhinitis/sinusitis in rodents by aspiration of formulation in gavage studies: Importance of evaluation of the nose. *Exp Toxicol Pathol* **61**, 410.

Marra, A. and Brigham, D. (2001). Streptococcus pneumonia causes experimental meningitis following intranasal otitis media infections via a nonhematogenous route. *Infect Immunol* **69**, 7316-7325.

Schachern, P.A., Paparella, M.M., Hybertson, R., Sano, S. and Duvall, A.J. (1992). Bacterial tympanogenic labyrinthitis, meningitis, and sensorineural damage. *Arch Otolaryngol Head Neck Surg* **118**, 53-57.

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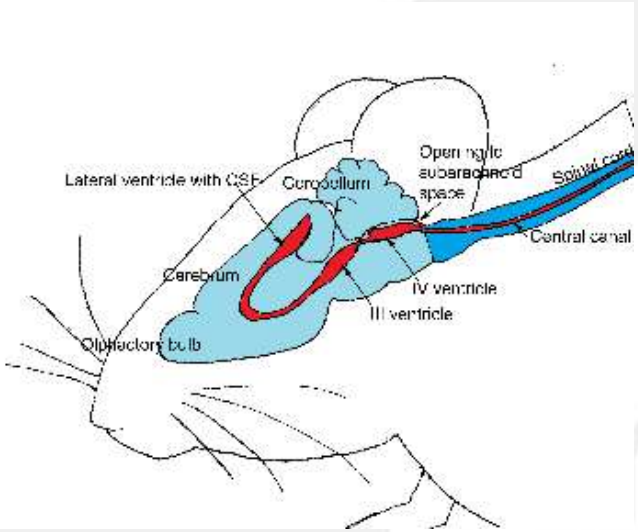
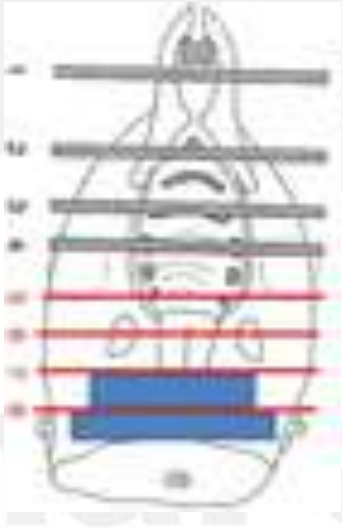
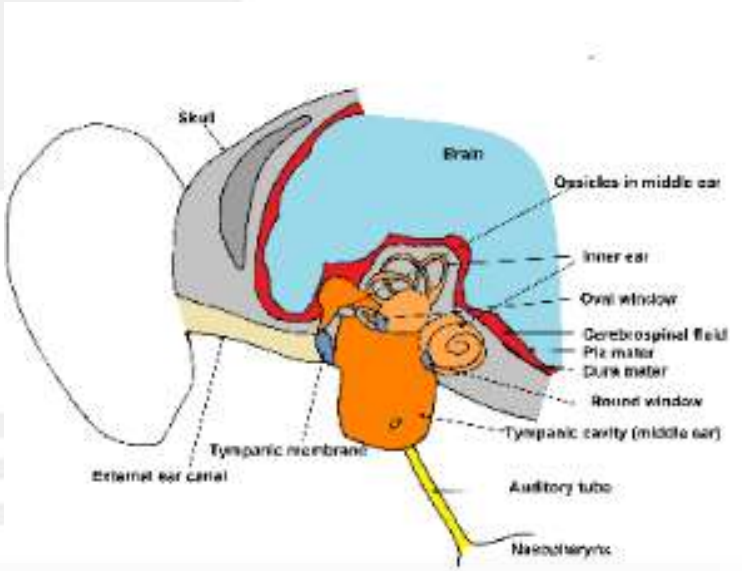
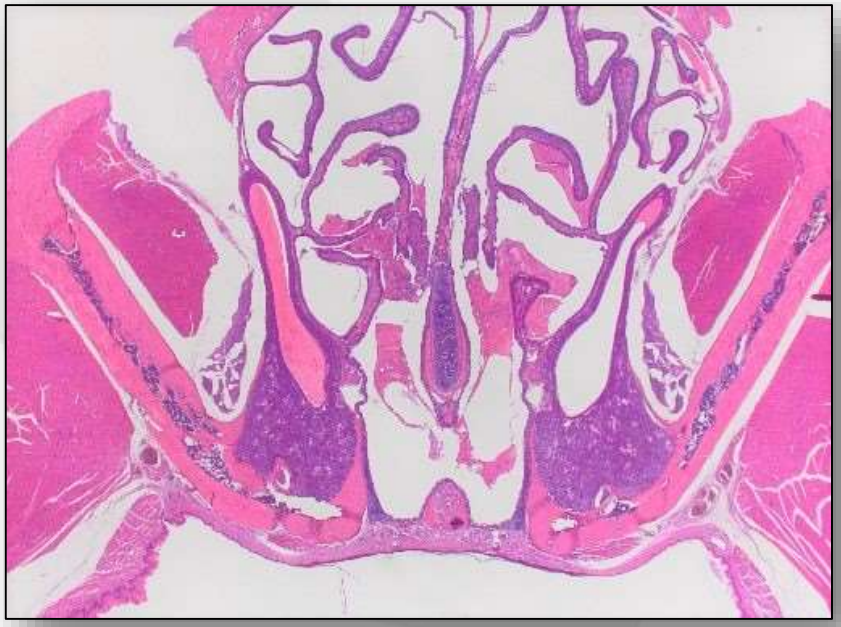
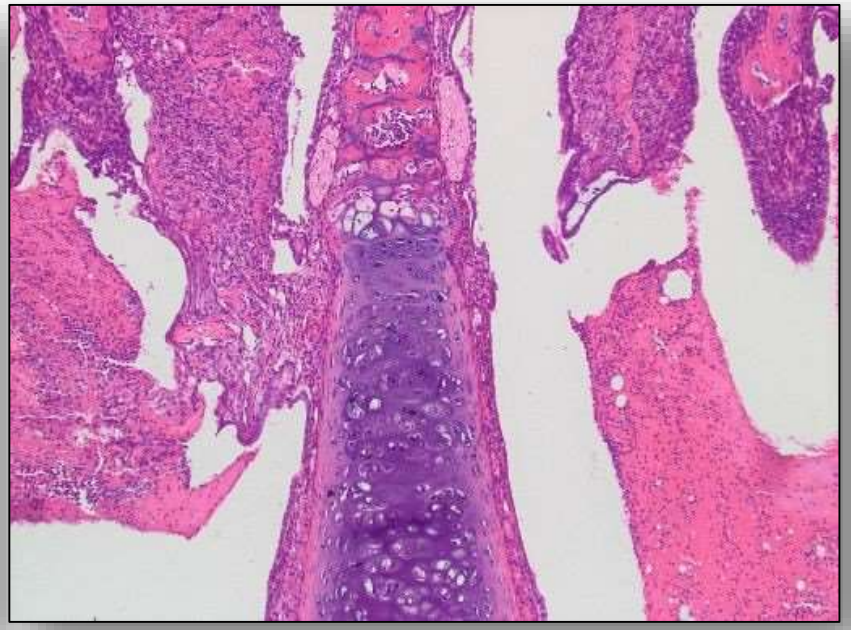
Reflux: Consequences in Other Organs











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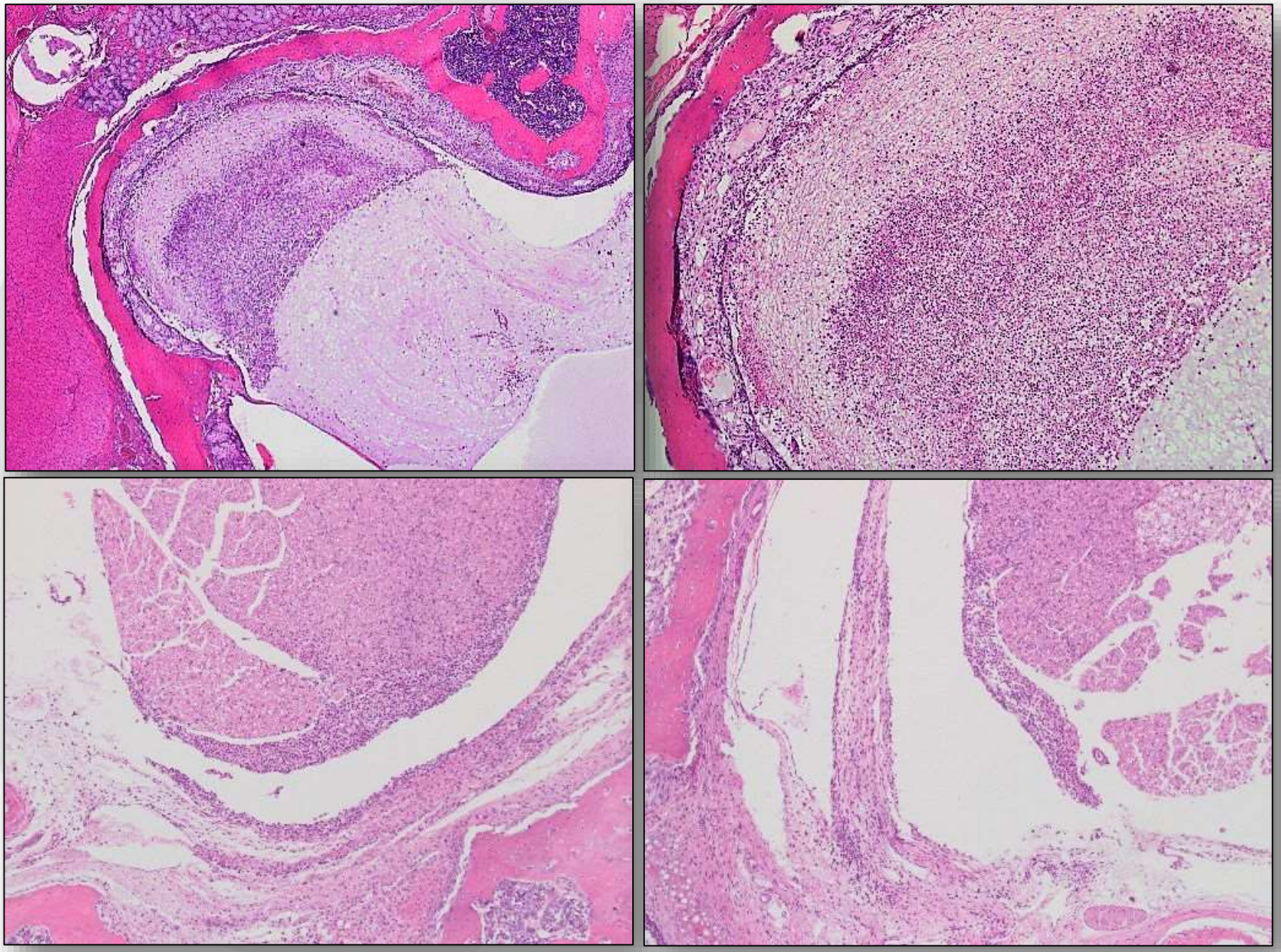
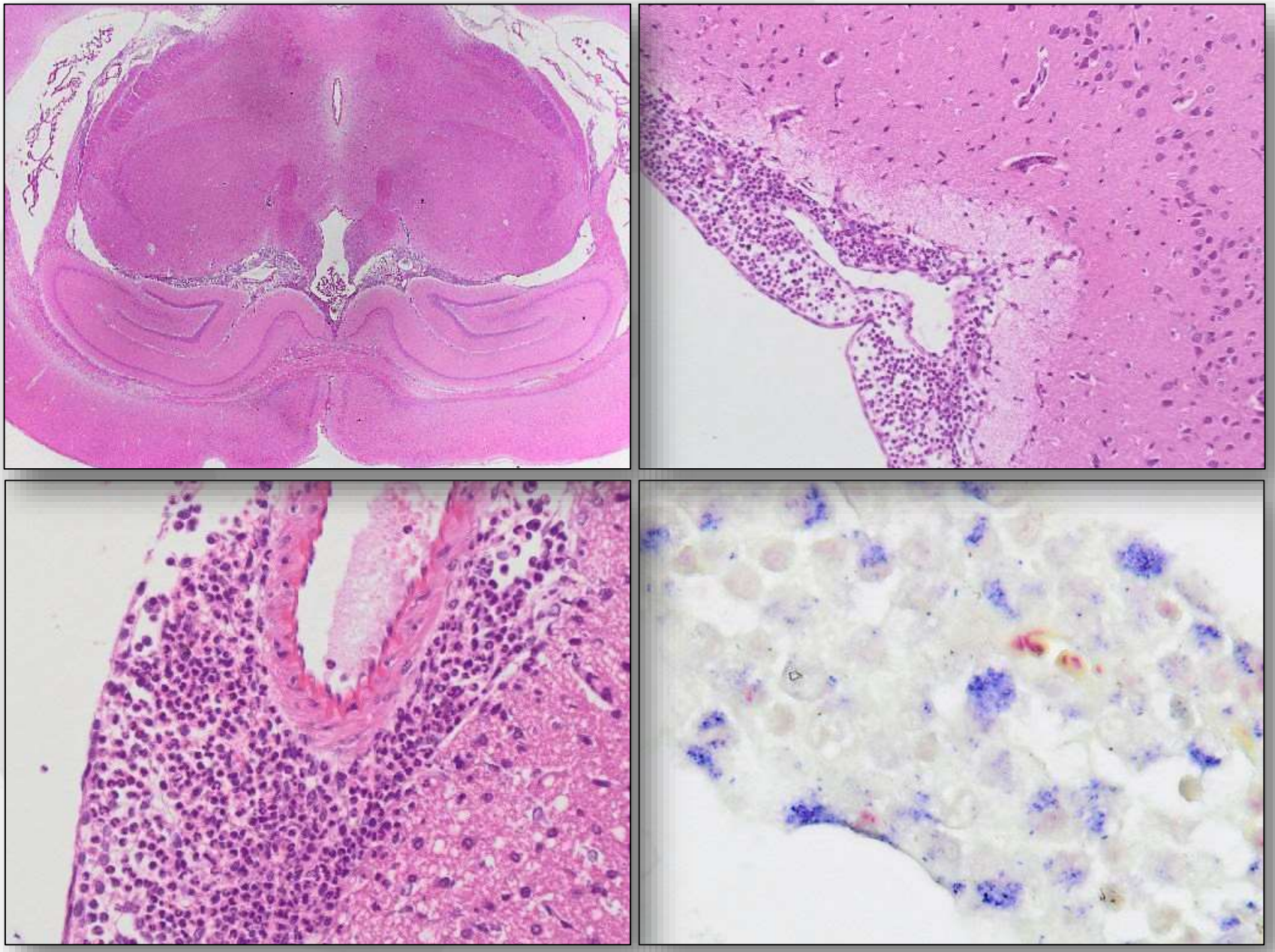


Reflux: Consequences in Other Organs



Summary

- **Exclude misgavage, i.e., pharyngeal, esophageal and gastric irritation/perforation due to incorrect technique or caustic test items**
- **Reflux might be test item or vehicle-related (CMC, Oils etc)**
- **Stress**
- **Passive reflux if excessive material was administered**
- **Pneumonia if test item was injected into the trachea or accidentally aspirated**

- **Check: thymus, heart, lungs, larynx, nasals, esophagus, stomach**
- **Consider other organs that might be affected**

ご清聴ありがとうございました